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Amidation of methyl-esterified oligogalacturonides: examination of the reaction products using MALDI-TOF MS

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Abstract

Amidation of methyl-esterified oligogalacturonides (oligoGalA) was studied to produce partly and fully amidated oligoGalA to be used as substrates and/or inhibitors for the characterization of pectolytic enzymes acting on the homogalacturonan backbone. The reactions were performed with varying concentrations of ammonia or methylamine and monitored in time using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) that allows sensitive monitoring of the reactions. MALDI-TOF MS reveals the degree of amidation (DAm) and extent of hydrolysis of methyl-esters. Using this technique the conditions for each of the reactions was optimized. Amidation was performed best under anhydrous conditions at a concentration of 4 M ammonia or methylamine at ambient temperature. Amidation using methylamine reached almost completeness (DAm 95) without hardly any hydrolysis of methyl-esters while amidation with ammonia reached a DAm of 70 on average. After an initial fast amidation, precipitation of the partly amidated oligoGalA reduced the reaction rate enormously. The use of ammonia in aqueous solutions instead off anhydrous ammonia resulted in 6–10% lower DAm values due to the hydrolysis of methyl-esters. Therefore, anhydrous conditions are preferred during amidation. Furthermore, methylamine is a better reagent for amidation of oligoGalA and pectins then ammonia, but also results in totally different products with other properties.

Keywords: Amidation; Oligogalacturonides; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

1. Introduction

Pectin is one of the most complex polysaccharides known and is found in the middle lamella of the primary cell wall of practically all higher plant tissue. Pectin consists of a homogalacturonan backbone of predominantly α-(1 → 4)-linked galacturonic acid (GalA) residues, interrupted by ramified rhamnogalacturonan regions (McNeil, Darvill, Fry & Albersheim, 1984; Schols & Voragen, 1996). The GalA residues can be methyl-esterified at the carboxyl group, which is essential for the applications of pectins (Pilnik & Voragen, 1970; Voragen, Pilnik, Thibault, Axelos & Renard, 1995).

Low methoxyl pectins (low content of methyl-esters) are widely used in food industry because they can form gels in the presence of calcium, like in low-sugar jams and jellies. However, amidated pectins often replace low methoxyl pectins, since amidated pectins need less calcium, are less sensitive to precipitation by high amounts of calcium, and their gels are claimed to be thermo-reversible (Gross, 1979). Amidated pectins have been studied quite extensive for their synthesis, their rheological properties of the gels, and their calcium binding properties (Racapé, Thibault, Reitsma & Pilnik, 1989), but hardly for their effect on enzyme specificity.

Several enzymes are known, which are capable of degrading homogalacturonans, although their activities are strongly influenced by the substituent on the carboxyl-group and the distribution of this substituent over the homogalacturonan backbone (Benen, Kester & Visser, 1999; Kester, Magaud, Roy, Anker, Doutheau, Shevchik et al., 1999; Pařenicová, Benen, Kester & Visser, 2000; Pilnik, Voragen & Rombouts, 1974; Versteeg, 1979; Voragen, Rombouts & Pilnik, 1971). Recently, the production and characterization of well characterized (partly) methyl-esterified oligogalacturonides (oligoGalA) was already described (Van Alebeek, Zabotina, Beldman, Schols & Voragen, 2000a,b). In order to gain more detailed information on the influence of amide groups on the specificity of the various pectic enzymes, it is

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Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; GalA, galacturonic acid; DP, degree of polymerization; PGA, polygalacturonic acid; DM, degree of methyl-esterification; DAm, degree of amidation

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essential to have well-characterized amidated oligoGalA as well.

In this paper, the amidation of purified methyl-esterified oligoGalA with ammonia or methylamine is examined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The generated substrates will be used for the investigation of the mode of action of pectolytic enzymes.

2. Experimental

2.1. Materials

PGA was purchased from ICN. Ammonia (25% (v/v)) was purchased from Merck. Anhydrous ammonia (10 M) was prepared by dissolving gaseous ammonia in methanol. Methylamine–HCl was acquired from Sigma and methylamine (33%) in ethanol from Fluka. 2,4,6-Trihydroxy acetophenone (THAP) was bought from Acros chimica. H^+-Dowex 50W X8 50–100 mesh was acquired from Fluka. H^+-Dowex AG 50W X8 100–200 mesh (Biotechnological grade) was 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. Preparation of saturated methyl-esterified oligoGalA

Saturated oligoGalA were isolated from an endo-PG1 digest of PGA subjected to anion-exchange chromatography as described before (Van Alebeek et al., 2000a). The purified oligoGalA were methyl-esterified completely using acid methanol and stored at −20°C (Van Alebeek et al., 2000a). Degradation of the oligoGalA during methyl-esterification was kept to a minimum and did not exceed 5% (w/w).

2.3. Amidation of methyl-esterified oligoGalA

Amidation of methyl-esterified triGalA (1% w/v) was performed with either 25% (v/v) ammonia or anhydrous ammonia in methanol. Ammonia was diluted to appropriate concentrations with anhydrous methanol to minimize side-reactions like saponification and β-elimination. During incubation precipitation of oligoGalA occurred. Therefore, samples taken during the incubation were centrifuged upon which the supernatant was neutralized (H^+-Dowex). Hereafter, both the supernatant and the pellet fraction were dried under a flow of air and dissolved in an appropriate volume of de-ionized water. Both the concentration of the ammonia and the time of incubation were varied for optimization of the reaction. Finally, methyl-esterified oligoGalA were amidated under the optimized conditions.

Alternatively, oligoGalA were amidated using methylamine. Either solid methylamine HCl dissolved in methanol, or a 33% (v/v) solution of methylamine in ethanol was used for amidation. Samples taken were dried under a stream of air and dissolved in de-ionized water. Desalting of the samples with H^+-Dowex was necessary. Concentration and time of incubation were varied using methyl-esterified triGalA. After optimization methyl-esterified oligoGalA were amidated under the optimized conditions.

All samples were subjected to MALDI-TOF MS.

2.4. Recovery of amidated oligoGalA

After completion of the amidation reaction with ammonia, the reaction mixture was centrifuged. The supernatant was aspirated and neutralized with H^+-Dowex (Fluka 50W X8 50–100 mesh). The pellet fraction was washed twice with isopropanol. Both the pellet and the supernatant were dried under a continuous flow of air. Subsequently, the pellet and supernatant were dissolved in water, freeze-dried and stored at −20°C.

Complete recovery of amidated oligoGalA using methylamine was accomplished by drying the mixture under a continuous flow of air. Subsequently, the amidated oligoGalA were solubilized in de-ionized water and desalted twice using H^+-Dowex and NH_4^+-Dowex, respectively. Hereafter, the oligoGalA were freeze-dried and stored at −20°C.

2.5. MALDI-TOF MS

THAP/nitrocellulose was used as matrix and prepared as described before (Körner, Limberg, Mikkelsen & Roepstorff, 1998). As soon as the matrix was applied (0.2 μl) to the sample plate it spread out and formed a thin-layer. On top of this layer the sample was applied (0.2 or 0.4 μl) and dried under a stream of air. When necessary, samples were desalted by addition of H^+-Dowex AG 50W X8 to the sample before drying. Alternatively, samples were treated with NH_4^+-loaded Dowex AG 50W X8.

The samples were analyzed with a 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 or negative 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.

2.6. Calculation of the degree of amidation

The degree of amidation (DAm) of an oligoGalA was determined from the mass spectra acquired in the positive mode revealing the number and relative amount of amide groups attached to e.g. triGalA. It was assumed that the amount of amide groups attached to the triGalA did not influence the response in the MALDI-TOF MS analysis using the positive mode. The ratio between the differently amidated triGalA was determined from the peak heights and

Table 1
Isotopic distribution of amidated oligoGalA calculated with IsoPro 3.0, an isotopic distribution simulator. In addition to the main peak (*m/z*), isotopic peaks are indicated as *m/z* +1, +2, +3, +4 and +5

OligoGalA	Isotope distribution (%)					
	m/z main peak	*m/z* + 1	*m/z* + 2	*m/z* + 3	*m/z* + 4	*m/z* + 5
DP3-3NH_2	100	22.1	5.5	0.9	0.1	
DP4-4NH_2	100	29.5	8.4	1.5	0.2	
DP5-5NH_2	100	36.9	11.9	2.7	0.5	
DP6-6NH_2	100	44.3	15.7	4.1	1.0	0.2
DP7-7NH_2	100	51.5	20.3	5.9	1.4	0.4
DP8-8NH_2	100	59.0	25.2	8.0	2.1	0.4

used to calculate the percentage of GalA residues occupied by amide groups. Amidation using methylamine resulted in an increase of mass of 13 *m/z* per methylamide group, whereas amidation with ammonia gave rise to mass increase of 1 *m/z*. As a consequence the mass spectra of amidation using ammonia were disturbed by isotopic peaks. Therefore, spectra needed to be corrected for these isotopic peaks. The isotopic distribution was simulated using IsoPro 3.0, an isotopic distribution simulator based on the algorithm of Yergey (Yergey, 1983). In Table 1 an overview is given of the results of the isotopic distribution simulation for oligoGalA amidated with ammonia. These data were used to correct the peak heights for isotopes starting from the smallest *m/z* as the main (100%) peak. After this first correction the next peak was taken as the main peak and correction was performed again and so on. In this manner all peaks were corrected and a real distribution could be determined. As a consequence the DAm could be calculated as mentioned above.

3. Results

3.1. Amidation with ammonia

Amides are commonly synthesized by reaction of an anhydride, an acid halide or an ester with ammonia. Since methyl-esterified oligoGalA can be prepared quite easily by acid-catalyzed esterification (Van Alebeek et al., 2000a) these were used as starting material. Amidation was performed with either anhydrous or water-containing ammonia at concentrations ranging from 0 up to 4 M at ambient temperature and 4°C, respectively. Ammonia was diluted with water-free methanol to reduce the amount of water and minimize hydrolysis of the esters. Dissolving methyl-esterified triGalA into the different ammonia solutions started the amidation reaction. After five days of incubation the reactions were stopped. Since a precipitate developed during incubation, both the insoluble pellet and supernatant fraction were neutralized with H^+-Dowex and subjected to MALDI-TOF MS analysis. The amidation with anhydrous or water-containing ammonia proceeded in a similar manner. MALDI-TOF MS analysis in the positive mode revealed the different amidated triGalA in their monosodiated form, *m/z* = 566, 581, 596 and 611, which corresponds to methyl-esterified triGalA with 3, 2, 1 or 0 amide groups replacing the methyl-ester, respectively. A gradual increase in amidation was seen with increasing concentration of ammonia, which was similar in both the pellet and supernatant fraction (Fig. 1A). Amidation was highest in the presence of 4 M ammonia resulting in partly and fully amidated triGalA, but the reaction still seemed to proceed. Hydrolysis of methyl-esters results in the formation of carboxyl groups, which can be monitored specifically using MALDI-TOF MS in the negative mode. Hardly any hydrolysis of methyl-esters was observed under anhydrous conditions, although this was the case when aqueous ammonia solutions were used. Here hydrolysis was found in the pellet fractions and increased with increasing ammonia concentration (not shown). Saponification of the samples removed all methyl-esters (14 Da each) that were still present, whereas the amide groups remained attached. Therefore, saponified triGalA with an increasing number of amide groups differ only 1 Da in *m/z* each. MALDI-TOF MS analysis in the positive mode clearly demonstrates the presence of non-, mono-, di- and tri-amide triGalA, which corresponds to a *m/z* of 569, 568, 567 and 566 Da, respectively (Fig. 1B). The distribution of these peaks shifts towards a higher DAm at higher ammonia concentrations. All other peaks, which were present before saponification (Fig. 1A), have disappeared from the spectrum. MS analysis in the negative mode confirmed these results (not shown). Since the isotopic distribution of each of the amidated peaks influences the interpretation of the spectrum, peak heights were corrected for isotopes to get a representative picture. The isotopic distribution simulation program, IsoPro 3.0, was used to simulate the distribution of isotope peaks and the ratio calculated was used to correct peak heights. Table 2 shows the peak height ratio before and after correction. It is clear that the peak height ratio shifts towards a higher DAm after correction, resulting in a higher DAm. The DAm values for the different ammonia concentrations are plotted in Fig. 2A. The DAm increases rapidly at lower ammonia concentrations and seems to level of at higher concentrations. However, still about 40% of the triGalA is not

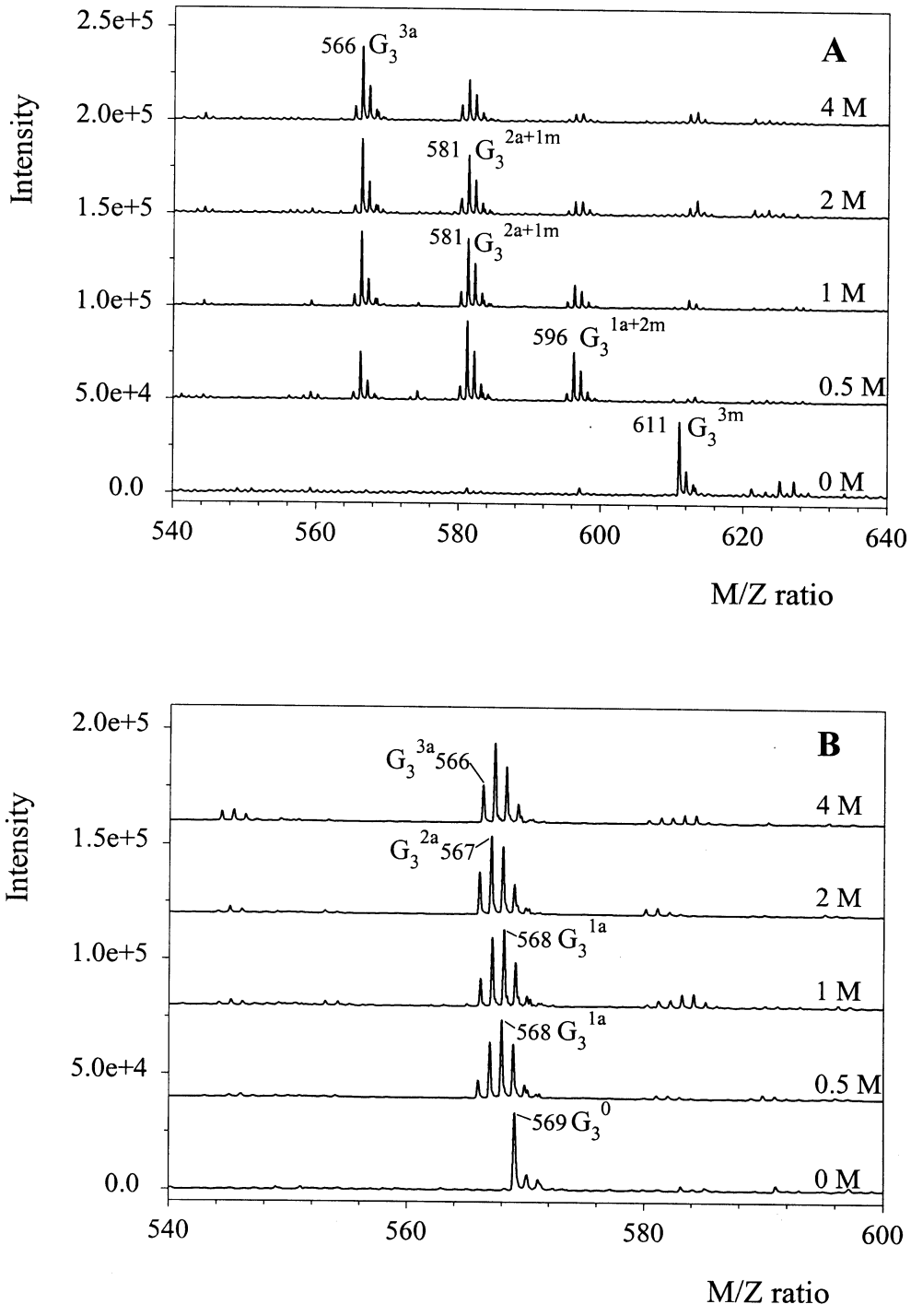

Fig. 1. MALDI-TOF mass spectra (positive mode) showing the amidation process of methyl-esterified triGalA at different ammonia concentrations (anhydrous) after five days of incubation at ambient temperature in the pellet fraction before (A) and after saponification (B). The signal intensity is plotted against the mass to charge ratio (*m*/*z*). The masses and the degree of esterification and amidation are indicated (Code G_3^{2a+1m}: a triGalA having 2 amide groups and 1 methyl-ester).

amidated yet. So it is clear that after five days of incubation amidation was not yet complete.

Therefore, amidation of tri-methyl triGalA was monitored in time using MALDI-TOF MS with either 2 or 4 M ammonia. Both anhydrous and water-containing ammonia were used at ambient temperature and 4°C, respectively. Analysis of the pellet fractions in the positive mode demonstrated a gradual increase in amidation using 2 M water-containing ammonia, whereas a very fast amidation was seen in the presence of 4 M water-containing ammonia.

Table 2
Peak height ratio of amidated triGalA (pellet fractions) incubated at different concentrations of anhydrous ammonia (five days, RT) before and after correction for isotopic distribution. The DAm was calculated as described in Section 2

TriGalA pellet fractions		Peak height ratio (%)				DAm (%)
Sample	Isotope correction	G_3^{3a} (566 *m/z*)	G_3^{2a} (567 *m/z*)	G_3^{1a} (568 *m/z*)	G_3^{0a} (569 *m/z*)	
[NH$_3$]						
0 M	−	0	0	0	100	0
0.5 M	−	8	28	38	26	39
1 M	−	13	31	36	21	46
2 M	−	19	37	30	14	54
4 M	−	20	41	29	10	57
0 M	+	0	0	0	100	0
0.5 M	+	10	31	39	20	44
1 M	+	16	34	35	15	50
2 M	+	24	40	27	9	60
4 M	+	25	45	25	5	63

Within 2.5 h most of the fully amidated triGalA has already been formed (Fig. 3A). However, hydrolysis was also fast as was demonstrated by MALDI-TOF MS analysis in the negative mode (not shown). Amidation using anhydrous ammonia resulted in a much slower amidation (Fig. 3B), but also in less hydrolysis (not shown). The mass spectra of the supernatant fractions were similar to that of the pellet fractions, except for the presence of some extra peaks with a *m/z* of 565 and 580 for which no explanation can be given. For the determination of the DAm all samples were saponified to remove methyl-esters and analyzed by MALDI-TOF MS in the positive mode. As a result of the saponification the peak height ratio of the amidated triGalA changed for all samples, indicating that methyl-esters were still present (not shown). The peak height ratio was corrected for the isotope distribution and subsequently the DAm was calculated and plotted against the time for 2 and 4 M anhydrous ammonia series (Fig. 2B). The DAm values for the 2 M ammonia time course was slightly lower compared to the 4 M ammonia time course. For both time courses the amidation proceeds in different stages. In the first stage the reaction is very fast. Within 15 h this stage is complete during which the amidated triGalA has precipitated. Hereafter, the amidation reaction proceeds very slowly. Probably, the precipitated triGalA is less accessible, which diminishes the reaction rate.

The same conditions were applied for amidation of larger oligoGalA (DP4–8). OligoGalA were incubated in 2 and 4 M water-containing ammonia. Precipitation occurred in all samples and increased with increasing concentration of the ammonia used and the size of the oligoGalA that were amidated, leaving hardly any amidated oligoGalA in solution. The use of 2 M ammonia resulted in partial amidation of oligoGalA. Amidation decreased with increasing size of the oligoGalA leaving most of the methyl-esters untouched. Hydrolysis was only a minor reaction. Using 4 M ammonia demonstrated a much better amidation but also hydrolysis was much higher. Upon saponification the 2 and 4 M series looked very similar and as was already shown for the triGalA. Amidation of oligoGalA (DP4–8) using 4 M anhydrous ammonia resulted in 4–10% higher DAm values compared to 4 M water-containing ammonia. Apparently, the rate of hydrolysis has a bigger effect in the amidation reaction of larger oligoGalA (DP4–8), since the rates of amidation should be equal at the same ammonia concentration. The hydrolysis rate will decrease considerably if anhydrous ammonia is used. Thus a higher DAm is reached, but never a full amidation. Some methyl-esters are still hydrolyzed, while others remain untouched since they are imbedded in the precipitate.

Although anhydrous ammonia would give the best results, for practical reasons 4 M water-containing ammonia was used in the amidation of oligoGalA on a larger scale. After 26 days of incubation at 4°C the reactions were stopped. Samples were taken and saponified to determine the DAm as described above (Table 3). In all cases the amidation was incomplete and the level of fully amidated oligoGalA decreased with increasing DP, except for hepta-GalA. The DAm varied between 64% (DP4) and 51% (DP8). Since the DM of the starting material is not exactly 100% (Table 3), the calculated conversion is 2–4% higher. As a result 34–45% of the methyl-esters is not amidated, but either hydrolyzed or still present.

3.2. Amidation with methylamine

Amidation of oligoGalA using methylamine results in the formation of methyl-amide at the C6 positions. This group resembles the methyl-ester to a high extent with respect to the structure and differs only 1 Da from the methyl-ester. Methylamine is available as a solid hydrochloride salt or as a liquid. The methylamine hydrochloric salt does not give any amidation in a solution of water or methanol. Only after raising the pH some amidation occurred. Therefore, liquid

methylamine in ethanol was used. Upon amidation of trimethyl-triGalA with three methylamine molecules the mass will decrease with, respectively, 1, 2 or 3 Da, while hydrolysis of the methyl-esters will give rise to a decrease of 14, 28 or 42 Da, respectively. Amidation of trimethyl-triGalA at different concentrations of methylamine was performed at 4°C and ambient temperature for 48 h. In contrast to amidation using ammonia, amidation with methylamine did not result in precipitation of the (partly) amidated triGalA. Amidation proceeded very efficiently as was shown with MALDI-TOF MS analysis in the positive mode (Fig. 4A). Already with 0.5 M methylamine the amidation of triGalA seemed to be rather complete, *m/z* 608, independent from the incubation temperature. MS analysis in the negative mode revealed hardly any hydrolysis of the methyl-esters during the reaction. Saponification of the samples resulted in complete removal of the methyl-esters, which is necessary for a good interpretation of the amidation reaction. MS analysis revealed a change in the peak height ratio towards dimethyl-amidated triGalA, indicating that the amidation was not complete yet (Fig. 4B). Again similar spectra are seen for incubations at 4°C and ambient temperature. The peak height ratio of fully and partly amidated triGalA were determined and the DAm was calculated (Table 4). The peak height ratio did not differ much upon change of the methylamine concentration. Only a slight increase in the fraction of fully methyl-amidated triGalA with increasing concentration of methylamine was seen. This is reflected in the DAm, which slightly increases to 94% at 4 M methylamine.

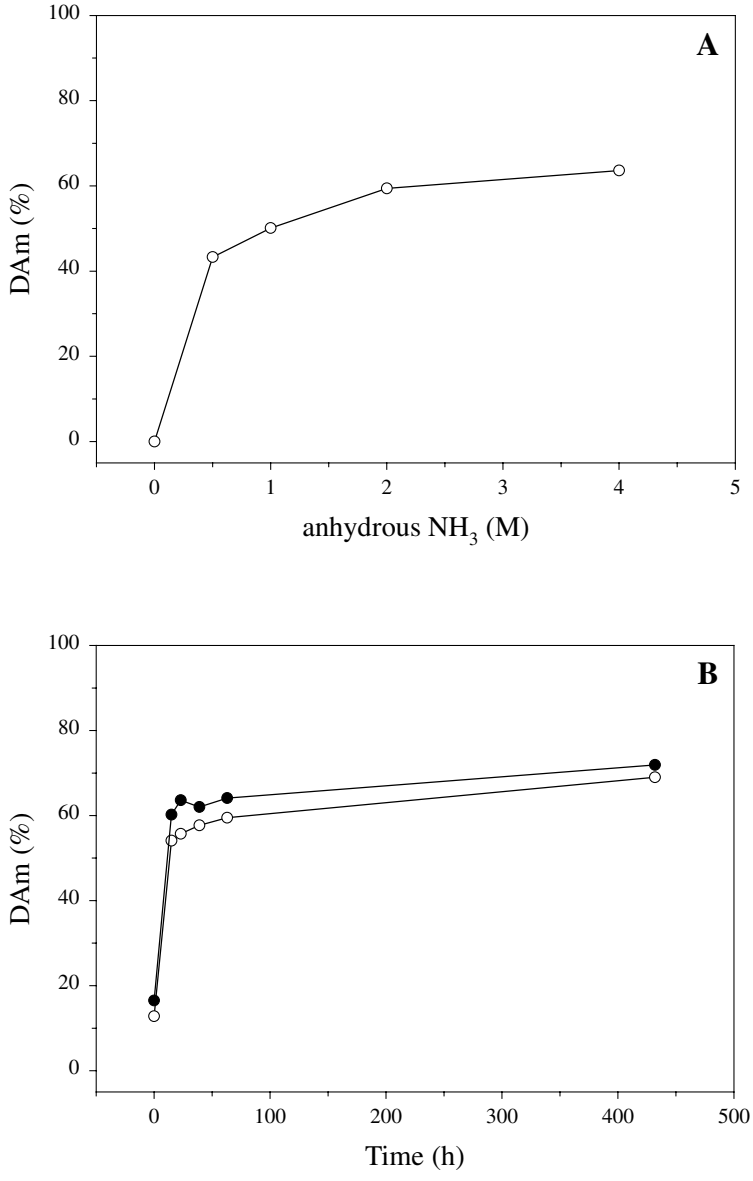

Fig. 2. The DAm of triGalA as a function of the concentration of anhydrous ammonia after five days of incubation (A) and as a function of time (B) at either 2 M (○) or 4 M (●) anhydrous ammonia.

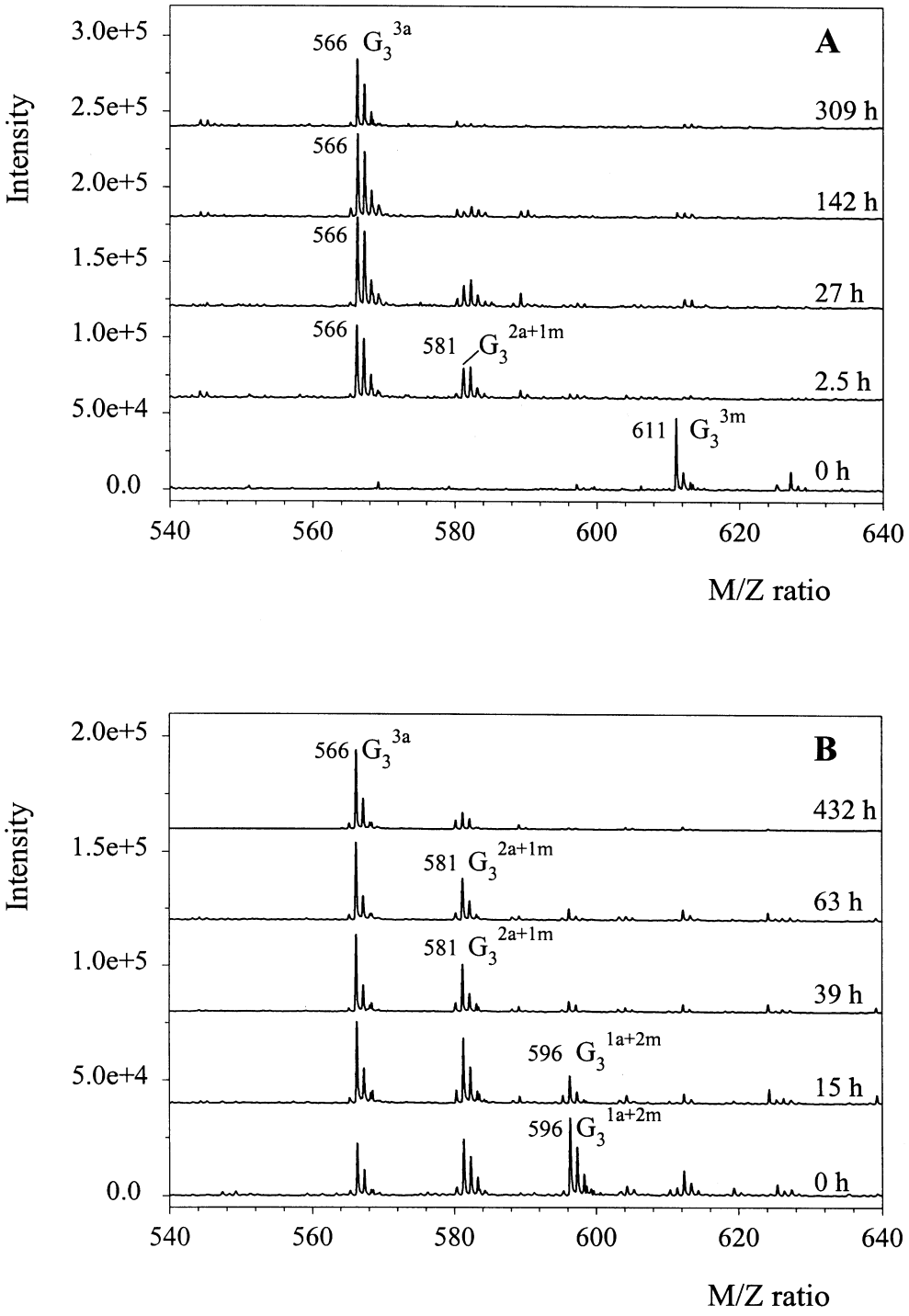

Fig. 3. MALDI-TOF mass spectra (positive mode) showing the amidation process of methyl-esterified triGalA in time as indicated either at 4°C using 4 M water-containing ammonia (A) or at ambient temperature using anhydrous ammonia (B). The signal intensity is plotted against the mass to charge ratio (*m*/*z*). The masses and the degree of esterification and amidation are indicated (see Fig. 1).

In addition, the amidation reaction with 4 M methylamine was monitored in time at 4°C and at ambient temperature. The reaction proceeded very fast. At both temperatures the reaction was almost complete after 4 h of incubation and the spectra changed only slightly (not shown). Upon saponification a slight increase in dimethyl-amide triGalA was seen in all spectra similar to Fig. 4B. The peak height ratio after saponification and the corresponding DAm were calculated. The peak height ratio slightly increases in time towards trimethyl-amidated triGalA. As a consequence a slight

Table 3
Peak height ratio of differently amidated oligoGalA (pellet fractions) after 26 days of incubation at 4°C using 4 M water-containing ammonia analyzed after correction for isotopic distribution. In addition, the DM of the starting material (to) was given and compared to the DAm calculated as described in Section 2

OligoGalA pellet fractions	Peak height ratio (%)									DM (%) before amidation[a]	DAm (%)	Conversion (%)[b]
DP (= n)	G_n^{na}	$G_n^{(n-1)a}$	$G_n^{(n-2)a}$	$G_n^{(n-3)a}$	$G_n^{(n-4)a}$	$G_n^{(n-5)a}$	$G_n^{(n-6)a}$	$G_n^{(n-7)a}$	$G_n^{(n-8)a}$			
4	20	35	28	15	2					97	64	66
5	6	27	34	22	9	2				95	59	62
6	4	17	34	25	16	3	1			97	59	61
7	5	18	25	31	15	6	0	0		96	64	67
8	0	6	13	23	25	16	11	3	3	92	51	55

[a] The DM was calculated from the peak height ratio of partly and fully methyl-esterified oligoGalA.
[b] The conversion was calculated as the percentage of methyl-esters converted to amide groups.

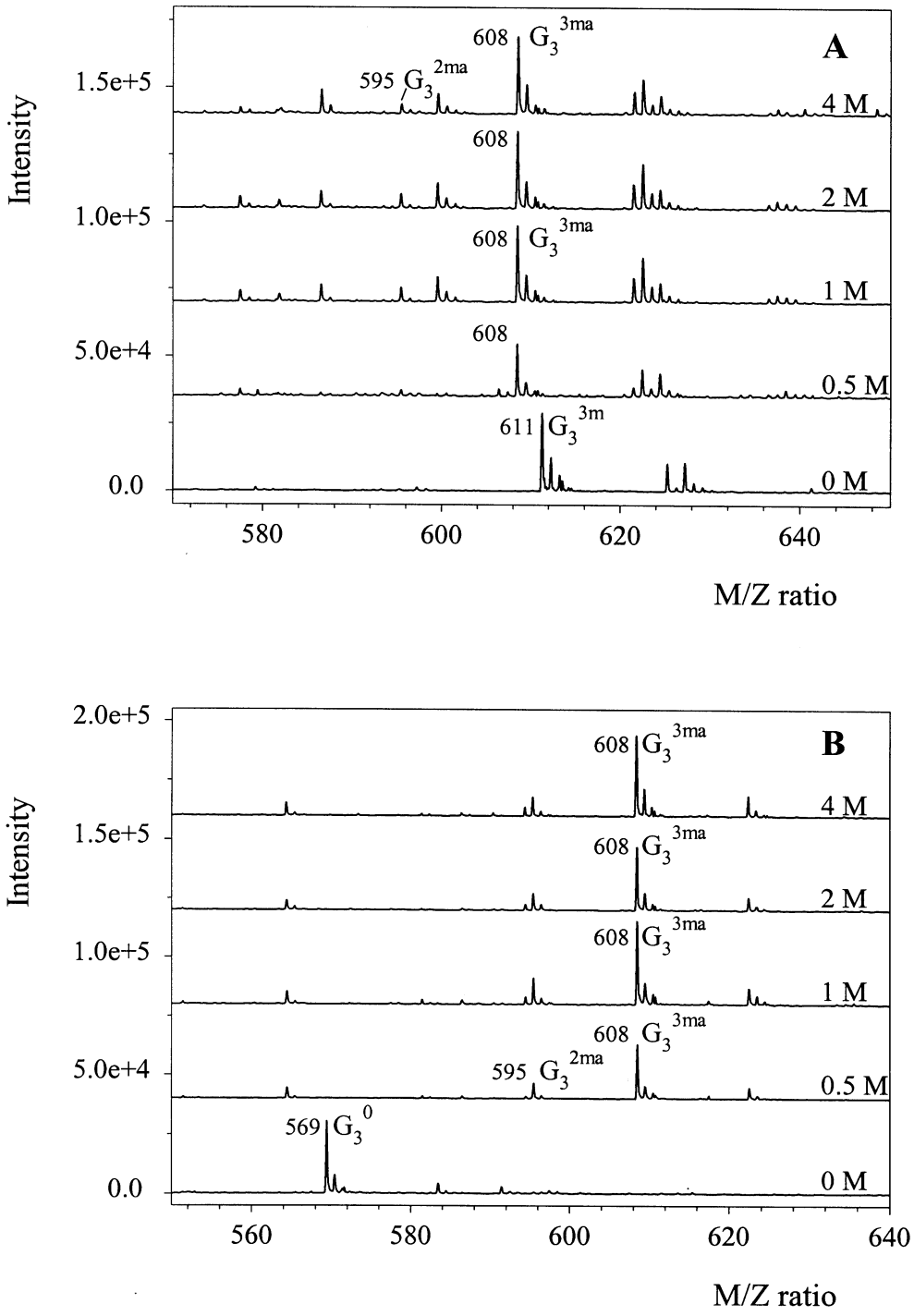

Fig. 4. MALDI-TOF mass spectra (positive mode) showing the amidation process of methyl-esterified triGalA at different methylamine concentrations after 48 h of incubation at ambient temperature before (A) and after saponification (B). The signal intensity is plotted against the mass to charge ratio (*m/z*). The masses and the degree of esterification and amidation are indicated (Code G_3^{2a+1m}: a triGalA having 2 methylamide groups and 1 methyl-ester).

increase in the DAm to 96–97% is seen also (Table 4). Optimal amidation seems to occur with 4 M of methylamine at ambient temperature for at least 50 h of incubation. These conditions were applied for amidation of oligoGalA with different DP (DP3–6). The samples were analyzed before and after saponification using MALDI-TOF MS in the positive mode. The peak height ratios were determined and the corresponding DAm were calculated (Table 5). In all cases the methyl-amidation was almost complete. The DAm varied between 94–97%, corresponding to 96–99%

Table 4
Peak height ratio of differently amidated triGalA after incubation at different concentrations of methylamine (48 h, RT) or at different points in time (4 M methylamine, RT) after saponification. The DAm was calculated as described in Section 2

TriGalA	Peak height ratio (%)				DAm (%)
Sample	G_3^{3ma} (608 *m/z*)	G_3^{2ma} (595 *m/z*)	G_3^{1ma} (582 *m/z*)	G_3^{0ma} (569 *m/z*)	
[CH_3NH_2] (48 h)					
0 M	0	0	0	100	0
0.5 M	77	23	0	0	93
1 M	75	25	0	0	92
2 M	79	21	0	0	93
4 M	81	19	0	0	94
Time (4 M)					
0 h	0	0	0	100	0
4 h	84	16	0	0	95
26 h	86	14	0	0	95
51 h	90	10	0	0	97
171 h	88	12	0	0	96

conversion of the methyl-esters. A small percentage of the GalA residues (1–4%) was not amidated, but still methyl-esterified since hydrolysis could not be detected at all using MALDI-TOF MS in the negative mode (not shown). So amidation of methyl-esterified oligoGalA using methylamine is a very efficient reaction, resulting in a high DAm, without hydrolysis taking place.

4. Discussion

Until now amidation has been restricted to polymeric pectins. This is the first time that amidation of methyl-esterified oligoGalA is described. Amidation of methyl-esterified oligoGalA can be monitored easily using MALDI-TOF MS in the positive mode. In addition, side reactions like hydrolysis and β-elimination can be detected simultaneously with MALDI-TOF MS. Amidation using ammonia was restricted to the solubility of the oligoGalA. Initially, amidation using ammonia takes place at a high rate. However, due to precipitation of the amidated oligoGalA the rate of amidation reduces enormously. Hydrolysis of methyl-esters is the only side reaction found and can be reduced by using anhydrous ammonia. Still, the DAm of 51–64% found for oligoGalA (DP4–8) would only increase 6–10% using anhydrous conditions. So amidation of methyl-esterified oligoGalA using ammonia can be performed best under anhydrous conditions at a concentration of 4 M at ambient temperature.

Amidation of pectins using either water-containing or anhydrous ammonia results in similar DAm values (up to 61%). However, these pectins become even partial insoluble in water, if the DAm rises above 41% (Pilnik et al., 1974; Reitsma, Thibault & Pilnik, 1986). Probably both the insolubility of these pectins in water and the insolubility of amidated oligoGalA in methanol are caused by interactions of amid groups with deprotonated carboxyl groups or hydroxyl groups.

Amidation of methyl-esterified oligoGalA using methylamine was optimal using 4 M anhydrous methylamine incubated for at least six days at ambient temperature resulting in a DAm of 95–97% with hardly any hydrolysis taking place. Precipitation of the oligoGalA does not occur, since the methyl-group attached to the nitrogen will prevent interaction with free deprotonated carboxyl-groups or hydroxyl groups. As a consequence amidation can continue and this is exactly what is seen. Recently, amidation of pectins with primary amines was reported (Sinitsya, Čopíková, Prutyanov, Skoblya & Machovič, 2000). DAm levels up

Table 5
Peak height ratio of differently amidated oligoGalA after 144 h of incubation at ambient temperature using 4 M methylamine analyzed after saponification. The DAm was calculated as described in Section 2. In addition, the DM of the starting material (t0) was given for comparison with the DAm

OligoGalA	Peak height ratio (%)							DM (%) before amidation[a]	DAm (%)	Conversion (%)[b]
DP (= *n*)	$G_n^{n\text{ma}}$	$G_n^{(n-1)\text{ma}}$	$G_n^{(n-2)\text{ma}}$	$G_n^{(n-3)\text{ma}}$	$G_n^{(n-4)\text{ma}}$	$G_n^{(n-5)\text{ma}}$	$G_n^{(n-6)\text{ma}}$			
3	84	16	0	0				98	95	97
4	76	24	0	0	0			98	94	96
5	72	28	0	0	0	0		97	95	98
6	80	20	0	0	0	0	0	98	97	99

[a] The DM was calculated from the peak height ratio of partly and fully methyl-esterified oligoGalA.
[b] The conversion was calculated as the percentage of methyl-esters converted to amide groups.

to 51% were found for ethylamine, which corresponded to 70% conversion of the original methyl-esters, although it is not clear if optimal conditions were used.

Methylamine is a better reagent for amidation of pectin and oligoGalA compared to ammonia. It probably could result in higher DAm values and would allow the pectin to remain soluble at higher degrees of amidation. Although these pectins would not be allowed in food industry it might be useful for applications outside this field. In addition, these kinds of pectins would be useful in all kind of mechanistic studies.

The amidated oligoGalA prepared here will be used in future research for the determination of substrate specificity of pectolytic enzymes. Together with the earlier prepared (partly) methyl-esterified oligoGalA (Van Alebeek et al., 2000a) a whole set of substrates is now available, which allows a thorough investigation.

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