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Acetylation and methylation of homogalacturonans 1: optimisation of the reaction and characterisation of the products

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Abstract

Tetrabutylammonium (TBA) salts of homogalacturonans, obtained by acid hydrolysis of demethylated citrus pectins, were acetylated and/or methylated. Methylation took place in DMSO, using CH_3I as the reagent, and was stoichiometric up to a degree of methylation (DM) of >60. The best acetylation results, with acetic anhydride as reagent, were obtained using formamide as solvent and pyridine as catalyst, giving degrees of acetylation (DAc) of up to 150% in a single step. The reaction was fast but demanded large excess of acetic anhydride. Acetylation occurred on both the O-2 and the O-3 position, with a slight preference for O-2, of galacturonic acid residues. The hydrodynamic volume of the molecules showed little change after derivatisation. Acetylation inhibited hydrolysis of homogalacturonans by endopolygalacturonase.

Keywords: Homogalacturonans; Methylation; Pectins

1. Introduction

Pectins are one of the main gelling agents used in food industry and are a major polysaccharide of the primary plant cell wall. Their major structural feature is a linear chain of (1,4)-linked α-d-galacturonic acid residues, which can be partially esterified by methanol on the carboxyl group and by acetyl on the secondary hydroxyls; native pectins are generally highly methylated but poor in acetyl groups. In plant cell walls, the homogalacturonan fraction of pectins is usually only slightly acetylated or not at all acetylated, and the acetyl groups seem to be concentrated in the rhamnogalacturonic backbone of the hairy regions (Ishii, 1995; Schols & Voragen, 1994; Rihouey, Morvan, Borissova, Jauneau, Demarty & Jarvis, 1995; Komavilas & Mort, 1989). However highly acetylated pectins, carrying acetyl groups on their homogalacturonan fraction, can be present, e.g. in beet (Rombouts & Thibault, 1986). It has long been recognised that pectins from sugar-beet are not able to form gels under the same conditions as apple or citrus pectins, and this has been attributed to presence of acetyl groups (Kertesz, 1951, and references cited therein).

Much work has been carried out on the effect of the degree of methylation (DM) and distribution of the methyl groups on binding of divalent cations, especially calcium (Powell, Morris, Gidley & Rees, 1982; Kohn, Markovic & Machova, 1983; Thibault & Rinaudo, 1985). There has been a number of attempts in the 1940s and the 1950s to understand the role of acetylation (Kertesz, 1951) better. Carson and Maclay (1946) succeeded in acylating pectin and pectic acid by swelling them in formamide prior to the reaction with acid anhydrides, using pyridine as a catalyst. Pippen, McCready and Owens (1950) used this method to obtain pectin acetate samples. As they did not indicate concentrations of galacturonic acid in their samples, it is not possible to calculate the degrees of acetylation (DAc) obtained. However, assuming a value of 750 mg GalA/g, which is realistic for commercial pectins, gelling was seriously hindered at about a DAc of 15, and completely inhibited at 20 (the DAc being the molar percent of acetyl groups relative to galacturonic acid). The influence of acetylation seemed independent of the DM of the pectins, and Pippen et al. (1950) were able to restore gelling properties by selectively removing acetyls. Solms and Deuel (1951) and Schweiger (1964) obtained highly acetylated pectic acids (DM < 5), up to a DAc of almost 150 and 172, respectively. In one study the viscosity of the acetylated pectic acids increased with the DAc (Solms & Deuel, 1951) and in the other increased up to about a DAc of 100 then plateaued (Schweiger, 1964). Acetylation inhibited coagulation and/or precipitation of pectic acid by most of the divalent cations

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tested in these studies, but not Cu^{2+} (Solms & Deuel, 1951; Schweiger, 1964), Cd^{2+} and Pb^{2+} (Schweiger, 1964) nor the mutivalent ions [Al^{3+} (Solms & Deuel, 1951; Schweiger, 1964), Fe^{3+}, Cr^{3+} and Sn^{4+} (Schweiger, 1964)]. Pectic acids of intermediary DAcs could form gels with sugar in acidic conditions while highly acetylated samples were not soluble in this medium (Solms & Deuel, 1951). Acetylation decreased the affinity for galacturonans and the extent of degradation catalysed by both *A. niger* endo-polygalacturonase (Rexova-Benkova, Mrackova, Luknar & Kohn, 1977) and carrot exo-galacturonase (Heinrichova & Kohn, 1980).

Studies on the effect of acetylation on pectins were hindered by a lack of an easy and controllable acetylation method and by difficulties in selectively removing acetyl groups. We wanted to vary the DAc independently of other parameters such as degree of methylation and rhamnose content. We have chosen to use homogalacturonans of DP $\sim$ 100, and we have further methylated them on the carboxyl group on C-6 or acetylated them on the secondary alcohols on C-2 and C-3. Two popular means of carboxyl group modification are the use of diazomethane (Deuel, Huber & Leuenberger, 1950; Klavons & Bennett, 1995) and esterification in acidic-methanol (Jansen & Jang, 1946), both of which take place in suspension. Matricardi, Dentini, Crescenzi and Ross-Murphy (1995) have shown that conversion of pectins to their tetrabutylammonium (TBA) salt allows their solubilisation in organic solvents such as DMSO, and easy substitution of the free carboxyls by dialkyl or dialkylaryl halides, without chain degradation. The main advantage of using TBA salts is their solubility in organic solvents, allowing modifications in the homogeneous solution. In this work, we have methyl esterified homogalacturonans using a variation on the procedure of Matricardi et al. (1995) for cross-linking of pectates, and studied the acetylation of their TBA salts.

2. Materials and methods

2.1. Materials

Tetrabutylammonium hydroxide (TBA-OH, 40% solution in water) was from Sigma. Monocomponent polygalacturonase (PG II) was a gift from Novo Nordisk A/S (Denmark). High DM citrus pectin was from SKW (Beaupte, France).

2.2. Preparation of homogalacturonans

The citrus pectin (at 20 g/l) was demethylated at 4°C and pH $>$ 13; after 2 h, the solution was brought to pH 6 and the pectate was precipitated in three volumes of ethanol, rinsed until the filtrate was devoid of Cl^- ions (absence of precipitation with 0.1 mol/l $AgNO_3$) and dried by solvent exchange (ethanol, acetone). The pectate was hydrolysed in 0.1 mol/l HCl at 80°C as described earlier (Thibault, Renard, Axelos, Roger & Crépeau, 1993). Homogalacturonans were separated from the oligosaccharides by centrifugation then dried by solvent exchange (ethanol, acetone). TBA salts were obtained by careful neutralisation of the homogalacturonans to pH 7.2.

2.3. Methylation

Methylation of homogalacturonans was carried out as described by Matricardi et al. (1995) using CH_3I as the reactant and 24 h as the reaction time at room temperature. DMSO was first eliminated by dialysis against water, and then the samples were concentrated by rotary evaporation. Partially methylated homogalacturonans were precipitated in two volumes of acidic ethanol (950 ml of 96% ethanol plus 50 ml of fuming HCl), and their filtration on G-3 sintered glass filters was attempted. Following this, they were redissolved in water and dialysed against water before freeze-drying.

2.4. Acetylation

Homogalacturonans in TBA form were dissolved at 20 g GalA/l. The catalyst was added followed by the acetic anhydride in four aliquots at 15 min intervals (to favour homogeneous distribution of acetyl groups). After a given time at room temperature, addition of water, thus destroying the excess acetic anhydride, stopped the reaction. Samples were then dialysed extensively, including once against 0.05 mol/l HCl, concentrated on a rotary evaporator and freeze-dried. The following conditions were tested: DMSO or formamide solvent, pyridine or *N*-methylimidazole as catalyst, room temperature or 50°C, pyridine:formamide ratios of 1:25–10:25 (volume to volume), reaction times up to 24 h and acetic anhydride:GalA ratios up to 20:1 (mole to mole).

2.5. Analytical

After saponification (2 h in KOH 0.5 mol/l at room temperature), uronic acids were measured by a modification of the method of Blumenkrantz and Asboe-Hansen (1973), using the heat of dilution to drive the reaction. Methanol was measured by an enzymatic oxidation method (Klavons & Bennet, 1986), and acetyl groups by HPLC on an HPX-87H column (Biorad) eluted by 5 mmol/l H_2SO_4 at 0.6 ml/min (Voragen, Schols & Pilnik, 1986).

2.6. Size-exclusion chromatography

HPSEC was carried out on a Shodex OHpack B-804 column (with SB-800 precolumn) eluted by $NaNO_3$ 0.1 M at 0.5 ml/h with RI detection.

2.7. Enzymic degradation

Partially acetylated homogalacturonans (2.5 mg/ml) were degraded by polygalacturonase at 10 μg protein/ml in 0.05 mol/l sodium acetate buffer pH 5 at 40°C. the extent

Table 1
Preparation of homogalacturonans: yields (in g/g pectin) and composition (mg/g; dry matter) of the substrates. (nd: not determined)

	Yields (GalA)	GalA	MeOH (DM)
Citrus pectin	—	747	0.104 (77)
Pectate	1.01 (0.99)	735	0.003 (2)
Homogalacturonans	0.63 (0.74)	878	nd

of degradation was monitored by an increase in the reducing end-groups (Nelson, 1944).

2.8. NMR

The sodium salts of partially acetylated or methylated homogalacturonans (obtained by careful neutralisation to pH 6.5) were exchanged twice with 99.9% D_2O then dissolved (∽ 20 mg/ml) in 100% D_2O. Their ^1H NMR spectra were measured at 353 K in a Bruker Aspect 3000 NMR or Bruker ARX 400 spectrometer.

3. Results

3.1. Preparation of homogalacturonans

The demethylation step was very specific (Table 1), resulting in an almost complete elimination of the methyl esters without loss of galacturonic acid. Replacement of the methyl groups by Na^+ led to a slight weight increase. Most of the GalA was collected as homogalacturonans, as observed earlier (Thibault et al., 1993); the soluble fraction has been shown to consist of small oligomers of Ara and Gal, rhamnogalacturonan oligomers and oligogalacturonans (Renard, Crépeau & Thibault, 1995).

3.2. Methylation

TBA salts of homogalacturonans dissolved readily in DMSO. Methylation (Fig. 1(a)) proceeded stoichiometrically, i.e. the DM obtained was the molar ratio of CH_3I to GalA initially present, up to a DM of 60. For the last two points (CH_3I to GalA ratios of 1 and 10), a precipitate appeared in the DMSO. The homogalacturonans of DM 95 were not soluble in water, while the homogalacturonans of DM 89 were soluble under neutral conditions. Yields were low (0.46–0.54 for the GalA), which might be caused by the problems encountered during the isolation procedure. These molecules, of relatively low DP, gave either very weak gelatinous or thin particulate precipitates, which could not be separated quantitatively by filtration. Dialysis was therefore chosen as the exclusive purification step for later reactions. HPSEC of partially methylated homogalacturonans (Fig. 2) showed limited degradation with increased elution times (from 26.8 min for the original homogalacturonan to 27.8 for DM = 98) and some peak widening. Presence of a shoulder at V_0 for the homogalacturonan of DM 89 might be due to aggregation.

Integration of the H-4 and non-esterified H-5 (H-5 n) signals in the NMR spectra (Fig. 3) of the methylated homogalacturonans made it possible to calculate DMs which were in good agreement with the values determined by the chemical methods (Table 2). As observed by Grasdalen, Bakoy and Larsen (1988), esterification resulted in a marked shift of the H-1 and H-5 signals (and, to a more limited extent, H-4), with in addition for H-1 splitting according to the nature of the neighbouring residue. Using the nomenclature of

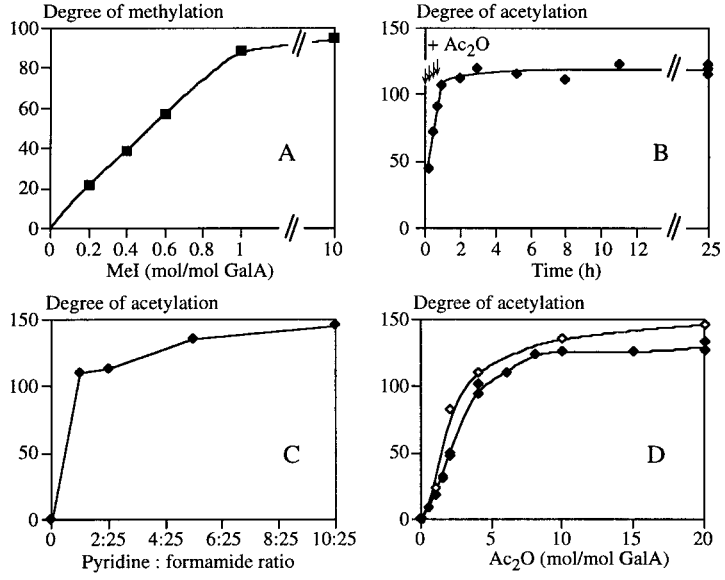

Fig. 1. Methylation and acetylation reactions: (a) extent of methylation of homogalacturonans according to the amount of CH_3I used; (b) time course of the acetylation reaction; (c) influence of the pyridine/formamide volume ratio on the acetylation reaction; and (d) extent of acetylation as a function of the amount of acetic anhydride. ◆ formamide:pyridine = 5:25; ◇: formamide:pyridine = 10:25.

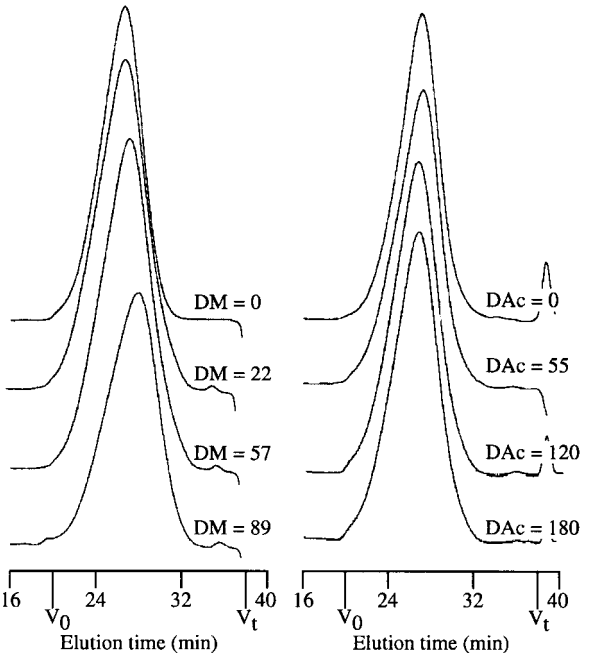

Fig. 2. HPSEC patterns of partially methylated (a) and partially acetylated (b) homogalacturonans.

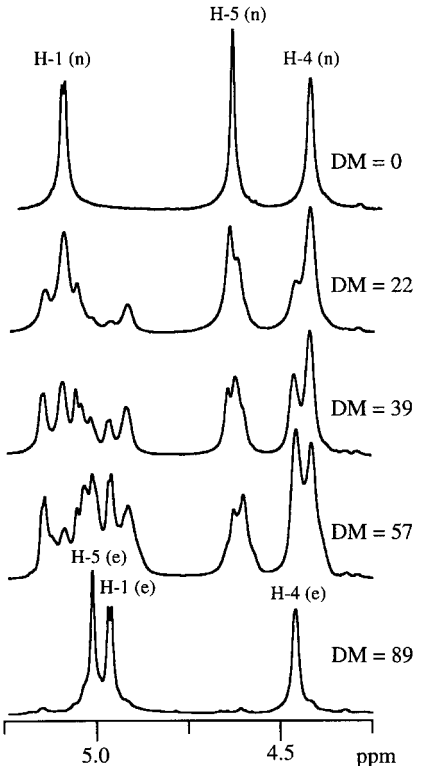

Fig. 3. Anomeric region of the ^{1}H NMR spectra of the partially methylated homogalacturonans: H-1, H-5 and H-4 regions. e: esterified (COOMe); n: neutral (COONa).

Table 2
Degrees of methylation of the partially methylated homogalacturonans by chemical methods and by NMR (1—ratio of H-5 (n) to H-4 signals)

Molar ratio of CH_3I to GalA for the reaction	DM by chemical means	DM by NMR
0.2	22	18
0.4	39	36
0.6	57	56
1	89	94
10	95	Not soluble

Grasdalen et al. (1988) and Andersen, Larsen and Grasdalen (1995) (G = GalA, E = GalA-Me), the signals for the H-1 can be interpreted as follows. The signals at 5.16 and 5.13 ppm are those of the non-esterified GalA, 5.16 ppm being that of a GalA residue with a GalA-Me neighbour (= diad GE or EG), and 5.13 ppm that of H-1 of a GalA residue with a GalA neighbour (GG diad). The signals at 4.97 and 4.92 ppm are those of the GalA-Me residues of EE and EG (GE) diads, respectively. In spite of the low resolution, leading to low accuracy of the integration, the areas of these signals fitted with the Bernouillian diad probabilities, indicating that the methylation had occurred randomly or at least not blockwise.

3.3. Acetylation

Three solvent–catalyst systems were tested with a large excess of acetic anhydride (Ac_2O:GalA ratio of 20:1) and 24 h as the reaction time (Table 3). The best results were obtained with pyridine in formamide, with a DAc of $\backsim$ 150 being reached in one stage. The reaction was fast (Fig. 1(b)), reaching a plateau in less than 2 h after the first introduction of Ac_2O. Raising the temperature to 50°C resulted in dark brown products without an increase of the DAc. The DAc increased with the amount of catalyst present (Fig. 1C). By varying the amount of Ac_2O, variable DAc could be obtained but the reaction plateaued at a DAcs of $\backsim$ 120 with a pyridine:formamide ratio of 5:25 (v:v) and $\sim$ 150 if the pyridine:formamide ratio was 10:25 (Fig. 1(d)). Reacetylation of the partially acetylated homogalacturonan made it possible to reach a DAc of 180. A side-reaction of Ac_2O with formamide has been shown to occur (Carson & Maclay, 1946), and might explain the low efficiency of the reaction. In addition, the presence of trace amounts of water is almost unavoidable, i.e. both residual water in solvents dried on molecular sieve and the water strongly bound to the

Table 3
Results of acetylation of homogalacturonans with acetic anhydride (20 mol/mol GalA) for 24 h at room temperature with varying solvents and catalysts

Solvent	Catalyst	Degree of acetylation	Yield (GalA)
DMSO	*N*-methylimidazole	135	0.50
DMSO	Pyridine	102	0.73
Formamide	Pyridine	154	0.73

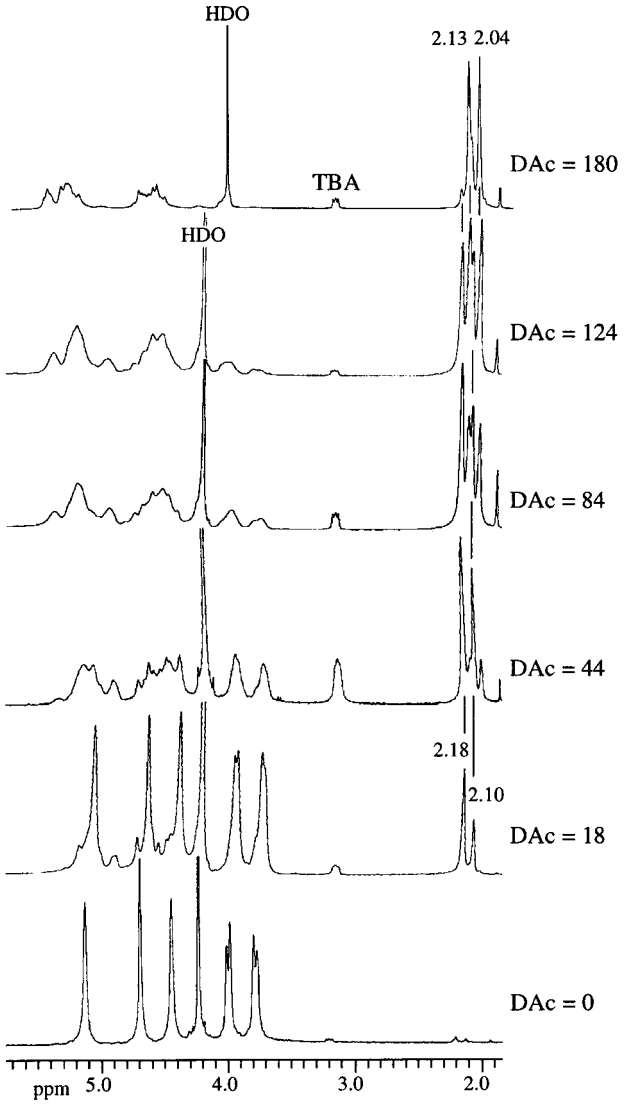

Fig. 4. ^{1}H nmr spectra of the partially acetylated homogalacturonans.

carbohydrate moiety, which leads to the unproductive use of acetic anhydride. Yields were good, ranging between 71 and 89% for the initial GalA. Once again there was no marked depolymerisation of the homogalacturonan polymer during the derivatisation (Fig. 2), except for the sample treated at 50°C (not shown).

3.4. NMR characterisation of the acetylated homogalacturonans

The NMR spectra of homogalacturonans with varying DAcs (Fig. 4) contained four distinct signals in the acetyl CH_3 range. There are two positions for acetylation on the galacturonic acids in the chain on each of the secondary hydroxyls, C-2 and C-3. Therefore, two of these signals probably corresponded to the single acetylation on either C-2 or C-3, the other two to C-2 and C-3 acetylation on a doubly substituted GalA residue, although more complex

Table 4
Interpretation of acetyl signals

Signal (ppm)	Interpretation	Position	Integration
2.18	Single acetylation,	A	A_s
2.13	Double acetylation	A or B	AB
2.10	Single acetylation	B	B_s
2.04	Double acetylation	A or B	AB

effects cannot be ruled out. Acetyl CH_3 signals at 2.18 and 2.10 ppm were preponderant at low DAcs (Fig. 4), with the 2.18 signal being always more intense. Signals at 2.13 and 2.04, with roughly identical intensities, increased at higher DAcs. The signals at 2.18 and 2.10 ppm arise from single substituted GalA residues, denoted without prejudging of the actual position A and B, while the signals at 2.13 and 2.04 indicated doubly substituted residues (AB). Of the two acetylation positions, the one resulting in an acetyl signal at 2.18 ppm was clearly favoured.

The areas of the acetyl signals were used to follow the course of acetylation. The total area for an acetyl signals was normalised by the DAc (measured chemically) to correct for intensity. A comparison with the integration of the carbohydrate signals, which would have been preferable, could not be used because of the interference of the HDO peak. The ratio of the area of individual signals to the total area of the acetyl signals could be described as follows (Table 4): 2.18 ppm representing A_s, single acetylation at position "A", 2.04 and 2.13 ppm representing AB, double acetylation at position "A" or "B" (of equal intensity), and 2.10 ppm representing B_s, single acetylation at position "B". As the signals at 2.13 and 2.10 were not resolved, they were integrated together, so that for each sample three measurements were obtained: A_s (2.18 ppm), AB (2.04 ppm), and B_s + AB (= B_{tot}) (2.13 + 2.10 ppm), this without assigning the signals at 2.04 and 2.13 ppm either to position "A" or "B". Total substitution at position "A" A_{tot} can be calculated from the sum of the signals as 2.18 (A_s) and 2.04 (AB)(Fig. 5). At all DAcs, position "A" is favoured. Double substitution was rare at first, in contrary to the conclusion of Rexova-Benkova et al. (1977), then increased rapidly. Double acetylation seemed to occur strictly as could be predicted from acetylation of "A" and "B" independently, i.e. AB $\backsim$ $A_{tot} \times B_{tot}$ (dotted line), except maybe for the highest DAc.

The carbohydrate part of the spectra very soon lost definition and the signals of the acetylated GalA could not be assigned. In rhamnogalacturonans (Ishii, 1995), acetylation of the O-2 of GalA resulted in a shift of the H-2 signal from 3.75 to 5.16 ppm, and in oligogalacturonates (Ishii, 1997) acetylation of the O-3 from 3.82 to 5.02 ppm. Similar shifts can be expected here and must result in an accumulation of all the carbohydrate signals below the HDO signal. The evolution of the areas of residual H-2 and H-3 signals at $\sim$ 3.7 and 4 ppm however can be used to identify the major

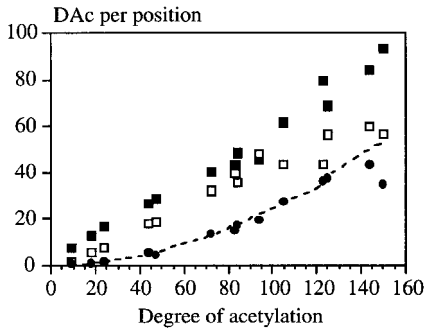

Fig. 5. Acetylation of the two secondary hydroxyl positions as a function of the total degree of acetylation. The data are presented as the ratio of the area of a given acetyl CH_3 signal to the total area of acetyl signals in that spectrum, weighted by the DAc: $(S/\sum S_{Ac}) \times$ DAc. ■: position "A", 2.18 + 2.04 ppm; □: position "B", 2.13 + 2.10 ppm; ●: measured double acetylation "AB", 2.04 ppm; ––––, calculated double acetylation (2.18 + 2.04) × (2.13 + 2.10).

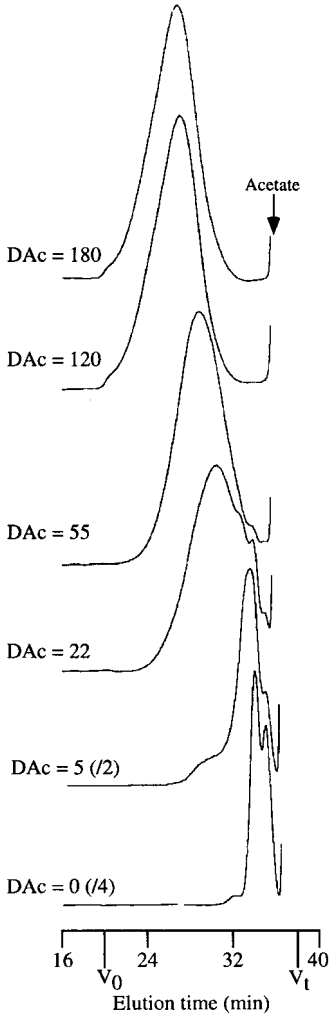

Fig. 6. Enzymic degradation of partially acetylated homogalacturonans: HPSEC of the homogalacturonans after treatment by *endo*-polygalacturonase.

acetylation position as the signal which decreased more rapidly; position "A" would thus most likely be C-2.

3.5. Enzymic degradation of acetylated homogalacturonans

Acetylation of the homogalacturonans strongly hindered their degradation by an *endo*-polygalacturonase from *A. niger*. While this enzyme cleaved 57% of the glycosidic bonds of non-acetylated homogalacturonan, only 46% of the glycosidic bonds could be cleaved at a DAc of 5, 22% at a DAc of 22, 8% at a DAc of 55 and about 1% at DAcs of 120 and 180. This effect was more marked than that obtained by Rexova-Benkova et al. (1977). After the enzyme treatment the non-acetylated homogalacturonan was converted almost completely to di- and mono-galacturonic acid (Fig. 6), and the homogalacturonan of DAc 5 was mostly converted to oligomers. Clear shifts for higher elution times were visible for DAc 22, with the presence of some oligomers, and DAc 55. For homogalacturonans of DAc 120 and 180 there was no visible effect of the enzyme treatment on the hydrodynamic volume. These HPSEC patterns, notably those obtained for DAc 22 and 55, also indicated that the acetyl groups were not concentrated on a specific population of molecules, and probably not in blocks of consecutive galacturonic acids.

4. Conclusion

Homogalacturonans with independently varying DMs and DAcs were obtained, without marked degradation of the polygalacturonic backbone. Reaction of homogalacturonans in homogeneous solutions lead to a homogeneous distribution of the methyl and acetyl groups, which might explain some differences with the earlier results where pectins were acetylated or methylated in suspension. NMR proved the existence, even preponderance of mono-acetylated galacturonic acid moieties at low DAc, in contrast to the conclusions of Rexova-Benkova et al. (1977) that acetylation of pectic acid leads, highly preferentially or exclusively, to diacetyl derivatives. Kohn and Furda (1969) have shown that esterification of pectins in acidic methanol leads to a blockwise distribution of the newly introduced esters unless precautions were taken to increase accessibility of the carboxyl groups. Amidation of pectins, which is carried out by treatment with ammonium in an ethanolic suspension, also results in blocks of amidated galacturonic acids (Racapé, Thibault, Reitsma & Pilnik, 1989).

Acetylation had a slight preference for the hydroxyl on C-2, probably due to better accessibility. In the 2_1 helical conformation of the homogalacturonan chain access to O-3 is hindered by the O-3– O-5′ hydrogen bond. In the derivatisation of cellulose (Pawloski, Sankar & Gilbert, 1987), the preferential position is the primary hydroxyl on C-6, absent in pectins.

These substrates and the acetylation method will be used

in the subsequent article to study the effect of acetylation on the binding of divalent cations and the conformation of the modified homogalacturonans.

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References

Andersen, A. K., Larsen, B., & Grasdalen, H. (1995). Sequential structure by ^{1}H NMR as a direct assay for pectinesterase activity. *Carbohydr. Res.*, *273*, 93–98.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Anal. Biochem.*, *54*, 484–489.

Carson, J. F., & Maclay, W. D. (1946). Acylation of polyuronides with formamide as dispersing agent. *J. Am. Chem. Soc.*, *68*, 1015–1017.

Deuel, H., Huber, G., & Leuenberger, R. (1950). Über das Geleirvermögen von Polygacturonsäuremethylester. *Helvetica Chimica Acta*, *33*, 1226–1228.

Grasdalen, H., Bakoy, O. E., & Larsen, B. (1988). Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by ^{1}H-NMR. spectroscopy. *Carbohydr. Res.*, *184*, 183–191.

Heinrichova, K., & Kohn, R. (1980). The role of secondary acetyl groups of d-galacturonan in its degradation with *exo*-d-galacturonase. *Coll. Czech. Chem. Commun.*, *45*, 427–434.

Ishii, T. (1995). Isolation and characterization of acetylated rhamnogalacturonan oligomers liberated from bamboo shoot cell-walls by Driselase. *Mokuzai Gakkaishi*, *41*, 561–572.

Ishii, T. (1997). O-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiol.*, *113*, 1265–1272.

Jansen, E. F., & Jang, R. (1946). Esterification of galacturonic acid and polyuronides with methanol-hydrogen chloride. *J. Am. Chem. Soc.*, *68*, 1475–1477.

Kertesz, Z. I. (1951). *The pectic substances*. New York: Interscience.

Klavons, J. A., & Bennet, R. D. (1986). Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food. Chem.*, *34*, 597–599.

Klavons, J. A., & Bennett, R. D. (1995). Preparation of alkyl esters of pectin and pectic acid. *J. Food Sci.*, *60*, 513–515.

Kohn, R., & Furda, I. (1969). Distribution of free carboxyl groups in the molecule of pectin after esterification of pectic and pectinic acid by methanol. *Coll. Czech. Chem. Commun.*, *34*, 641–648.

Kohn, R., Markovic, O., & Machova, E. (1983). Deesterification mode of pectin by pectin esterases of *Aspergillus foetidus*, tomatoes and alfalfa. *Coll. Czech. Chem. Commun.*, *48*, 790–797.

Komavilas, P., & Mort, A. J. (1989). The acetylation at O-3 of galacturonic acid in the rhamnose-rich portion of pectins. *Carbohydr. Res.*, *189*, 261–272.

Matricardi, P., Dentini, M., Crescenzi, V., & Ross-Murphy, S. B. (1995). Gelation of chemically cross-linked polygalacturonic acid derivatives. *Carbohydr. Polym.*, *27*, 215–220.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, *153*, 375–380.

Pippen, E. L., McCready, R. M., & Owens, H. S. (1950). Gelation properties of partially acetylated pectins. *J. Am. Chem. Soc.*, *72*, 813–816.

Powell, D. A., Morris, E. R., Gidley, M. J., & Rees, D. A. (1982). Conformation and interactions of pectins II. Influence of residue sequence on chain association in calcium pectate gels. *J. Mol. Biol.*, *155*, 517–531.

Pawloski, W. P., Sankar, S. S., & Gilbert, R. D. (1987). Synthesis and solid state ^{13}C-NMR studies of some cellulose derivatives. *J. Polym. Sci. A: Polym. Chem.*, *25*, 3355–3362.

Racapé, E., Thibault, J. -F., Reitsma, J. C. E., & Pilnik, W. (1989). Properties of amidated pectins II polyelectrolyte behaviour and calcium binding of amidated pectins and amidated pectic acids. *Biopolymers*, *28*, 1435–1448.

Renard, C. M. G. C., Crépeau, M. -J., & Thibault, J. -F. (1995). Structure of the repeating units in the rhamnogalacturonic backbone of apple, beet and citrus pectins. *Carbohydr. Res.*, *275*, 155–165.

Rexova-Benkova, L., Mrackova, M., Luknar, O., & Kohn, R. (1977). The role of sec-alcoholic groups of d-galacturonan in its degradation by endo-d-galacturonase. *Coll. Czech. Chem. Commun.*, *42*, 3204–3213.

Rihouey, C., Morvan, C., Borissova, I., Jauneau, A., Demarty, M., & Jarvis, M. (1995). Structural features of CDTA-soluble pectins from flax hypocotyls. *Carbohydr. Polym.*, *28*, 159–166.

Rombouts, F. M., & Thibault, J. -F. (1986). Enzymic and chemical degradation and the fine structure of pectins from sugar beet pulp. *Carbohydr. Res.*, *154*, 189–203.

Schols, H. A., & Voragen, A. G. J. (1994). Occurence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydr. Res.*, *256*, 83–96.

Schweiger, R. G. (1964). Acetyl pectates and their reactivity with polyvalent metal ions. *J. Org. Chem.*, *29*, 2973–2975.

Solms, J., & Deuel, H. (1951). Unterschungen an acetylieter Pektinsaüre. *Helvetica Chimica Acta*, *34*, 2242–2249.

Thibault, J. -F., & Rinaudo, M. (1985). Interaction of mono- and divalent couterions with alkali- and enzyme-deesterified pectins. *Biopolym.*, *24*, 2131–2144.

Thibault, J. -F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crépeau, M. -J. (1993). Studies on the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydr. Res.*, *238*, 271–286.

Voragen, A. G. J., Schols, H. A., & Pilnik, W. (1986). Determination of the degree of methylation and acetylation of pectins by H.P.L.C. *Food Hydrocolloids*, *1*, 65–70.