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Carbohydrate Polymers 43 (2000) 39–46

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Esterification and glycosydation of oligogalacturonides: examination of the reaction products using MALDI-TOF MS and HPAEC

G.-J.W.M. van Alebeek, O. Zabotina, G. Beldman, H.A. Schols, A.G.J. Voragen*

Department of Food Technology and Nutritional Sciences, Chair of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD, Wageningen, Netherlands

Accepted 13 December 1999

Abstract

Methyl-esterified and methyl-glycosydated oligogalacturonides (oligoGalA) were produced to be used as substrates for the characterization of pectinolytic enzymes acting on the homogalacturonan backbone. The reactions were monitored with recently developed techniques like high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) that allow sensitive monitoring of the reactions. MALDI-TOF MS reveals the degree of esterification and or glycosydation. HPAEC at neutral pH separates not only oligogalacturonides with a different degree of esterifcation but seems to separate some isomers of e.g. monomethyl- and dimethyl-triGalA as well. Breakdown products formed by hydrolysis side reactions were revealed and could even be quantified by HPAEC pH 12 analysis. Using these techniques the conditions for each of the reactions were optimized. Esterification was performed best at concentrations of maximal 0.02 N sulfuric acid in anhydrous methanol at 4°C. Hardly any glycosydation occurs and the level of hydrolysis of the oligoGalA was less than 5%. Methyl-glycosydation and simultaneous esterification was performed best in 0.1 N sulfuric acid in anhydrous methanol at room temperature. HPAEC revealed only a limited hydrolysis (<11%).

Keywords: Methyl-esterification; Methyl-glycosydation; Oligogalacturonides; HPAEC; MALDI-TOF MS

1. Introduction

Pectin is located in the primary cell wall and the middle lamella of practically all the higher plant tissues. Pectin consists of a backbone, in which 'smooth' homogalacturonan regions are interrupted by 'hairy' ramified rhamnogalacturonan regions. Homogalacturonan is a polymer composed of predominantly α-(1 → 4)-linked galacturonic acid (GalA) residues, which can be methyl-esterified at the carboxyl group (McNeil, Darvill, Fry & Albersheim, 1984; Pilnik & Voragen, 1970; Schols & Voragen, 1996; Voragen, Pilnik, Thibault, Axelos & Renard, 1995).

Several enzymes are known, which are capable of degrading homogalacturonans, but their activities are strongly influenced by the amount and distribution of the methyl-ester groups in the homogalacturonan (Benen, Kester & Visser, 1999; Versteeg, 1979; Voragen, Rombouts & Pilnik, 1971). To obtain more detailed information on the exact mechanism and specificity of the various pectic enzymes, it is essential to have well-characterized (methyl esterified) oligoGalA, which can be used within the analytical range of modern instrumental methods. In addition, substrate analogues like fully methyl-esterified oligoGalA, which are also methyl-glycosydated on the reducing end, are very useful in this study.

Both the esterification and the glycosydation reaction have been described extensively (Chanez & Sag, 1959; Jansen & Jang, 1946; Edstrom & Phaff, 1964; McCready & Seegmiller, 1954; Voragen, 1972; Wood, 1963), although usually, the more traditional techniques like titration (Jansen & Jang, 1946; Voragen, 1972), paper-chromatography (Edstrom & Phaff, 1964; McCready & Seegmiller, 1954; Voragen, 1972), and TLC (Koller & Neukom, 1964; Voragen, 1972) were used to monitor the reaction.

In this paper, the esterification and glycosydation of purified oligogalacturonides is re-examined using the latest techniques, e.g. MALDI-TOF MS and HPAEC. Using MALDI-TOF MS it is possible to determine the reaction

Abbreviations: MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPAEC, High-performance anion-exchange chromatography; GalA, Galacturonic acid; DP, Degree of polymerization; endo-PG, endo-polygalacturonase; PGA, Polygalacturonic acid; PAD, Pulsed amperometric detector

* Corresponding author. Tel.: + 31-317-483209; fax: + 31-317-484893.

E-mail address: fons.voragen@chem.fdsci.wau.nl (A.G.J. Voragen).

0144-8617/00/$ - see front matter
PII: S0144-8617(99)00207-6

products at any point in time and distinguish between the degree of esterification of the oligogalacturonides. HPAEC at pH 5 appeared to differentiate between isomers of oligogalacturonides with a low degree of methyl-esterification. HPAEC at pH 12 reveals the extent of hydrolytic breakdown, which will take place during the process of esterification and/or glycosydation.

The techniques, the generated substrates and the results described here are essential for the investigation of the mode of action of pectinolytic enzymes.

2. Experimental

2.1. Materials

PGA was purchased from ICN. Cloned endo-PG 1 (*Aspergillus aculeatus*) was a gift from NOVO Nordisk A/S (Copenhagen, Denmark). Methanolic HCl (3 N) was acquired from Supelco. Molecular sieve (0.4 nm) was purchased from Merck for the preparation of anhydrous MeOH. H^+-Dowex 50W X8 50–100 mesh was acquired from Fluka. H^+-Dowex AG 50W X8 100–200 mesh (Biotechnological grade) is from Bio-rad and used for the preparation of NH_4^+-loaded Dowex (Körner, Limberg, Mikkelsen & Roepstorff, 1998). All other chemicals used were analytical grade.

2.2. Large-scale preparation and purification of saturated oligoGalA

PGA (1% (w/v) in 2 l 10 mM NaOAc pH 5) was degraded with endo-PG 1 (80 U) for 45 min at 40°C to oligoGalA. The enzyme was heat-inactivated (30-min boiling step). The high molecular weight oligoGalA were removed by acid precipitation (6 N HCl) at pH 2 (Spiro, Kates, Koller, O'Neill, Albersheim & Darvill, 1993). The supernatant was recovered and the pH set with NaOH (5 N) to 12 to dissolve aggregates (Mort, Moerschbacher, Pierce & Maness, 1991). The pH was then set to 5 using H^+-Dowex 50W X8. The oligoGalA were separated on a Source 15Q (Pharmacia) column (1.2 l) equilibrated with 50 mM NH_4OAc (pH 5) using a gradient of 50–200 mM in two column volumes (CV). Subsequently, the concentration was increased in 5 CV to 400 mM followed by an 8 CV gradient to 1 M. Fractions of 150 ml were collected and analyzed by HPAEC pH 12 to visualize the presence of oligoGalA. Fractions containing pure oligoGalA were pooled and concentrated to approximately 100 ml with a rotating evaporator (50°C). The oligoGalA were precipitated with an equal volume of $BaCl_2$ (100 mM) solution and 300 ml EtOH (96%) (Robertsen, 1986). The precipitate was washed twice with EtOH (60%) and dissolved in 50 ml Millipore water containing 5 g of H^+ loaded cation-exchanger (Dowex 50W X8), to exchange Ba^{2+} for H^+. Finally, the Dowex was removed by filtration and the filtrate was frozen and freeze-dried. This Dowex treatment was repeated once in order to get a complete removal of Ba^{2+}-ions. The individual freeze-dried oligoGalA (DP 2–8) were analyzed using HPAEC at pH 12 and MALDI-TOF MS to check the purity.

2.3. Esterification and glycosydation of oligoGalA

Methyl esterification and glycosydation was performed essentially as described before (Jansen & Jang, 1946; Voragen, 1972). Purified oligoGalA (DP 2–6; 1% (w/v)) were incubated for 14 days in either anhydrous methanolic 0.02 N HCl at 4°C (esterification) or in anhydrous methanolic 0.1 N HCl at ambient temperature (esterification and glycosydation). Alternatively, 0.02 or 0.1 N anhydrous methanolic H_2SO_4 were used for esterification or esterification and glycosydation, respectively. To monitor the reaction, samples were taken at different time points and neutralized by either solid Ag_2CO_3 (HCl) or $BaCO_3$ (H_2SO_4). The soluble fraction of the samples was analyzed using MALDI-TOF MS and HPAEC at pH 12 and pH 5 with post-column NaOH addition.

2.4. Recovery of glycosydated and/or esterified oligoGalA

After completion of the glycosydation and/or esterification reaction, the oligoGalA were recovered. Since the oligoGalA are not solubilized completely in methanolic HCl or H_2SO_4, the insoluble part of the oligoGalA was separated from the solution by centrifugation and washed twice with isopropanol and dried under a stream of air. The soluble part was neutralized with solid $BaCO_3$ or Ag_2CO_3 and also centrifuged. The supernatant was collected and the solid material washed with anhydrous MeOH. These supernatants were combined and dried under a stream of air at ambient temperature. Both the soluble and insoluble part were dissolved in water and freeze-dried. The freeze-dried material was analyzed again on MALDI-TOF MS and stored at −20°C until used.

2.5. Chromatographic analysis of esterification and or glycosydation reaction

HPAEC at pH 5 was performed as described before (Daas, Arisz, Schols, De Ruiter & Voragen, 1998). Partly methyl-esterified oligoGalA were separated on a CarboPac PA-1 column (Dionex corp) using a linear NaOAc (pH 5) gradient of 0.05–0.7 M for 65 min at a flow rate of 0.5 ml/min, followed by 10 min gradient to 1 M NaOAc. After 5 min of washing with 1 M NaOAc the column was equilibrated in 15 min with 0.05 M NaOAc pH 5. Detection of the compounds was accomplished by the post-column addition of NaOH to the column eluent to raise the pH (>12) before it entered the PAD (Electrochemical detector ED40, Dionex).

Hydrolytic breakdown during the glycosydation and or esterification reactions was determined with HPAEC at pH 12 as described by Daas et al. (1998). Samples were

separated on a CarboPac PA-100 column preceded by a CarboPac PA-100 guard-column. A 40-min linear gradient of 0.3–0.7 M NaOAc in 0.1 M NaOH at a flow rate of 1 ml/min was used, followed by a 5-min gradient to 1 M NaOAc. After 5 min of washing with 1 M NaOAc in 0.1 N NaOH the column was equilibrated in 0.3 M NaOAc in 0.1 N NaOH. Due to the high pH (12.7) all methyl-esters were removed and a separation of oligoGalA was achieved. Pure oligoGalA (DP1-3) were used as standards for external calibration of the system.

In order to isolate individual compounds separated by HPAEC at pH 5 large amounts of partly methyl-esterified trigalA were prepared: trigalA was incubated for 3 h and 2 days with methanolic 0.02 N H_2SO_4. After neutralization using $BaCO_3$, 80 μl sample was injected on a CarboPac PA-1 column using a modified gradient: 10 min isocratic elution (10 mM NaOAc pH 5), followed by a 60 min linear gradient to 0.5 M NaOAc, a 20 min linear gradient to 1 M and 5 min isocratically at 1 M at a flow rate of 0.5 ml/min. Na^+-ions were replaced on-line with H^+-ions using the Carbohydrate Membrane Desalter (CMD™;Dionex corp). The first analysis was performed with the PAD on-line to determine the position of the peaks. During the second analysis the post-column NaOH delivery system and the PAD were disconnected to avoid saponification of the compounds and fractions of 250 μl were collected. The fractions were analyzed using MALDI-TOF MS and HPAEC at pH 12 (off-line detection) for identification and confirmation of the localization of the compounds, respectively. Subsequently, the fractions were pooled, desalted a second time as described before (Körner et al., 1998), freeze-dried, dissolved in a small volume (20 μl) and analyzed again by MALDI-TOF MS.

2.6. MALDI-TOF MS

2,4,6-Trihydroxy acetophenone (THAP, Acros chimica) and THAP/nitrocellulose were used as matrix. THAP was prepared in 50% acetonitrile (10 mg/ml). The matrix was applied to the sample plate (1 μl). Subsequently, the sample (1 μl) was added and mixed with the matrix. THAP/nitrocellulose was prepared as described before (Körner et al., 1998). As soon as the matrix was applied (0.2 μl) it spread out and formed a thin-layer. On top of this layer the sample was applied (0.2 or 0.4 μl). After application the gold plate was dried under a stream of air. When necessary, samples were desalted by addition of H^+-Dowex AG 50W X8. Alternatively, samples were treated with NH_4^+-loaded Dowex AG 50W X8.

The samples were analyzed with the Voyager DE™-RP MALDI-TOF MS (Perseptive Biosystems) equipped with a nitrogen laser of 337 nm and a 3 ns pulse. The mass spectrometer was selected for positive ions. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12,000 V. Hereafter, the ions were detected using the reflector mode. The lowest laser power required to obtain good spectra was used and at least 50 spectra were collected. The MALDI-TOF MS was externally calibrated using a mixture of oligoGalA.

3. Results

3.1. Methyl-esterification

Methyl-esterification of individual oligoGalA (DP 2–6) was performed with acidic methanol (0.02 N) using either hydrochloric acid or sulfuric acid at 4°C. Since no differences in the process of esterification were observed while using different oligoGalA, the methyl-esterification of triGalA will be described. MALDI-TOF MS analysis in positive mode (Fig. 1) revealed the different methyl-esterified triGalA in their monosodiated form, $m/z = 569$, 583, 597 and 611, which corresponds to triGalA with 0, 1, 2 or 3 methyl-esters, respectively. The constant mass difference of 14 Da between major peaks corresponds to a methyl-ester. Methyl-esterification using sulfuric acid (Fig. 1) is almost identical to methyl-esterifcation using hydrochloric acid (not shown). In the first few days, the reactions proceed relatively fast, but only after 10–14 days the methyl-esterification is completed.

During methyl-esterification two side reactions may occur depending on the conditions used. HPAEC pH 12 analysis showed that less then 5% (w/w) of triGalA is hydrolyzed to diGalA and GalA if methanol 0.02 N sulfuric acid is used, while 10% (w/w) is hydrolyzed using methanol 0.02 N hydrochloric acid. After completion of the methyl-esterification using 0.02 N acid, no methyl-glycosydation (methyl-group at C1 at the reducing end) could be detected with MALDI-TOF MS. This was confirmed by saponification of the fully methyl-esterified triGalA, which resulted only in the original triGalA, $m/z = 569$.

Furthermore, the methyl-esterification of oligoGalA was followed in time by HPAEC at pH 5 with post column pulsed amperometric detection. Although the reaction proceeded faster if hydrochloric acid was used, similar elution patterns were gathered with sulfuric acid in the reaction mixture but at a later point in time. Typical elution patterns for triGalA are shown in Fig. 2. The reaction starts with the non-methyl-esterified triGalA with a retention time of 40 min (A). As the degree of methyl-esterification increases the overall charge of the molecule decreases and the molecule will bind less strongly to the column. As the reaction proceeds peaks appear with a decreasing retention time: 29 min (B), 20 min (C), 8 min (D), 7 min (E), 5 min (F), and 3 min (G). After 11 days all the triGalA has been fully methyl-esterified (Fig. 1) and elutes in peak G. While only four different peaks could be expected based upon the overall degree of methyl-esterification, seven peaks appeared. This suggests that isomers of partly methyl-esterified triGalA were separated using this CarboPac PA-1 column. Peak C has already been identified before as a

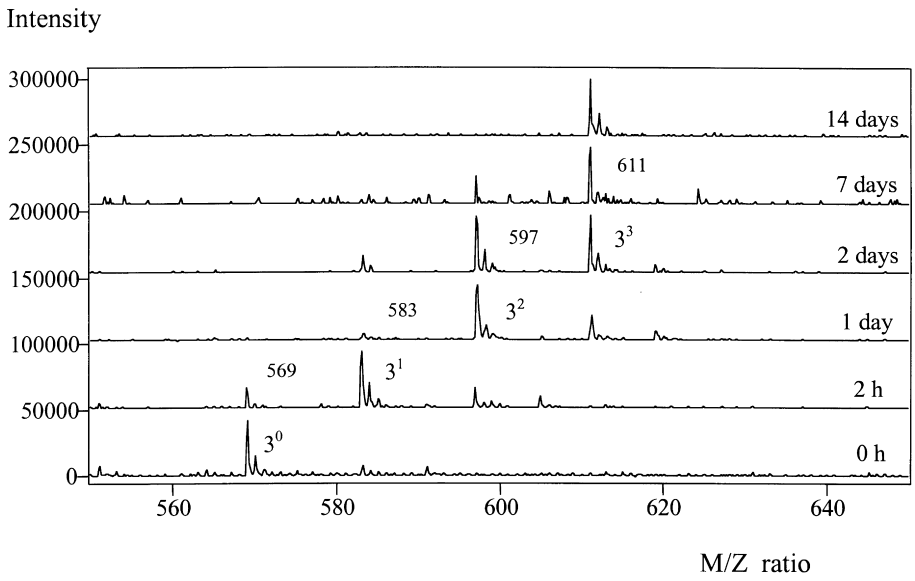

Fig. 1. MALDI-TOF mass spectra showing the methyl-esterification process of triGalA in time (0, 2 h, 1 day, 2 days, 7 days, and 14 days of incubation in 0.02 N sulfuric acid in anhydrous methanol at 4°C). The signal intensity is plotted against the mass to charge ratio (*m/z*). The masses and the degree of esterification are indicated (Code 3^3: a triGalA having three methyl groups). THAP was used as a matrix.

trimer with 1 methyl-group (Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999). Since this trimer is the main component after 2 h of incubation (Fig. 1) and peaks B and C are the main peaks in the chromatogram, it is likely that component B and C are isomers of the triGalA with one methyl-group.

3.2. Off-line HPAEC–MALDI-TOF MS

In order to identify the different peaks in the elution profiles (Fig. 2) partly methyl-esterified triGalA (3 and 48 h of incubation) were separated by HPAEC at pH 5. Fractionation of these two time points covers all the peaks at relatively high concentration. Since a more extended gradient was used the retention times of the peaks shifted towards a later point of elution and peaks E and F seem to have merged (Fig. 3a and c). In order to confirm the presence of triGalA in the most predominant peaks, all fractions collected from the HPAEC pH 5 separation were also analyzed individually by HPAEC pH 12 (Fig. 3b and d). This not only confirms the elution behavior of the triGalA peaks, but allows quantification of the reaction products as well. A striking observation is that both elution patterns at pH 5 contain a peak with a retention time of 65 min, which seems to be a ghost peak because no compound could be detected by HPAEC pH 12. In addition, peak G contains

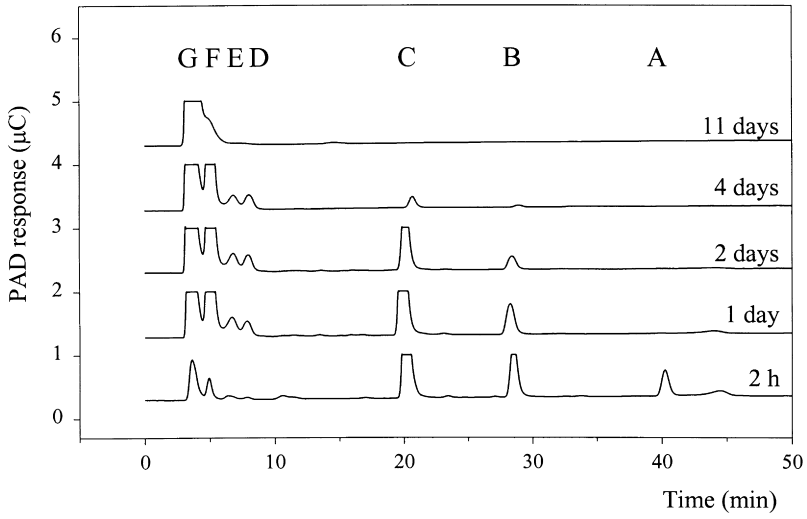

Fig. 2. HPAEC (pH 5) elution patterns showing the methyl-esterification process of triGalA in time (2 h, 1 day, 2 days, 4 days, and 11 days of incubation in 0.02 N sulfuric acid in anhydrous methanol at 4°C).

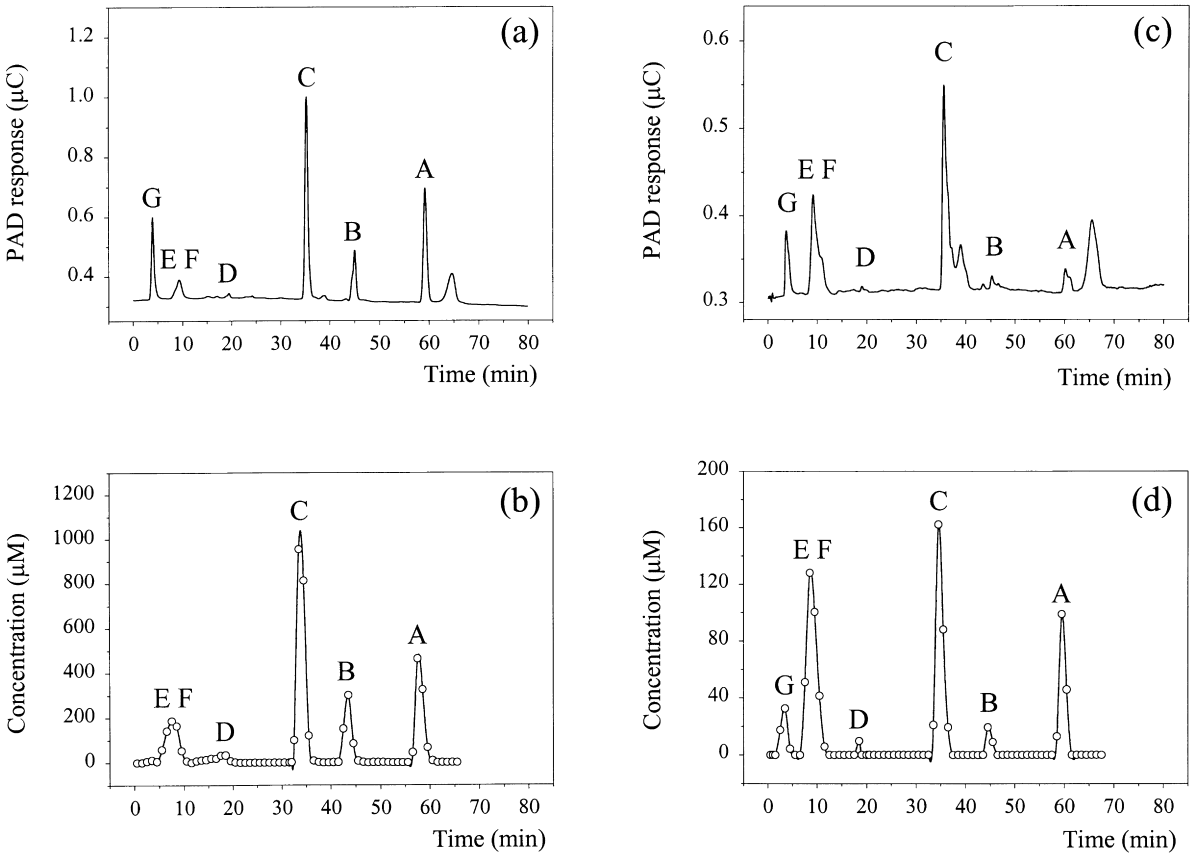

Fig. 3. HPAEC (pH 5) elution profiles showing methyl-esterified triGalA after 3 h (a) and 48 h (c) of incubation (4°C) in 0.02 N sulfuric acid in anhydrous methanol. The PAD response is plotted against the retention time. In addition, the triGalA content of the fractions determined with HPAEC (pH 12) is plotted against time: 3 h (b) and 48 h (d) of incubation.

both salt and triGalA, since triGalA in peak G was only present after 48 h of incubation (Fig. 3c and d). All the fractions were subjected directly to MALDI-TOF MS analysis using THAP/nitrocellulose as a matrix. Since not all peaks could be identified clearly, fractions were pooled, desalted, freeze-dried and dissolved in a small volume and analyzed again. As expected, peak A was identified as non-methyl-esterified triGalA. The identity of peak B was uncertain, since it contained triGalA with and without one methyl-ester. Peaks C and D contained triGalA with one methyl-ester, but in peak D the triGalA with two methyl esters was also present. Peaks EF and G were identified as triGalA with two and three methyl-esters, respectively. Summarizing, all peaks could be identified unambiguously except peaks B and D. The presence of triGalA with different amounts of methyl-esters in peaks B and D is very peculiar since these compounds elute at different retention times. Spontaneous de-esterification after separation could be an explanation of this observation. For this reason the pooled peak fractions were re-analyzed by HPAEC at pH 5. Peaks A, C, EF, and G eluted at the same position as before. However, re-injection of peak B revealed two peaks, peak B and peak A, the non-methyl-esterified triGalA. Re-analysis of peak D also gave peak C, stable monomethyl-triGalA. So it seems that peaks B and D contain unstable isomers of monometyl- and dimethyl-triGalA, respectively, which are converted to stable triGalA and monomethyl-triGalA, respectively, during storage and/or handling of the fractions. So the stability amongst partly methyl-esterified oligoGalA differs greatly. For this reason the stability of fully methyl-esterified oligoGalA was investigated as well.

3.3. Stability of fully methyl-esterified oligogalacturonides

The influence of pH, buffer, time and temperature on the stability of fully methyl-esterified oligoGalA (DP 3–6) was investigated using MALDI-TOF MS. Fully methyl-esterified oligoGalA (10 mM) was dissolved in HEPES, TRIS, acetate and phosphate buffers covering a pH range of 3–8. Only when phosphate buffer was used de-esterification was found already within a period of 4 h at ambient temperature for hexaGalA (Table 1). Somehow phosphate buffer induced de-esterification of fully methyl-esterified hexaGalA. This de-esterification was more pronounced after heating (5 min, 100°C) and seemed to be related to the size of the oligoGalA (Table 1). The other buffers tested did not demonstrate this de-esterification after this treatment. The mechanism involved is not known. De-esterification also

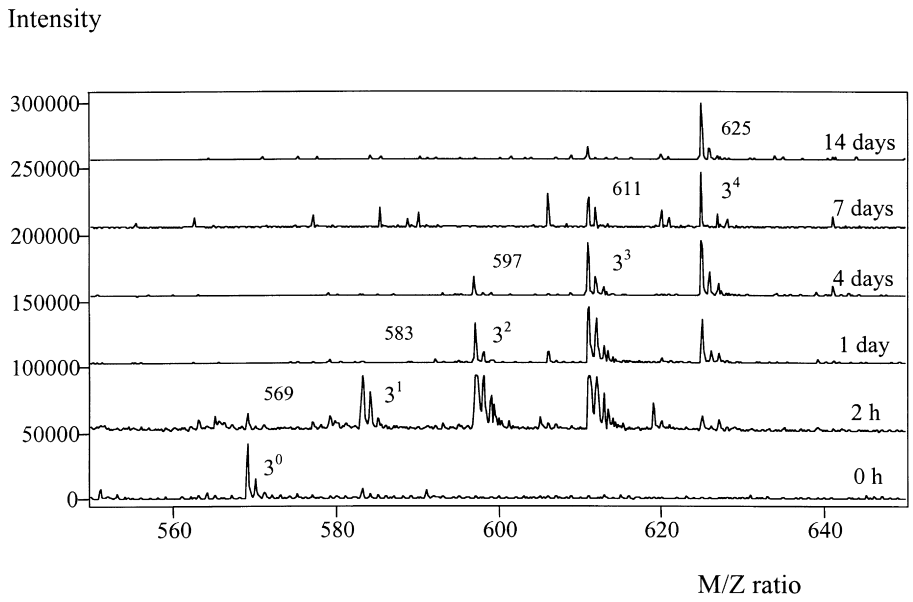

Fig. 4. MALDI-TOF mass spectra showing the methyl-esterification and -glycosydation process of triGalA in time (0, 2 h, 1 day, 4 days, 7 days, and 14 days of incubation in 0.1 N sulfuric acid at ambient temperature). The signal intensity is plotted against the mass to charge ratio (*m/z*). The matrix and coding used are as indicated in Fig. 1.

proceeds above pH 8, while under acid conditions (24 h, 2 N acid, ambient temperature) fully methyl-esterified oligoGalA appeared to be very stable. In addition to de-esterified products, unsaturated oligogalacturonides, indicative for β-elimination, have been found only after heat treatment (5 min, 100°C) of hexaGalA in phosphate buffer.

To investigate long-term storage freeze-dried samples are stored at −20°C. MALDI-TOF MS analysis showed that only minor de-esterification had taken place during five months of storage.

3.4. Methyl-glycosydation and -esterification

Methyl-esterification and methyl-glycosydation of oligoGalA (DP 2–6) will occur simultaneously if the acid concentration in the methanol is raised (Jansen & Jang, 1946). Again sulfuric and hydrochloric acid were used, but now a 0.1 N solution was used and incubation took place at ambient temperature. The methyl-glycosydation resulted in an additional methyl-group as can be seen very clearly with MALDI-TOF MS for triGalA (Fig. 4). Already after one day the clear peak of fully methyl-esterified and -glycosydated triGalA, $m/z = 625$, can be seen. HPAEC pH 5 analysis revealed that the methyl-esterification proceeded much faster as before (Figs. 1 and 2) and was already completed after 4 days of incubation (not shown), while full methyl-esterification and -glycosydation of triGalA is reached only after 14 days of incubation. So the esterification is the initial reaction and the methyl-glycosydation proceeds at a much lower rate. Saponification of the fully methyl-esterified and -glycosydated triGalA results in the complete removal of all methyl-esters, but the methyl-glycoside will remain unaffected. MALDI-TOF MS analysis indeed demonstrated the presence of one methyl group, while HPAEC (pH 5) analysis showed the presence of a peak eluting around the position of non-esterified triGalA. Since the separation is based on charge it is expected that both the methyl-glycosydated triGalA and the non-esterified triGalA elute at the same position. So HPAEC pH 5 confirms the absence of methyl-esters therefore the methyl-group found by MALDI-TOF MS is glycosydated at the reducing end.

During the methyl-esterification and methyl-glycosydation the hydrolysis of the triGalA is an important side reaction (HPAEC pH 12). Using sulfuric acid resulted in a hydrolysis of 11% (w/w) of the triGalA, while usage of

Table 1
The influence of phosphate buffer and heating on methyl-esterified oligoGalA. Samples were analyzed by MALDI-TOF MS in positive mode after 4 h of incubation at ambient temperature in water and in phosphate buffer with and without heating

Oligomer		Ratio between de-esterified peaks (%)[a]		
		Water	70 mM phosphate pH 7.5	70 mM phosphate pH 7.5:5 min 100°C
hexaGalA	6^6	83	51	0
	6^5	17	46	65
	6^4	0	3	35
	6^3	0	0	0
pentaGalA	5^5	89	89	57
	5^4	11	11	41
	5^3	0	0	2
	5^2	0	0	0

[a] The peak heights were used for the determination of the percentage of de-esterification.

hydrochloric acid resulted in more then 30% (w/w) breakdown.

4. Discussion

In the past, methyl-esterification of oligogalacturonides was monitored with traditional techniques, like thin-layer and paper chromatography and titration. Titration determines the amount of free carboxylic acids, but nothing can be concluded about the reaction products. Although thin-layer and paper chromatography can reveal the reaction products of esterification, it is elaborate and will not reveal small amounts of impurities. Using MALDI-TOF MS the esterification reaction can be monitored very quickly and not only the (partly) methyl-esterified product will be seen, but side products as well. So at any moment during the reaction the status of the reaction can be determined rather quickly. Analysis of the reaction using HPAEC at pH 5 not only differentiates between oligoGalA with a different degree of methyl-esterification, as MALDI-TOF MS does, but seems to differentiate between some isomers of monomethyl- and dimethyl-triGalA, also. Such a separation of isomers has not been demonstrated before. Apparently, the position of the methyl-ester influences the charge distribution in such a way that the isomers are bound differently.

Apart from the esterification reaction, side reactions can be monitored as well: hydrolysis can be quantified using HPAEC at pH 12 and methyl-glycosydation can be revealed with MALDI-TOF MS. Up till now hydrochloric acid was used for esterification and glycosydation of oligoGalA. But analysis at pH 12 demonstrated that the use of sulfuric acid in the esterification and glycosydation reaction resulted in less hydrolysis and should be preferred above hydrochloric acid. Esterification was performed best with 0.02 N sulfuric acid in anhydrous methanol at 4°C for two weeks. Glycosydation and esterification was performed optimal in 0.1 N sulfuric acid in anhydrous methanol at ambient temperature using an incubation time of two weeks.

Furthermore, it is clear that the process of esterification is not at random. The ratio between monomethyl-triGalA peaks B and C clearly demonstrates a preference for one of the GalA residues. The position of this methyl-group has not been elucidated yet, but it has been suggested before that there is a preference for the residue at the reducing end at the start of methyl-esterification (Tjan, Voragen & Pilnik, 1974).

Stability of methyl-esterified oligoGalA is dependent on the degree of methyl-esterification. Fully methyl-esterified triGalA appeared to be very stable under various conditions. But some isomers of monomethyl- and dimethyl-triGalA were unstable and were converted to a stable triGalA and monomethyl-triGalA, respectively. It is most likely that in both cases the same methyl-esterified GalA residue is involved. This residue forms an unstable ester, but in fully methyl-esterified triGalA neighboring methyl-esters probably stabilizes it. If these unstable partly methyl-esterified oligoGalA are formed during enzymatic degradation of pectin, they will probably de-esterify further and will not show up in the HPAEC pH 5 elution patterns (Daas et al., 1998, 1999) although this may depend significantly on the sample preparation. Interpretation of pectin digests in studies aiming in elucidating the mode of action of pectic enzymes or the chemical fine structure of pectins, which contain partially methyl-esterified oligoGalA, should take instability of partially methyl-esterified oligoGalA into account. Furthermore, the use of phosphate buffer should be avoided at all times.

MALDI-TOF MS and HPAEC have proved to be very good tools to monitor the methyl-esterification reaction and the stability of (partly) methyl-esterified oligoGalA. These techniques will have the same value in the study of the enzymatic conversions of the prepared (partly) methyl-esterified oligoGalA, which will reveal the mode of action of these pectic enzymes.

Acknowledgements

The EU Biotechnology Program financially supported this work under contract number ERBBIO4CT960685.

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