Resolution of the structural isomers of partially methylesterified oligogalacturonides by polysaccharide analysis using carbohydrate gel electrophoresis

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Pectins differing in their degree and pattern of methylesterification are important in diverse aspects of plant physiology and also in many industrial applications. Determination of methylesterification fine structure and knowledge of enzyme specificities in modification and fragmentation of pectin are key to understanding the relationship between structure and function. The development of methodologies for the detection, separation and sequencing of different partially methylesterified oligogalacturonides (Me-OGAs) is consequently very important. Polysaccharide analysis using carbohydrate gel electrophoresis (PACE) has been shown to be powerful for the quantitative resolution of species different in degree of polymerization (DP) and/or degree of methylesterification (DM). Mass spectrometry (MS) has, to date, been the only tool with which to obtain isomeric information. However, it is not quantitative, and the presence of isobaric species makes the interpretation of the fragmentation patterns complicated. Here, we present evidence that Me-OGAs with the same DP and DM but different patterns of methylesterification (structural isomers) can easily be separated and quantified using PACE.

Key words: methylesterification/PACE/pectin fine structure

Introduction

The plant cell wall is a complex biological matrix in which hemicelluloses, pectins, cellulose, proteins, and lignin all play a role in determining structure and properties (Fry, 2000). Despite this complexity, the enzymatic remodelling of the pectin component that accompanies many physiological changes in which the mechanical properties of the wall must be controlled highlights the important role played by the methylesterification (level and distribution) of polygalacturonan (PGA). Understanding the functional role of pectic methylesterification patterns is also crucial to select and design fine structures for specific industrial application (Hoondal et al., 2002). Indeed the effect of the pattern of methylesterification on the rheological (Powell et al., 1982) and gel forming (Ralet et al., 2001a,b) properties of pectin has been the subject of many studies.

To obtain such structural information, a fragmentation approach can be used in which endo-polygalacturonase (endo-PG) is used to digest the polysaccharide and its subsequent (methyl-ester sequence dependent) digest pattern is determined. The separation, detection and quantification of partially methylesterified oligogalacturonides (Me-OGAs) therefore play a key role in the elucidation of the fine structure of pectin, as well as in the characterization of enzyme specificity. Although the size and charge of oligosaccharides have classically been distinguished by methods such as high-performance anion-exchange chromatography (HPAEC; Daas et al., 1999; Van Alebeek et al., 2000), two additional methods have recently been developed to achieve this: capillary electrophoresis (Williams et al., 2003) and polysaccharide analysis using carbohydrate gel electrophoresis (PACE; Goubet et al., 2003). To obtain the maximum information on the structure of the digest fragments, not just their size and total degree of methylesterification (DM) are required but also the position of the esterified residues. Such information can be obtained using multiple fragmentation strategies in electrospray ionization-ion trap MS (ESI-ITMS; Körner et al. 1999, Quéméner et al., 2003), although the interpretation of fragmentation patterns is complicated by the presence of multiple isobaric species and quantification is not possible. Although it has been suggested that HPAEC was able to partially separate one form of singly methylesterified-(GalU)3 from another (Van Alebeek et al., 2000), there is, to date, no reported detailed chromatographic methodology for the separation of structural isomers of Me-OGAs.

PACE involves a derivation of the sugars by a fluorophore and then a separation by gel electrophoresis. The method has been shown to be useful to characterize polysaccharide structure (Goubet et al., 2002, 2003) and enzyme characteristics (Hogg et al., 2003) of different neutral or acidic oligosaccharides. Previously, Goubet et al. (2003) have shown that Me-OGAs of different degree of polymerization (DP) and DM can be resolved using this methodology. Furthermore, sugar quantification is possible as previously demonstrated (Goubet et al., 2002, 2005). Here, we show for the first time that PACE can, in addition, separate the different isomers of partially Me-OGAs.
Results

We previously showed that PACE can be used to resolve methylesterified and unmethylesterified OGAs derivatized with 2-aminoadridone (AMAC), and this work raised the question of whether Me-OGA structural isomers could also be resolved (Goubet et al., 2003). To investigate this issue, multiple isomers of Me-OGA species were first generated by partially demethylesterifying pure standards chemically. For example, although the base saponification of the species GGMMG (G is for the GalU residue, and M is for the GaUMe residue) will eventually yield only GGGGG, during the deesterification process both GGMMG and GGGMG are expected to be present. The timecourse of such deesterification measurements was recorded using PACE to observe the number of discrete resolved bands.

Demethylesterification of pure and identified Me-OGAs

Figure 1A shows the demethylesterification of a single (GalU)5Me sample believed to be one isomer, namely GGMMG (based on it being a digest product of endo-PG II from Aspergillus niger and previous characterization of the products of this enzyme by MS [Limberg et al., 2000b]). This (GalU)5Me species corresponds to the one band observable in the PACE experiment at the start of the demethylesterification process. As deesterification proceeded the intensity of the (GalU)5Me band decreased, as another band, known to be (GalU)5, the ultimate saponification product, increased.

Also shown in Figure 1A is the demethylesterification of a (GalU)6Me2, once again believed to be a single isomer, GGMMG, based on its production by endo-PG II (Limberg et al., 2000b). In complete demethylesterification, only one band was detected and corresponds once again to GGGGG as expected. However, it is clear that there are two intermediate and transitory bands detected during the timecourse of the experiment, labelled 5,1a and 5,1b. 5,1b clearly labels species migrating in the same fashion as the (GalU)4Me previously examined and is hence assigned GGGAM. By deduction the band labelled 5,1a corresponds to GGMMG. Thus, the presence of two different separated isomers of (GalU)4Me can be inferred from the results of the PACE experiment.

In a similar vein the demethylesterification of the tetramer GMGG also exhibited two intermediate and transitory bands, before ultimately displaying the presence of only one band known to be GGGGG (data not shown). Once again one of these transitory bands migrated in the same place as an already known form of (GalU)4Me, namely GGMMG, and the other by implication was GMGG. Clearly, as with the pentamic species, two different isomers of (GalU)6Me could be resolved.

It has previously been suggested that multiple isomers of (GalU)6Me2 might be digestion products of pectin with endo-PG II, in contrast to the single (GalU)5Me2 and (GalU)4Me2 isomers produced by the enzyme and furthermore that such multiple isomers would comigrate in HPAEC experiments (Limberg et al., 2000b). As the (GalU)6Me2 species studied here was obtained by exactly this method, we initially supposed that this sample was unlikely to migrate as only one band. However, on separating the oligosaccharides in the collected fraction by PACE only one band was detected, as can be seen in Figure 1B. This suggested that either only one isomer of (GalU)6Me2 was present in the collected sample or that some isomers can comigrate by PACE. However, it is clear that upon demethylesterification only two transitory bands were observed enroute to (GalU)6. The simplest explanation of this observation is that there are only two (GalU)6Me isomers generated by partial demethylesterification and thus, that the parent oligogalacturonide was only one doubly methylesterified isomer. This result is in agreement with previous data (Quéméner et al., 2003). We conclude that, once again, with hexamers, two different isomers of (GalU)6Me can be resolved.

Quantification of Me-OGAs

Quantification of all the bands detected at each stage of demethylesterification showed a good correlation in total OGA quantity between the start and all other points. This was true for all Me-OGAs tested: (GalU)4Me (Figure 2A), (GalU)5Me (Figure 2B), (GalU)5Me2 (Figure 2C), and (GalU)6Me2 (Figure 2D) and gives further confidence in the band assignments.

It is interesting to note that the rates of appearance and/or disappearance of the two (GalU)5Me isomers were different (Figure 2C). This suggests that the demethylsterification is partially selective. A similar observation is true of (GalU)6Me isomers (Figure 2D). Indeed, the forms labelled 5,1a and 6,1a were reproducibly present in a slightly greater quantity as intermediate species compared to 5,1b and 6,1b, respectively.
Demethylesterification of a highly Me-OGA

To investigate the degree to which different isomeric forms might be resolved a highly Me-OGA, (GalU)_4Me_2 was targeted for deesterification. This sample had initially been produced as (GalU)_4Me_4 by methylesterification of (GalU)_4 but was demethylesterified at the GalU at the reducing end after significant storage time. Such methylesterification sensitivity is well known (Van Alebeek et al., 2000). To establish the isomeric purity of the putative (GalU)_4Me_3 species, the derivatized (GalU)_4Me_3 structure was obtained by ESI-ITMS to assign the methylester groups to GalU residues. The full positive MS spectrum of (GalU)_4Me_3-AMAC corresponded mainly to a tetramer of GalU bearing three methyl ester groups (Figure 3A). As previously reported with AMAC derivatives prepared from chitooligosaccharides and analyzed by matrix assisted laser desorption ionization time-of-flight MS (MALDI TOF MS), the sodiated pseudomolecular ions were the main components observed (Bahrke et al., 2002). This result is also in agreement with the affinity order of different alkali metal ions for carbohydrates previously ranked as Cs > K > Na > Li > H (Mohr et al., 1995). The predominant pseudomolecular ion produced was the dehydrated sodiated (GalU)_4Me_3-AMAC at m/z 963 [958 + Na – 18]”. This water loss was not observed in MALDI TOF MS and seems to be related to the ESI process. The ion observed at m/z 981 corresponds to the same sodium-cationized species but before the water loss [958+Na]^+. The ion at m/z 1003 is the respective sodium salt species in which one hydrogen atom is replaced by sodium in one acidic group [958+Na+Na-H]^+. These three kinds of pseudomolecular sodiated ions were also observed on the ESI-ITMS spectrum of the (GalU)_4Me_2-AMAC at m/z 773, 791, and 813, respectively, and roughly in the same proportions (data not shown). MS^2 is generally performed by selecting the parent ion (by ejecting the undesired ions) and by the subsequent fragmentation of the selected parent by collisionally induced dissociation (CID). This MS^2 experiment performed on the parent ion may be sequentially performed on daughter ions, and we can obtain MS^3, MS^4 up to MS^10 stages of mass analysis. The MS^2 and MS^4 experiments performed on the predominant [M+Na-H-0] pseudomolecular ion at m/z 963 (Figure 3B) and on the fragment product ion at m/z 583 (Figure 3C), respectively, revealed major fragment ions of Y_j series (Domon and Costello, 1988) because of consecutive neutral losses of 190 Da (methylesterified GalU residue mass). The smallest Y_j fragment ion obtained was [Y_1] and had to correspond to the nonesterified GalU residue at the reducing end linked to AMAC. CID was also carried out on the nondehydrated sodiated ion at m/z 981. Major fragment ions of Y_j series were again produced with a difference of 18 (water molecule mass) compared to the respective fragment ions obtained from the dehydrated sodiated species at m/z 963. All these results confirm that the (GalU)_4Me_3-AMAC structure used in this work was MMMG-AMAC. Negative MS^n experiments were in agreement with this assignment, and in particular, the full MS spectrum which was dominated by the deprotonated (GalU)_4Me_3-AMAC at m/z 957 (data not shown). ESI-ITMS analysis performed on the nonderivatized (GalU)_4Me_3 delivered the same chemical structure (data not shown) and allows us to conclude that the derivatization process did not cause the demethylsterification.

The partial chemical demethylsterification of MMMG produced a complex fingerprint with eight bands being detected in the PACE experiment (Figure 4). Starting with...
cies. The detection of eight bands clearly suggests that all these (GalU)4Me2 isomers, three different (GalU)4Me isomers find, in addition to the starting species, three different erate all possible species of lower DM, we would expect to MMMG and assuming that demethylesterification can gen-

Fig. 3. Positive ion mode mass spectrometry (MS) and MS2 spectra of the sodium-cationized [(GalU)4Me]-2-aminoacridone (AMAC) confirms the structure as MMMG-AMAC. (A) Full MS spectrum; (B) MS2 spectrum of the pseudomolecular ion at m/z 963 (963 > products); (C) MS4 spectrum of the fragment ion at m/z 583 (963 > 773 > 583 > product). M, [(GalU)4Me]-AMAC; Y3, [(GalU)3Me]-AMAC; Y2, [(GalU)2Me]-AMAC, and Y1, (GalU)-AMAC.

Fig. 4. Complex mixture separation of methylesterified oligogalactur-

and (GalU)4, indeed corresponding to a total of eight spe-
cies can be resolved. Three of the seven product bands (band 8, GGGG; band 7, GGMG; and band 4, GMMG) were identified using standards produced by hydrolysis of methylesterified pectins by endo-PG from A. niger (Williams et al., 2002), as with the previous work. Band 6 was identified as an intermediate in deesterification of GMMG along with band 7, and is therefore GMGG. The correspondence of 3 bands to particular species was unknown (labelled 2, 3, and 5 and corresponding to some combination of MMGG, MGMG, and MGGG). Bands 2 and 3 are likely to be (GalU)3Me2 isomers, as they migrate near to each other and other highly methylesterified species, whereas band 5 is likely to be the singly methylesterified oligomer, MGGG. The oligosaccharides derivatized with AMAC were extracted from gel bands 2–6 and further analyzed by MALDI TOF MS. Post source decay (PSD) experiments identified band 2 as MMGG, band 3 as MGMG, band 5 as MGGG and confirmed the identity of bands 4 and 6 (data not shown).

It is also interesting to note that the intensities of bands 2, 3, and 4 (all three (GalU)4Me2 isomers) are different. This is consistent with the data shown in Figure 2 and clearly supports the hypothesis that not all the GalU residues have the same chemical sensitivity to base saponification.

Analysis of exo-polygalacturonase by PACE

We further used the PACE technique to examine the exo-polygalacturonase (exo-PG) sensitivity of the different isomers we had generated and resolved. The purpose of this was 2-fold. Firstly, because the specificity of the enzyme was known (Limberg et al., 2000a) (it requires two methylester-free residues at the nonreducing end of the OGA to remove a single GalU residue), it could be used to check the band assignment, by noting which bands were sensitive to the enzyme. Secondly, it demonstrates how being able to resolve isomeric Me-OGA species can be used to study enzyme characteristics, by revealing enzyme
Resolution of partially methylesterified oligogalacturonides by PACE

The partial base saponification of a specific (GalU)₄Me₃ species (MMMG) was used to generate a sample that exhibits eight bands corresponding to the initial MMMG, three isomers of (GalU)₄Me₂, three isomers of (GalU)₄Me and the completely deesterified GGGG. For Me-OGAs of greater DP and DM the possible number of isomeric species quickly increases. The degree to which further isomers are resolvable forms part of ongoing work. The fact that so few bands have been detected in previous studies of pectin fragmentation (Goubet et al., 2003) is testament to the specificity of the enzymatic processes generating the fragments. It should be stressed, however, that having a technique with the potential to quantify different isomeric forms, if indeed they are present in a mixture, advances the area significantly.

The detailed study of position-resolved demethylesterification sensitivity is an interesting chemical problem that could be studied further using this technique. Although partial demethylesterification conditions were selected here to generate all possible isomeric compounds from our starting Me-OGAs, it is clear from the different rates of disappearance of transitory bands that the process is not completely random. In the case of esterification, Tjan et al. (1974) suggested that there is a preference for the GalU at the reducing end for the start of methylesterification. In our preliminary experiments, the methylester-GalU at the reducing end was the most sensitive for demethylesterification. After storage (GalU)₄Me₄, (GalU)₃Me₃, and (GalU)₂Me₂ species were all found to have lost the methylester group from the reducing end of the molecules (data not shown for the last two Me-OGAs).

It is clear that the Me-OGAs are relatively prone to demethylesterification. It has previously been found that keeping these species for a short time in water could induce partial demethylesterification and by increasing the pH and temperature, the reaction rate is increased (Van Alebeek et al., 2000). This highlights the importance of performing control experiments to confirm that the substrates are not modified during the sample preparation before the PACE experiments.

Previously, an exo-PG has been shown to cut PGA and OGAs from DP 2–7 (Kester et al., 1996). We were able to show simply and quickly using PACE that the enzyme can cut only OGAs that have at least two nonesterified GalUs at their nonreducing end, as previously observed (Limberg et al., 2000a). This highlights the importance of performing control experiments to confirm that the substrates are not modified during the sample preparation before the PACE experiments.

Discussion

We have shown that partially Me-OGAs of the same DP and DM but with different methylesterification patterns (structural isomers) can be separated and quantified by using PACE. Thus, this technique is more informative than current methodologies. Furthermore, Me-OGAs derivatized with AMAC can be studied by ESI-ITMS and MALDI TOF MS. Individual unknown bands can be extracted from gels and their methylesterification pattern determined by MS. Therefore, a lack of complex standards need not unduly limit the analysis.

Materials and methods

Materials

Acrylamide/N,N'-methylenebisacrylamide (29:1) was purchased from Biorad (Hertfordshire, UK). (GalU)₃ and GalU were purchased from Sigma (Poole, UK). Me-OGAs of DP, DM 4.1; 4.2; 5.1; 5.2; 6.2 were obtained by preparative HPAEC and characterized by ESI-ITMS (Quéméner et al., 2003). AMAC was purchased from Molecular Probes (Leiden, The Netherlands). Exo-PG (EC 3.2.1.67) from Aspergillus tubingensis was a gift from Jacques Benen.
Production and purification of Me-OGAs

Fractions of partially methylated Me-OGAs with known patterns of methyl esterification were obtained by fraction collection from pectin digests by endo-PG from *A. niger* using HPAEC as described previously (Williams *et al.*, 2002). (GalU)₄Me₃ was produced by esterification of the (GalU)₄ sample using methanolic sulphuric acid (Héri *et al.*, 1961). After extended storage, it was found that the GalU at the reducing end was deesterified. The sample identity as (GalU)₄Me₃ was inferred from the products obtained on subsequent deesterification and was confirmed unequivocally by ESI-ITMS.

Demethylesterification of oligosaccharides

Me-OGAs were demethylated using 1 μL of NaOH solution (1 M) in a total volume of 100 μL of water. The treatment was performed at room temperature from 1 to 60 min before neutralization with HCl (0.5 M) using a pH paper. After that the solutions were dried using a centrifugal vacuum evaporator (ThermoQuest, Hampshire, UK).

OGA hydrolysis by exo-PG

Digests with exo-PG were carried out by incubating partially deesterified (GalU)₄Me₃ with 5 μL (65 U mL⁻¹), in 2 mL of 100 mM ammonium acetate buffer pH 4.3, for 5 hours at 20°C. The reaction was stopped by boiling for 10 min. The samples were then dried using centrifugal vacuum evaporator before analysis by PACE.

PACE analysis

Derivatization was carried out in the tubes containing dried polysaccharides, oligosaccharides or monosaccharides. The derivatization and the analysis were performed as previously described for pectin analysis using AMAC, as fluorophore (Goubet *et al.*, 2003). All gels were performed at least three times with samples made at least two times. The figures given in this publication are a representative example of gels obtained. Gels were scanned and quantified as previously described (Goubet *et al.*, 2005) using GalU and (GalU)₃ as the standards. Analyses were performed using a 16-bit image files and GeneTools software (Syngene, Cambridge, UK).

Preparation of Me-OGAs derivatized by AMAC for MS analysis

The Me-OGAs derivatized by AMAC and dried were resuspended in 500 μL of 10 mM acetic acid to adjust the pH to ~4, as monitored using pH paper. Initially a study was carried out in the presence of water but a partial demethylesterification of the Me-OGAs was observed. An acetic acid solution (10 mM) was required to stabilize the methylesters. The samples were subsequently filtered to eliminate all precipitated compounds. The solutions were dialyzed using a dialysis tubing of molecular weight cutoff (MWCO) of 500 Da (Spectrum Laboratories, Rancho Dominguez, CA), once against 10 mM acetic acid and three times against distilled water. These samples were analyzed by ESI-ITMS.

To determine the identity of oligosaccharides in specific bands, a preparative gel with four adjacent lanes loaded with 6 μL of AMAC-derivatized oligosaccharides was prepared. Bands were excised whilst viewing the gel under a UV transilluminator (wavelength 360 nm), and suspended in 1 mL of 10% (v/v) glacial acetic acid. To extract the oligosaccharides, acrylamide gel slices were partially crushed (and the pH verified to be acid to reduce any demethylesterification process) and subjected to three cycles of freeze/thawing. To eliminate any polyacrylamide fragments, the samples were filtered using the Nanoprep system (MWCO of 10,000 Da; Pall, East Hills, NY) and then dialyzed against water using dialysis tubing (MWCO of 500 Da). The solution was dried and the pellet was suspended in 10–20 μL of water. Aliquots of the specific derivatized-Me-OGA samples were loaded onto a gel to estimate their quantity. These samples were analyzed by MALDI TOF MS.

ESI-ITMS analysis

ESI-ITMS analysis of Me(GalU)₂-, Me₂(GalU)₃-, Me₃(GalU)₄-, AMAC, and Me₃(GalU)₄ were achieved on a LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, Cergy Pontoise, France) using positive and negative electrospray as the ionization processes. Lyophilized sample solutions (20 μL) were diluted 200 times by 50% methanol to favour the spray formation into the electrospray source. Infusion was performed at a flow rate of 2.5 μL/min. Nitrogen was used as sheath gas (20 arbitrary units). The MS analyses were carried out under automatic gain control conditions, using a typical needle voltage of 4 kV and a heated capillary temperature of 200°C. For MS² experiments, the various parameters (collision energy, qz activation value, and activation time) were adjusted for each sample to optimise the signal and get maximal structural information from the ion of interest. About 1 min of cumulative spectra was recorded for each MS² spectrum.

MALDI TOF MS

MALDI TOF MS analyses of AMAC-derivatized OGAs extracted from the PACE gel were carried out using a 4700 Proteomics Analyzer operated in positive ion mode (Applied Biosystems, Warrington, UK). This MALDI1 tandem mass spectrometer uses a 200 Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. The diazoyl OGAs were diluted 20 times with 50% aqueous methanol and mixed 1:1 with 2.5-dihydroxybenzoic acid (Fluka, Buchs, Switzerland), which was prepared as a saturated solution in 50% methanol in water. This solution (0.6 μL) was spotted onto the MALDI target and dried rapidly in a desiccator. MS spectra were acquired in reflector mode with an average of 1000 laser shots. Sequence information was provided by post source decay (PSD) experiments on selected molecular ions [M + H]⁺, using an average of 5000 laser shots.

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Abbreviations
AMAC, 2-aminoacridone; DM, degree of methylesterification; DP, degree of polymerization; endo-PG, endo-polygalacturonase; ESI-ITMS, electrospray ionization-ion trap mass spectrometry; exo-PG, exo-polygalacturonase; HPAEC, high-performance anion-exchange chromatography; MALDI TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; Me-OGA(s), methylesterified oligogalacturonide(s); MS, mass spectrometry; MWCO, molecular weight cutoff; OGA(s), oligogalacturonide(s); PACE, polysaccharide analysis using carbohydrate gel electrophoresis.

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