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Elucidation of Pectin Methylester Distributions by Capillary Electrophoresis

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Evidence is presented that the electrophoretic mobility of the polysaccharide pectin in typical capillary electrophoresis (CE) experiments is determined largely by its chain-averaged charge density, irrespective of how that charge is distributed. This was found to be the case for both high and low calcium sensitive sister fractions separated from a mother sample on the grounds of calcium affinity and for blocky methylester distributions generated by a processive demethylating enzyme. The blockiness of the generated *intramolecular* methylester distribution was substantiated experimentally, also by CE, by observing the oligomeric digest fragments produced by incubation with *endo*-polygalacturonase II from *Aspergillus niger*. The conclusion that the CE migration time is largely invariant to the intramolecular methylester distribution supports the idea that the CE peak shapes can be used to give a useful indication of the *intermolecular* methylester distribution.

KEYWORDS: Pectin; degree of methylesterification; *endo*-polygalacturonase digests; CE

INTRODUCTION

One of us has previously reported that capillary electrophoresis (CE) can be successfully used to measure the degree of methylesterification (DM) of a pectin sample, because there is a linear relationship between the electrophoretic mobility and the average charge per residue (*1*−*3*). Although many other methods perform this DM measurement equally well (*4*−*8*), an advantage of the electrophoretic method is its inherent separation quality. For chains with lengths in excess of around 15 residues, a symmetrical scaling of charge and hydrodynamic friction coefficient with the degree of polymerization (DP) is found. This means that larger polymeric chains, regardless of their DP, elute according to their average charge density and, therefore, that each CE migration time marks species with a unique degree of methylesterification. Peak shapes thus reflect the intermolecular methylesterification distribution (the distribution of degree of methylesterification among chains) of the sample. This aspect of the CE methodology has also been investigated previously (*2*), and although some differences were found between the detailed shapes of intermolecular DM distributions obtained by CE when compared with the results of a more conventional ion-exchange chromatography−size exclusion chromatography (IEC-SEC) methodology, average DM values and, moreover, the widths of the extracted distributions showed good agreement between techniques.

It is worth noting that the validity of such a methodology for obtaining intermolecular DM information from CE hinges crucially on two major assumptions. The first is that contributions to peak widths arising from chromatographic factors are small compared to the breadth of the intermolecular mobility (charge) distribution; second, the mobility is not significantly dependent on the intramolecular distribution of charge (methylesterification), so the migration behavior is determined simply according to the chain-averaged charge density. The first point has been largely addressed by observing the invariance of peak widths to changes in injection time, monitoring the co-injection and subsequent resolution of discrete samples, and performing calculations of the relative contributions expected from the relevant band-broadening mechanisms (*1*, *2*). In this previous work an effort was also made to address the second point by studying commercial pectin samples deemed by the manufacturers (owing to the way in which they had been generated from a mother pectin on the grounds of calcium affinity) to have different arrangements of methylesterification within single chains. Those with increased calcium sensitivity were taken to be likely to contain longer runs of galacturonic acid along the backbone, uninterrupted by methylesterification. It was concluded that there was no statistically significant difference between the electrophoretic mobilities of pectins of equivalent DM with differing intramolecular arrangements of methylesterification.

However, recent work (*9*) has claimed that the intramolecular DM distribution does, in fact, significantly influence the migration time of pectin samples in CE. In an attempt to clarify these issues, we first present further evidence for the usefulness

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of CE in determining the intermolecular DM distribution of commercial high and low calcium sensitive fractions. Second, we examine pectin samples that have been molecularly engineered, using base-catalyzed or enzymatic demethylation, to have the same average DM but different methylester distributions. To demonstrate that a blocky intramolecular methylester distribution was, in fact, generated, the assumed fine structures were substantiated experimentally by observing the oligomeric digest fragments produced by incubation with *endo*-polygalacturonase II from *Aspergillus niger* (*10*).

MATERIALS AND METHODS

Substrates. Several commercial pectin samples derived from lemon peel were supplied as powder by Copenhagen Pectin (www.cpkelco.com). Initial characterization of the degrees of methylesterification was carried out by the manufacturer using a titration method, and these were found to be 31.1, 51.7, 54.2, 55.8, and 77.8%, respectively. The 51.7 and 54.2% samples were produced as high and low calcium sensitive sister fractions from the same mother pectin, separated on the grounds of calcium affinity. A further set of pectin samples included in our work, also consisting of two daughter fractions of high and low calcium sensitivity, but here including the mother pectin (70, 74, and 71% DM, respectively), have been characterized in more detail by Daas (*11*).

Molecularly Engineered Distributions. To have the best possible control over the intramolecular methylester distribution, a highly methylesterified starting material was first constructed so that the subsequent demethylation was, as much as possible, generated by the chosen de-esterification method and did not originate from the properties of the starting pectin. A 65% DM citrus pectin was extensively esterified with methanolic sulfuric acid at 4 °C for 7 days, using a method based on that of Heri (*12*). This yielded a polymer that by both CE and titration had a DM of ~90%. The procedure was found to reduce the molecular weight from around 120 to 44 kDa.

Randomly Distributed Methylesterification. To generate a random distribution of methyl esterification along the polymeric chains, de-esterification was carried out by base saponification as described previously (*13*). Maintaining the pH at 10.5 and the temperature between 2 and 4 °C meant that the reaction was slow (1 day), but any degradation of the chains by β-elimination was effectively prevented, as confirmed by SEC.

Blockwise Methylesterification. To generate an extreme blockwise distribution of methyl esterification along the polymeric chains, de-esterification was carried out by using a PME derived from orange peel. A 2% solution of the highly esterified starting substrate was prepared as before, held at 30 °C with constant stirring, and titrated to pH 7.0. The enzyme was supplied as powder with a quoted activity of 350−700 units mg^{-1} and was diluted in 1.7 M NaCl; 9 mg was added to 225 mL, giving a solution of activity between 14 and 28 units mL^{-1} (assay conditions from the Sigma website). A volume of this enzyme stock equivalent to ~200 units per gram of pectin was then added to the pectin solution, and as de-esterification took place the pH was maintained at 7.0 with the addition of NaOH. This was continued until the amount of base that had been added corresponded to the release of the desired amount of protons and, hence, the required amount of de-esterification. When the desired DM value (30%) has been reached, a small amount of sulfuric acid was added to change the pH to 3.5 to inactivate the enzyme. Subsequently, the protein was denatured by heating the solution to 75 °C for 10 min. The material was then alcohol precipitated, filtered, washed, pressed and dried as described previously (*13*). No molecular weight changes were observed during this de-esterification procedure.

Solutions of the final pectic materials were prepared by heating in deionized water at 60 °C for 20 min or by stirring at room temperature overnight. Repeat experiments revealed no difference between the results obtained with either preparation methodology.

Enzymes. The PME (EC 3.1.1.11) used for blockwise de-esterification was a commercial sample from Sigma (www.sigma-aldrich.com) derived from orange peel.

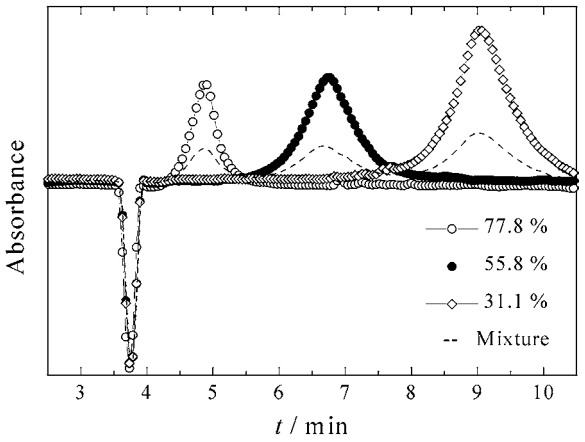

Figure 1. Typical electropherograms obtained from commercial pectin samples of various DM (absorbance at 191 nm).

The pure *endo*-PG II (EC 3.2.1.15) isoform was prepared as described previously (*14*). Digests were carried out by incubating 2.5 mL of 0.5% w/w substrate with 1.9 μg of protein, in 50 mM acetate buffer at pH 4.2, for 16 h at room temperature.

Buffers. Phosphate buffer at pH 7.0 was used as a CE background electrolyte (BGE) and was prepared by titrating aqueous NaH_2PO_4 of the required ionic strength (50 or 80 mM) with 1 M NaOH. Sodium acetate at pH 4.2 was used as the enzyme digest medium and was prepared by titrating 50 M NaOAc with 50 M HOAc. All buffers were filtered through 0.2 μm filters (Whatman).

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 of 50 μm and a total length of 46.5 cm (40 cm from inlet to detector), unless otherwise stated. The capillary incorporated an extended light-path detection window (150 μm) and was thermostated at 25 °C. 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 the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with water, and 2 min with BGE. 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 volumes 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. Electrophoretic mobilities, μ, are related to the migration times of the injected samples relative to a neutral marker, t and t_0, respectively, by

$$\mu = \mu_{obs} - \mu_{eo} = (lL/V)(1/t - 1/t_0)$$

where L is the total length of the capillary, l is the distance from the inlet to detector, V is the applied voltage, μ_{obs} is the observed mobility, and μ_{eo} is the mobility of the electroosmotic flow (EOF) (*15*, *16*).

RESULTS AND DISCUSSION

Figure 1 shows typical electropherograms obtained from 20 s injections of 0.5% w/w solutions of commercial pectin samples of (a) 77.8%, (b) 55.8%, and (c) 31.1% DM. [These measurements were carried out in a capillary of internal diameter of 50 μm and a total length of 64.5 cm (56 cm from inlet to detector) using a voltage of 30 kV.] The absorbance dip, here at ~4 min, results from the refractive index change caused by the passage of water from the injection plug past the detection window and indicates the position of the EOF. This has been verified in an independent experiment using mesityl oxide as a UV-absorbing neutral marker. It can clearly be seen that the migration time increases with decreasing DM (increasing charge). At pH 7.0 the galacturonic acid groups, $pK_a = 3-4$, are fully charged,

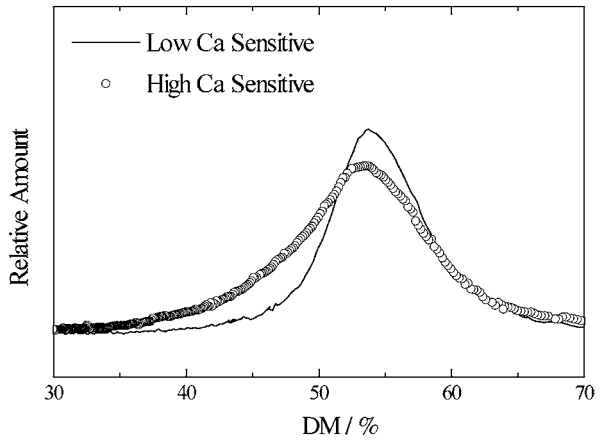

Figure 2. Intermolecular DM distributions (calculated from the CE peak shape as described in the text) for high and low calcium sensitive pectin fractions that have titration-determined average DM values of 51.7 and 54.2% respectively.

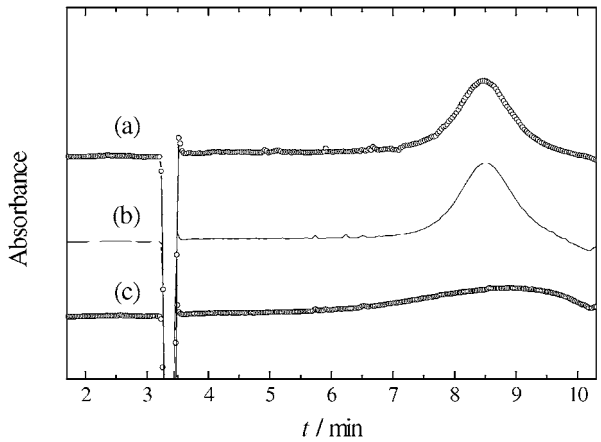

Figure 3. Typical electropherograms of (a) a commercial 31.1% DM sample, (b) a base de-esterified sample of DM 30% produced as described under Materials and Methods, and (c) a plant PME de-esterified sample of DM 30%, also produced as described under Materials and Methods (absorbance at 191 nm).

and although pectin is susceptible to base-catalyzed β-elimination above pH 4.5, no problems were encountered during run times of <20 min, at 25 °C, in the CE capillary. All of these anionic polysaccharides migrate after the neutral marker. The observed mobility μ_{obs} is the vector sum of μ_{eo} and μ (see eq 1), and because μ is negative and smaller in magnitude than μ_{eo}, the anions having the most negative mobility have the smallest μ_{obs} and thus the longest migration times.

Also shown is the result obtained following a consecutive 7 s injection of all three samples in one run. It can clearly be seen that the individual peak shapes are maintained in the mixed sample and, as reported previously, the peak widths are invariant to injection length, demonstrating that peak variance arising from the length of the injection plug is minimal. These data are in good agreement with previous work (*1*−*3*). Using eq 1 and the appropriate value for the EOF, the migration time axis can be transformed to a mobility axis. Subsequently, the number-average electrophoretic mobilities are found to a yield a linear relationship with sample-averaged charge density (as measured by the manufacturers), and hence such calibrations can be used to construct a simple transform that maps electrophoretic mobility onto degree of methylesterification (*1*−*3*).

Figure 2 shows the comparison of the intermolecular DM distributions, obtained by CE using such a transform, as described above, for commercial high and low calcium sensitive pectin samples of similar DM. In each case the number-average DM values obtained by CE are found to be in good agreement with those obtained by the manufacturers (within 2%). It is particularly interesting to note that there is a low DM tail in the distribution obtained from the highly calcium sensitive sample. A similar result was found from the comparison of the distributions obtained from further commercial samples of high and low calcium sensitivity of DM around 65% (data not shown). This is clearly what would be expected for a highly calcium sensitive daughter fraction obtained by separation on the grounds of calcium affinity. It can be presumed that the mother sample will contain chains with different chain-averaged DM and different intramolecular distributions. A fractionation based on calcium sensitivity will surely pick out more blocky chains but will also be inclined to preferentially retain the chains with low DM. Furthermore, the fact that the number-averaged DM values agree so well with those measured by other bulk methods (even for substantially asymmetric peaks) lends credence to the fact that the extracted DM distribution is a good representation of reality and, thus, that it is reasonable to assign a particular electrophoretic mobility to a particular chain-averaged charge density, irrespective of its intramolecular charge distribution. This is in good agreement with previous work on commercial high and low calcium sensitive pectin fractions (*1*). Attention is now turned to the samples with more extreme blockiness, generated by PME.

Figure 3 shows the electropherograms of (a) a commercial 31.1% DM sample, (b) a base de-esterified sample of DM 30% produced as described under Materials and Methods, and (c) a plant PME de-esterified sample of DM 30% also produced as described under Materials and Methods. Preliminarily, it seems that the designed polymers are similar in average migration time to the commercial sample, which suggests that they are indeed of the planned nominal DM (30%). It can be seen that the PME-generated sample has a significantly broader peak, which could be interpreted as reflecting a broader distribution of chain-averaged methylesterification values between individual polymers. It seems reasonable to suggest that such an outcome could be a simple consequence of the kinetics of demethylation. Processive behavior, coupled with the fact that it is the sample-averaged DM that is monitored by pH changes, means that, when the desired sample-averaged DM value is reached, although many chains are severely demethylated, others may have yet to be substantially de-esterified. Perhaps more importantly within the context of the work described here are the relative DM distributions obtainable from these data. To ensure transparency, the steps taken in the processing of the data shown in **Figure 3** are elaborated forthwith. First, a simple small translation of the data in the *y* direction is made to ensure that the baseline around the peak is assigned a value of close to zero. This is important with regard to the normalization of the peak shape that forms the next step. The absorbance values are divided by their respective migration times, thus ensuring that the relative intensities are normalized for the different amounts of time spent in the detector by species with different electrophoretic velocities (*17*). Equation 1 is then used to generate an electrophoretic mobility data set corresponding to the time values, and thus a plot of normalized intensity (relative amount) versus electrophoretic mobility can be formed.

Figure 4 shows the resultant electrophoretic mobility distributions for the DM 30 samples produced with differing patterns of intramolecular methylesterification. As a last step the electrophoretic mobilities can be mapped onto DM by using a calibration. However, it is worth noting that as the relationship

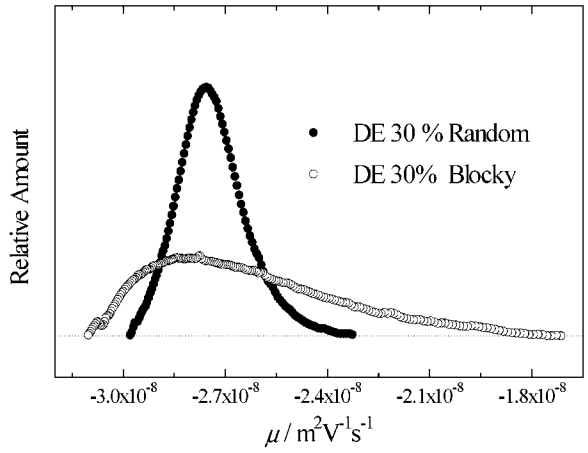

Figure 4. Resultant electrophoretic mobility distributions for the DM 30 samples produced with differing patterns of intramolecular methylesterification.

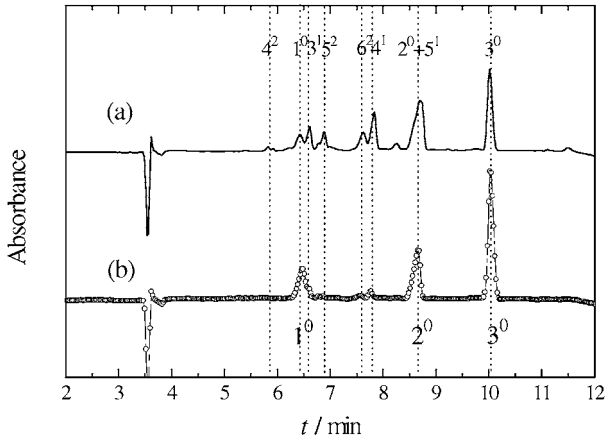

Figure 5. Electropherograms showing the results of incubating the engineered pectic polymers: (a) base de-esterified; (b) PME de-esterified, with *endo*-PG II. The notation n^m is used to indicate an *n*-mer of galacturonic acid with *m* groups methylesterified (absorbance at 191 nm).

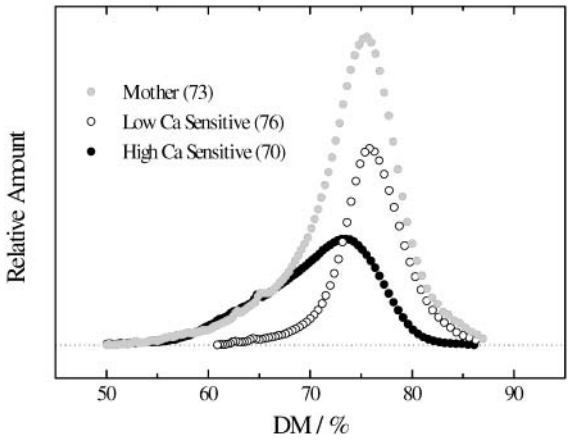

Figure 6. Intermolecular DM distributions (calculated from the CE peak shape) for a mother pectin and high and low calcium sensitive daughter fractions.

is known to be of the form DM = $A(\mu) + C$, then the number-average DM is equivalent to A (number average μ) + C. That is to say, two samples with very similar number-average μ will possess very similar number-average DM values. Excluding any form of calibration for simplicity, the number-average electrophoretic mobilities obtained from the distributions shown in **Figure 4** are -2.70×10^{-8} and -2.74×10^{-8} m^2 V^{-1} s^{-1}, respectively, for the random and blocky pectins. These values are indistinguishable within experimental uncertainty, estimated from several repeat experiments at 2%. This suggests that there is no difference in this experiment between the electrophoretic mobility of pectin molecules with different intramolecular DM distributions, as long as they possess the same chain-averaged charge density. This result is then consistent with the previous work on the commercial high and low calcium sensitive fractions (that might have been argued to possess more limited blockiness) and strongly argues for an interpretation of the electropherogram in terms of an intermolecular distribution of methylesterification where, for molecules in excess of DP ~15, every migration time does mark species of a particular chain-averaged charge density, irrespective of how that charge is distributed.

It is worth noting that different PME attack mechanisms have been proposed to be operative at different pH values (some of which would produce considerably less blocky materials) (*18*), and as a check on the validity of the assumptions made about generated fine structure, it was decided to seek experimental evidence that the engineered substrates did indeed have different distributions of methylesterification. Parts a and b of **Figure 5** show the results of incubating the engineered pectic polymers with *endo*-PG II. It has previously been shown that the separation of the resultant oligomeric digest fragments can be performed by CE (*10*), and it is reassuring to find that the digest pattern of randomly distributed pectin looks similar to that obtained from a commercial 31% DM pectin (*19*). The peaks are labeled according to the assignments made in previous work. (The notation n^m is used throughout to indicate an *n*-mer of galacturonic acid with *m* groups methylesterified.) The use of such digests in inferring the intramolecular methylesterification properties of the predigested substrate has been the subject of much recent interest (*3*, *10*, *11*, *13*, *20*−*25*). It is not the purpose of this study to discuss, or to model in detail, the enzymatic degradation process, rather to use this methodology to demonstrate that the PME-engineered pectin substrate is indeed substantially more blocky in its distribution of methylesters compared with that produced by base saponification. It is generally accepted that more blocky pectic polymers release more unmethylated fragments as digest products when compared to randomly distributed substrates of the same DM. Indeed, a blockiness scale based on the amount of galacturonic acid released in the form of unmethylated mono-, di-, and trimers (*20*) has been proposed. It is clear from **Figure 5** that, purely on the basis of this criteria, the PME de-esterified substrate is indeed substantially more blocky than its base-produced counterpart, and yet, as shown in **Figure 4**, the polymers do not seem to have hugely different number-average electrophoretic mobilities. This result appears to be contrary to that reported recently in the literature (*9*), although it is difficult to comment too much further as only migration times and not electrophoretic mobilities were reported.

Finally, it was decided to examine a further set of high-DM pectins, namely, high and low calcium sensitive daughter fractions, this time together with the mother sample from which they had been extracted. The rationale here was that if the extracted distributions were a realistic representation, then it should be possible to reconstruct the mother pectin distribution from those of the daughters. **Figure 6** shows the intermolecular DM distributions measured for this sample set by CE, as described herein. The number-average DM calculated from the distributions was in all cases within 2% of that found by Daas

[(73 ± 3) cf. 71, (70 ± 3) cf. 70, and (76 ± 3) cf. 74 for the mother and the high and low calcium sensitive fractions, respectively]. As expected from the results obtained from other commercial samples, the highly calcium sensitive fraction does indeed show a low DM tail. In lieu of information regarding the relative amounts of the two sister fractions derived from the mother sample, the relative magnitudes of the distributions have been scaled arbitrarily, to demonstrate how the distribution measured for the mother pectin can be well approximated by a sum of the distributions obtained for the two daughter samples. Once again, this clearly supports the interpretation of the CE peak shape as a real distribution that reflects a physical property of the sample.

CONCLUSIONS

Further evidence has been presented that the electrophoretic mobility of the polysaccharide pectin in typical CE experiments is determined largely by its chain-averaged charge density, irrespective of how that charge is distributed. This was found to be this case for both high and low calcium sensitive fractions separated by calcium affinity and for experimentally substantiated more extreme block distributions, generated by a processive demethylating enzyme. This strongly supports the interpretation of the electropherogram peak shape in terms of an intermolecular distribution of methylesterification.

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