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Acetylation and methylation of homogalacturonans 2: effect on ion-binding properties and conformations

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Abstract

Acetylated citrus pectin and pectate (DAc of 213 and 150, respectively) were prepared; they were soluble in ethanol (up to 90%) or acetone and did not precipitate or form gels in the presence of calcium, zinc or lead. Binding isotherms indicated a loss of the cooperative behaviour with acetylation, particularly for zinc and calcium. Partially methylated and acetylated homogalacturonans showed different effects of methyl and acetyl substitution on binding of calcium, zinc and lead. Calcium and lead salts of partially methylated homogalacturonans adopted different conformations in the dry state, as indicated by their ^{13}C CP-MAS NMR spectra: whereas calcium homogalacturonans could adopt (partially) a 2_1 conformation which was lost at higher degrees of methylation, lead salts kept the same, 3_1, conformation for all degrees of methylation. ^{13}C CP-MAS NMR spectra of partially acetylated homogalacturonans showed the presence of a wide range of conformations with marked differences between the ions. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Homogalacturonans; Acetylated pectin; Conformations

Abbreviations:

Dac degree of acetylation;
DM degree of methylation;
CP-MAS
cross polarisation magic angle spinning;
TBA tetrabutyl ammonium;
M^{2+} divalent metal cation

1. Introduction

The ability of pectins to bind divalent cations, notably calcium, is of paramount importance both for their roles in cell walls and for their technological properties after extraction. The intensity and mode of binding are influenced by the presence of methyl groups but also of acetyl substituents on the secondary alcohol functions. Much work has been carried out on the effect of the degree of methylation 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). Acetylation also modifies the properties of pectins, but has been less well explored. Solms and Deuel (1951) and Schweiger (1964) obtained highly acetylated pectic acids (DM < 5), up to a DAc of almost 150 and 172, respectively. Acetylation inhibited coagulation and/or precipitation by most divalent cations tested (Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+} (Solms & Deuel, 1951), Ca^{2+}, Mg^{2+}, Zn^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Schweiger, 1964)), but not Cu^{2+} (Solms & Deuel, 1951; Schweiger, 1964), Cd^{2+} and Pb^{2+} (Schweiger, 1964) nor any of the multivalent ions (Al^{3+} (Solms & Deuel, 1951; Schweiger, 1964), Fe^{3+}, Cr^{3+} and Sn^{4+} (Schweiger, 1964)). With calcium and zinc, as the DAc increased the gelatinous precipitates gave way to lumpy, grainy gels, then at DAc > 109 no more precipitation was obtained (Schweiger, 1964). Acetylation decreased the degree of binding of calcium by pectin and pectic acid (Kohn & Furda, 1967; Kohn & Malovikova, 1978); this effect was attributed to inhibition of intermolecular chelate binding (Kohn & Malovikova, 1978). Citrus (DM 54, DAc) and beet pectins (DM 58, Dac 14) differed in their degrees of cooperativity of binding of Ca^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} (Dronnet, Renard, Axelos & Thibault, 1996).

The selectivity scale of pectins for various metal cations ($Cu \sim Pb \gg Zn \sim Ni > Ca$) (Dronnet et al., 1996) cannot easily be related to any property of these ions such as hydrated radius. To understand better the mode of binding and this variation in the affinity, we need to be able to identify further the conformation of pectin salts. The ^{13}C

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chemical shifts of polymers in solid state NMR may vary with the conformation because conformational effects are no longer averaged by fast motion. In carbohydrates, the chemical shifts of the C-1 and C-4 depend on the actual Φ and Ψ angles of the glycosidic bond. This is notably the case for pectins, for which Jarvis and Apperley (1995) have shown dependence of the C-1 and the C-4 signals on the presence of 2_1 or 3_1 helices.

In the accompanying article, we have modified the procedure of Matricardi, Dentini, Crescenzi and Ross-Murphy (1995) to methyl-esterify homogalacturonans and described a procedure for acetylation of homogalacturonans in solution as their tetrabutyl ammonium salts, leading to a homogeneous distribution of acetyl groups without backbone degradation. This procedure was used to obtain partially methylated and/or partially acetylated homogalacturonans and acetylated pectins and pectate, to study the ability of these substrates to bind three divalent cations with, respectively, low (Ca^{2+}), intermediate (Zn^{2+}) and high (Pb^{2+}) affinity for pectins. Conformation of the calcium and lead salts of partially methylated and partially acetylated homogalacturonans was studied by ^{13}C CP-MAS NMR spectroscopy.

2. Materials and methods

2.1. Materials

Tetra butyl ammonium hydroxide (TBA-OH, 40% solution in water) was from Sigma. High DM citrus pectin was from SKW (Beaupte, France). Pectate was obtained by saponification of this citrus pectin 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 $0.1\ mol\ l^{-1}$ $AgNO_3$) and dried by solvent exchange (ethanol, acetone). Some of the pectate was hydrolysed in $0.1\ mol\ l^{-1}$ HCl at 80°C as described earlier (Thibault, Renard, Axelos, Roger & Crépeau, 1993) to obtain homogalacturonans.

2.2. Preparation of salt forms

Pectins and pectate were converted to their acidic form by incubating overnight in acidic ethanol (96% ethanol, water and concentrated hydrochloric acid 700:300:30 ml). TBA or Na salts were obtained by careful neutralisation of the acid form of pectins, pectic acid or homogalacturonans to pH 7.2. The Na salts of (partially methylated and/or partially acetylated) homogalacturonans were dialysed overnight against $0.1\ mol\ l^{-1}$ $Ca(NO_3)_2$ or $0.05\ mol\ l^{-1}$ $Pb(NO_3)_2$, followed by dialysis once against distilled water to obtain the calcium or lead salts, respectively, for CP-MAS NMR.

2.3. Chemical modification

Methylation of homogalacturonans was carried out as described by Matricardi et al. (1995) using CH_3I as the reactant and 24 h reaction time at room temperature, as described in Renard and Jarvis (accompanying article). For acetylation, samples in TBA form were dissolved at 20 g GalA/L in formamide. Pyridine was added at a pyridine:formamide volume ratio of 5:25 then acetic anhydride in four aliquots at 15 min intervals. For pectin and pectate a large excess of acetic anhydride was used, while for homogalacturonans the amount was varied to obtain a range of DAcs. After 2 h at room temperature, the reaction was stopped by adding water, thus destroying the excess acetic anhydride. Methylated and acetylated samples were dialysed extensively, including once against $0.05\ mol\ l^{-1}$ HCl, concentrated on a rotary evaporator and freeze-dried.

2.4. Analytical

After saponification (for 2 h in KOH $0.5\ mol\ l^{-1}$ 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 $5\ mmol\ l^{-1}$ H_2SO_4 at $0.6\ ml\ min^{-1}$ (Voragen, Schols & Pilnik, 1986). The cation-exchange capacity was determined by titrimetry (Dronnet et al., 1996).

2.5. Solubility

Aliquots of ~ 20 mg of the acidic forms of acetylated pectin and pectic acid were mixed with 5 ml of ethanol:-water or acetone:water mixtures. Solubility was assessed visually.

2.6. Ion-binding experiments

Samples in acidic form were dissolved in water or $0.1\ mol\ l^{-1}$ $NaNO_3$ then the pH was adjusted to 7.2 with $0.1\ mol\ l^{-1}$ NaOH and the volume was adjusted to give $2 \times 10^{-3}\ mol\ l^{-1}$ of free COO^- groups. Varying amounts of divalent metal ion solution (at $0.02\ mol\ l^{-1}$) were added to 10 or 20 ml aliquots of the sample solutions and gently stirred for 2 h at room temperature (20–22°C). Activities of Ca^{2+} and Zn^{2+} were measured by the dual wavelength spectrophotometric method, using tetramethylmurexide as an activity probe (Dronnet et al., 1996); activity of Pb^{2+} was quantified using a lead specific electrode (Orion Pb 9482SC) and a reference electrode (Orion 90-02) with 10% KNO_3 as outer filling solution. Activities (in standard solutions) and free ion concentrations were calculated by the Debye–Huckel formula (Koryta, Dvorak & Kavan, 1993).

2.7. NMR

For solid-state NMR, Na, Ca and Pb salts of partially methylated and partially acetylated homogalacturonans were dried over P_2O_5 at ~ 35°C (except Na salts of partially methylated homogalacturonans, which were freeze-dried,

Table 1
Characteristics of the native and acetylated pectins

	GalA (mg g^{-1})	DM	DAc
Citrus pectin	747	77	nd[a]
Acetylated citrus Pectin	510	75	213
Pectate	735	2	nd[a]
Acetylated pectate	533	nd[a]	150

[a] nd: not determined.

and the homogalacturonan of DM 95, which was not soluble in water). They were rehydrated to a 1:0.2 ratio of sample:H_2O just before measurement. Their CP-MAS spectra were measured on a Varian Unity + spectrometer operating at 75.34 MHz for ^{13}C, with MAS rates of 4–5 kHz and 0.5 ms contact time.

3. Results

3.1. Preparation of substrates

The demethylation step was very specific (Table 1), resulting in almost complete elimination of the methyl esters without loss of galacturonic acid. The high DM pectin was difficult to dissolve in formamide even as its TBA salt, a clear solution being observed only after the first addition of Ac_2O. Yields (76 and 80% of the GalA, for pectin and pectic acid, respectively) and DAcs (213 and 150) were high. The DAcs might be overestimated as neutral sugars were not taken into account in their calculation. The acetylation did not modify significantly the DM of the pectin. Homogalacturonans and homogalacturonans with low DMs (13 and 46) were acetylated at varying extents. There was a slight decrease of the DM, to DM = 12 for DAc = 113 (starting from DM 13) and to DM = 40 for DAc = 129 starting from DM = 46.

The acidic form of the acetylated pectic acid and pectin dissolved completely in mixtures containing up to 80% ethanol or acetone, and partially at 90%.

3.2. Ion-binding properties

The ability of homogalacturonans and their derivatives to bind calcium, zinc and lead ions was estimated as the proportion of bound cation for $[M^{2+}] = [COO^-] = 2$ meq l^{-1} (Table 2). Results obtained for the non-acetylated substrates were as expected from data on pectins and oligogalacturonates (Thibault & Rinaudo, 1985; Kohn & Luknar, 1977). All three ions were strongly bound by the non-methylated samples in the absence of supporting salt. The intensity of the binding was decreased by the presence of competing Na^+ ions and by methylation, retaining the selectivity scale Pb > Zn > Ca. The decrease with DM was much more pronounced for Ca^{2+} and very limited for Pb^{2+}.

Acetylation had drastic effects on the binding of Ca^{2+} and Zn^{2+}, specially in the presence of a supporting salt (Table 2). Kohn and Malovikova (1978) noted an increase in free calcium in solutions of pectinate of increasing DAc, with a plateau for DAc of about 100. Zn^{2+} binding was affected as strongly by acetylation as Ca^{2+} binding. The effect was less pronounced for Pb^{2+} but at DM 46 its binding was also severely impaired. As acetylation does not directly affect charge density, this effect is due to a modification of the complexation possibilities, either by blocking hydroxyls active in the chelation mechanisms, by physically impeding access of the ions or by preventing H-bonds between adjacent galacturonic acid residues, thus changing the chain conformation. Another possibility might be the existence of an entropic barrier to the formation of cation-mediated intermolecular associations which is due to the restriction in conformational mobility by the acetate substituents.

In water, precipitates formed for all three ions for the non-acetylated samples but for the acetylated samples, only the homogalacturonans with DM 0, DAc 48 and DM 13, DAc 59 could be precipitated by Pb^{2+}. Schweiger (1964) observed precipitation of acetylated pectates with Pb^{2+}

Table 2
Effect of acetylation upon binding of calcium, zinc and lead ions by homogalacturonans and partially methylated homogalacturonans: $[Me^{2+}]$bound/$[COO^-]$ for $[Me^{2+}]$total/$[COO^-] = 1$ at pH = 7.2 and 2 meq l^{-1}

Homogalacturonan	DAc	In the absence of supporting salt			In 0.1 mol l^{-1} $NaNO_3$		
		Ca^{2+}	Zn^{2+}	Pb^{2+}	Ca^{2+}	Zn^{2+}	Pb^{2+}
DM = 0	0	0.95[a]	0.95[a]	0.96[a]	0.51[a]	0.70[a]	0.80[a]
	48	0.61	0.78	0.95[a]	0.14	0.22	0.75
	85	0.56	0.61	0.88	0.15	0.12	0.66
	105	0.57	0.61	0.88	0.12	0.13	0.62
DM = 13	0	0.85[a]	0.92[a]	0.96[a]	0.39	0.62[a]	0.83[a]
	63	0.46[a]	0.55	0.78	0.06	0.14	0.61
	113	0.50	0.52	0.77	0.06	0.08	0.38
DM = 46	0	0.46[a]	0.73[a]	0.89[a]	0.14	0.27	0.78[a]
	59	0.35	0.44	0.72	0.10	0.07	0.38
	129	0.31	0.32	0.43	0.07	0.08	0.15

[a] Precipitation.

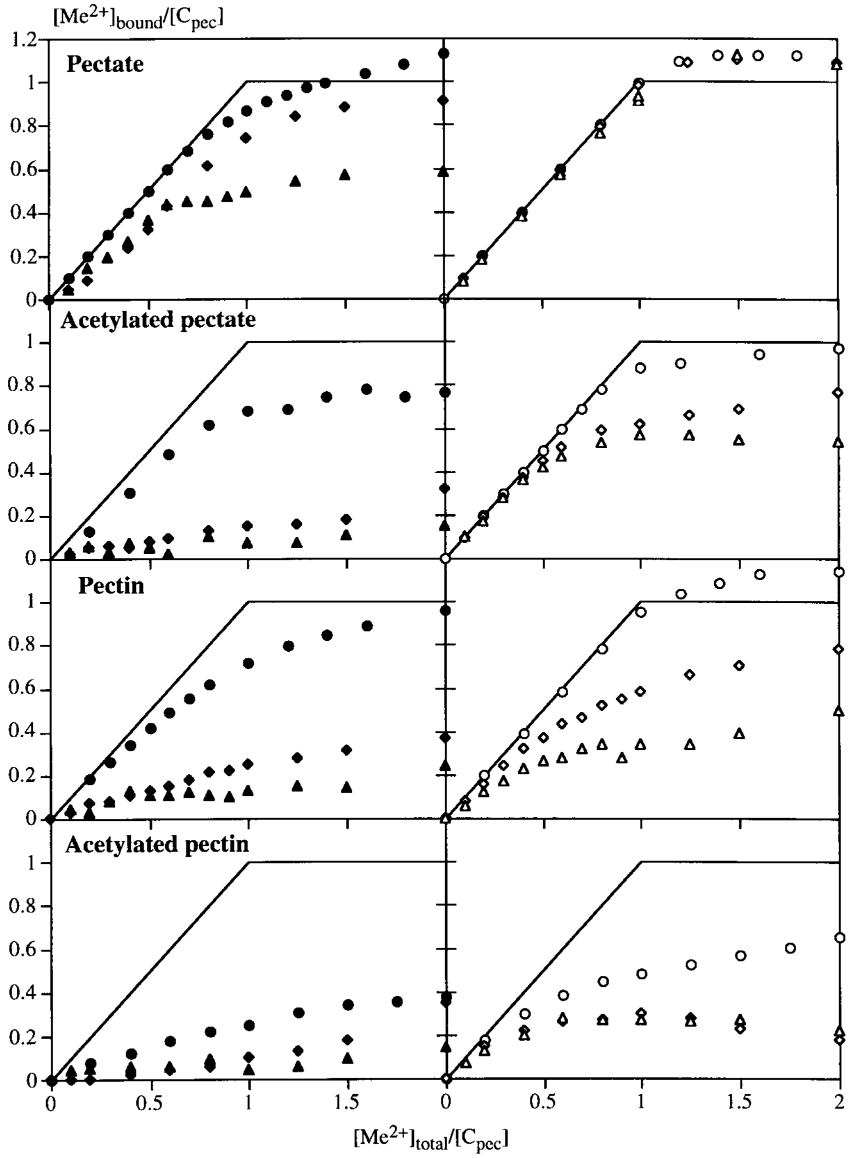

Fig. 1. Ion-binding isotherms for citrus pectin, pectate and their acetylated derivatives. In presence of supporting salt ($NaNO_3$ 0.1 mol l^{-1}): ● Pb^{2+}, ◆: Zn^{2+} and ▲: Ca^{2+}; In absence of supporting salt: ○: Pb^{2+}, ◇: Zn^{2+} and Δ: Ca^{2+}.—: stoichiometric isotherm.

whatever the DAc whereas Ca^{2+} and Zn^{2+} only gave precipitates for DAc < 100.

3.3. Binding of ions by modified pectin and pectate

The effect of acetyl groups on the binding of Ca^{2+}, Zn^{2+} and Pb^{2+} was further investigated using acetylated pectate and high methoxy pectin. Binding isotherms were established at pH 7.2 for a non-esterified galacturonic acid concentration of 2 meq l^{-1} (Fig. 1). For all three cations and all substrates, higher binding intensities were obtained in water than in the presence of supporting salts because of the competition between the Na^+ and M^{2+} ions, as observed earlier (Dronnet et al., 1996). The scale and intensity of binding with the pectin were comparable to the results obtained for a citrus pectin of DM 54 (Dronnet et al., 1996). In water, the binding isotherm of Pb^{2+} followed the stoichiometric isotherm, whereas for Ca^{2+} a plateau was reached at $[M^{2+}]_b/[COO^-] \sim 0.4$ and for Zn^{2+} at $\sim$0.6. In the presence of supporting salt, the plateau reached for Ca^{2+} was very low. Higher levels of binding were reached for pectate (DM < 5); Garnier, Axelos and Thibault (1994) had noted an increase in the level of Ca^{2+} binding isotherms of pectins with increased charge densities. In the absence of supporting salt, levels of binding higher than the free GalA equivalence were observed for all three cations in the case of pectate and for Pb^{2+} in the case of pectin. Binding levels higher than that of the theoretical cation exchange capacity was observed for polypectate with Ca^{2+} (Mattai & Kwak, 1986), and for beet pulp with Cu^{2+} or Pb^{2+}, particularly after saponification (Dronnet, Renard, Axelos & Thibault, 1997; 1998). As gelatinous precipitates formed

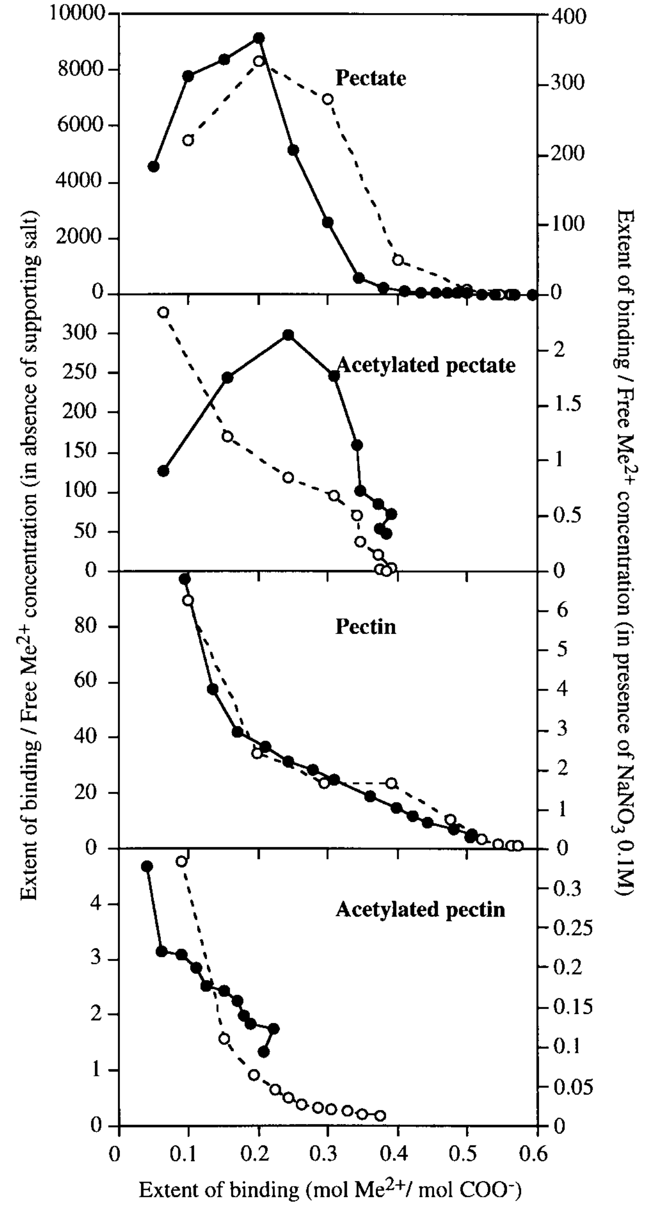

Fig. 2. Influence of methylation and acetylation on Scatchard representations of the binding isotherms for Pb^{2+}. ●: in the presence of supporting salt ($NaNO_3$ 0.1 mol l^{-1}); ○: in the absence of supporting salt.

in these conditions upon mixing of the pectin/pectate and M^{2+} solutions, this could be an artefact due to the retention of free M^{2+} ions in the precipitates. However long equilibration times were used and although precipitates were eliminated by centrifugation for measurement of Ca^{2+} and Zn^{2+} activities, for Pb^{2+} the measurements were done directly in the suspension. Another possibility is the formation of monovalent cation-nitrate or cation-hydroxide complexes, which could form chelates involving only one carboxyl group i.e. one galacturonic acid residue. Precipitation of hydroxides can be ruled out because of low overall metal concentrations and because it would occur with all samples, especially those with limited metal binding.

Acetylation led to a marked decrease in the capacity of the pectin and pectate to bind the metal ions, with different effects on Pb^{2+} than on Ca^{2+} and Zn^{2+}. Acetylated pectin and pectate did not form precipitates, in agreement with the data of Schweiger (1964) and Solms and Deuel (1951). With pectate, binding of Ca^{2+} and especially Zn^{2+} were decreased, the extent of binding of these two ions becoming virtually identical. Pb^{2+} was still bound by acetylated pectate, in contrast to Ca^{2+} and Zn^{2+}. Binding of Pb^{2+} was strongly decreased only with both high DAc and high DM, as observed for the homogalacturonans. Differences in effects of acetylation on the binding of Ca^{2+} and Pb^{2+} were also observed for alginates (Lee et al., 1996).

The binding process can be further characterized by its cooperative or anti-cooperative mode, i.e. an increased or decreased affinity of the ligand (cation) for the polymer as the binding proceeds. Convex-shaped Scatchard plots (Scatchard, 1949) are indicative of cooperativity, while anti-cooperativity results in concave shapes. The plots, shown here for Pb^{2+} (Fig. 2), had clear convex curvatures for the binding of all three cations by pectate, indicating a cooperative mode in water as well as in the presence of supporting salt. All three cations were bound in an anti-cooperative mode by pectin, again both in the presence or absence of supporting electrolyte. Dronnet et al. (1996) observed cooperative binding in the presence of supporting salt (and non-cooperative in water) for Ca^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Cu^{2+} with a citrus pectin of DM 54, i.e. having about twice the charge density of the one used here, while Malovikova, Rinaudo and Milas (1994) reported cooperative binding of Ca^{2+} by polygalacturonate even in salt-free solutions. The lower charge density caused by the higher DM of the citrus pectin used here might be the cause of the disappearance of cooperativity. Using a slightly acetylated (DAc = 14) sugar-beet pectin of DM 58, Dronnet et al. (1996) obtained a cooperative behaviour only for strongly bound cations, i.e. Pb^{2+} or Cu^{2+}. Here also after acetylation a cooperative behaviour was only observed for binding of Pb^{2+} in the presence of supporting salts. These results support the interpretation by Dronnet et al. (1996) that the difference of behaviour in citrus and sugar-beet pectin is due to the presence of acetyls in sugar-beet pectin. Acetylated pectin, like its non-acetylated counterpart, showed anti-cooperative behaviour for the binding of all three cations.

3.4. ^{13}C CP-MAS NMR of homogalacturonan salts

The CP-MAS NMR spectra of Na^{+}, Ca^{2+} and Pb^{2+} salts of partially methylated homogalacturonans are shown in Fig. 3, and those of partially acetylated homogalacturonans in Fig. 4. Walkinshaw and Arnott (1981a,b) have shown by X-ray diffraction that the Na^{+} and Ca^{2+} salts of pectate (DM < 5) adopt a 3_1 conformation in the dry state, and that the same was probably true of the acid and methyl forms. On the basis of circular dichroism and by analogy with polyguluronate segments of alginate, Ca pectate is thought to form antiparallel dimers of polygalacturonate

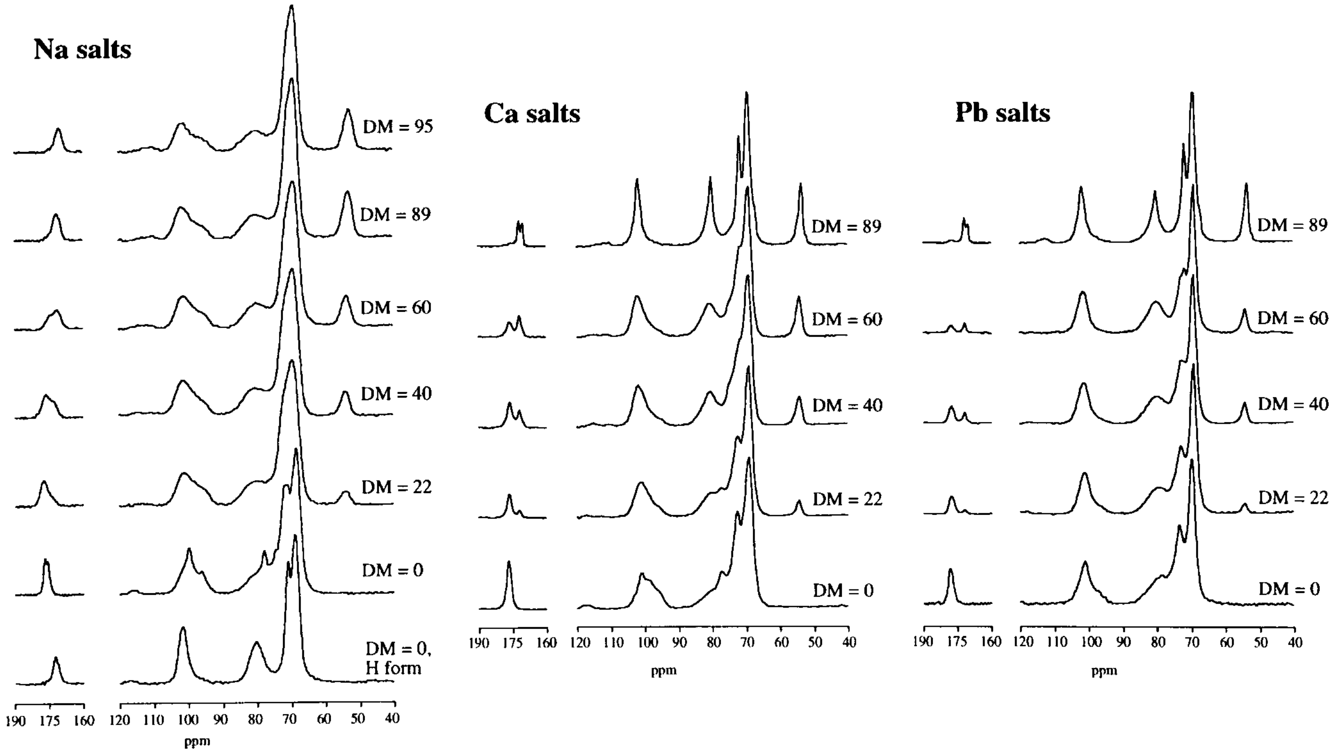

Fig. 3. ^{13}C CP-MAS NMR spectra of Na^+, Ca^{2+} and Pb^{2+} salts of partially methylated homogalacturonans.

chains in the 2_1 helix conformation in dilute gels, and these helices would further aggregate according to the "egg-box" model (Grant, Morris, Rees, Smith & Thom, 1973). Upon drying a polymorphic phase transition would take place, leading to 3_1 helices (Morris, Powell, Gidley & Rees, 1982). Molecular modelling indicated that three-fold helices, whether right or left handed, two-fold helices and even four-fold helices can be generated with low energy values and easy interconversion without noticeable modification of the length of the macromolecule (Cros, Hervé du Penhoat, Bouchemal, Ohassan, Imberty & Pérez, 1992). Jarvis and Apperley (1995), on concentrated pectin gels, observed a C-1 resonance centered on 101 ppm and the C-4 signals at 80–81 ppm for pectins forming 3_1 helices, while 2_1 helices had their C-4 at ca. 77 ppm.

The sodium homogalacturonate (DM 0) presented at least two different, relatively ordered conformations, with two signals for the C-1, at 100 and 96 ppm, two signals for the C-6 at 177 and 176 ppm and a C-4 signal at 78 ppm superimposed on a broad band with a shoulder at 80–81 ppm. The H form of the homogalacturonan had single signals for C-6, C-1 and C-4 at, respectively, 172, 102 and 80 ppm. For the Ca salts at DM = 0 the C-1 peak indicated varied conformations, with a dominant C-4 signal at 77 ppm, although other conformations are present. Comparison with the spectra of Jarvis and Apperley (1995) would indicate the presence of 2_1 helices. A different spectrum was obtained for the Pb salt, with a sharper C-1 signal at 100–101 ppm, indicative of a more ordered structure, and a C-4 signal shifted towards 80 ppm, with only a limited signal at 77 ppm. The 2_1 conformation appears much less abundant if not absent in the Pb salt of homogalacturonans.

Increasing degrees of methylation are reflected as an increase in the intensity of the peak at 55 ppm (C of the methyl) and for the C-6 signal by a displacement from ~177 to ~170 ppm. The C-4 and C-1 signals vary according to the cation present, with the differences being especially noticeable at low degrees of methylation. Very similar spectra were obtained for all the highly methylated samples: with a DM of 89, about 1 galacturonic acid in 10 is charged and conformation is probably determined by the nine in ten methyl-esterified residues. For Ca and Pb salts of the homogalacturonan of DM 89, the C-6 gave two signals at 171 and 170 ppm, while the C-4 gave one sharp signal at 101 ppm and the C-4 at 80 ppm. Partially methylated homogalacturonans gave lower resolution reflecting more variable conformations with reciprocal effects of ions and methyl groups, particularly for the Na form, although that might also be an artefact from the drying method. The evolution of the spectra of the Ca salts of partially methylated homogalacturonans show the disappearance of the 2_1 helices as the DM increases, with a displacement of the C-4 peak to 80–81 ppm at DM 89. All through the range of DM the conformation obtained for Pb salts was more similar to that observed for the H and the methyl form, i.e. presumably 3_1 helices.

The variation in the DAcs was reflected in the increasing intensity of the peak at 21 ppm for the CH_3 carbon of acetyl substituents. The signal for the carboxy carbon was at 176 ppm, i.e. superimposed on the C-6 of the galacturonic acid residues, except for the Pb form for which two signals

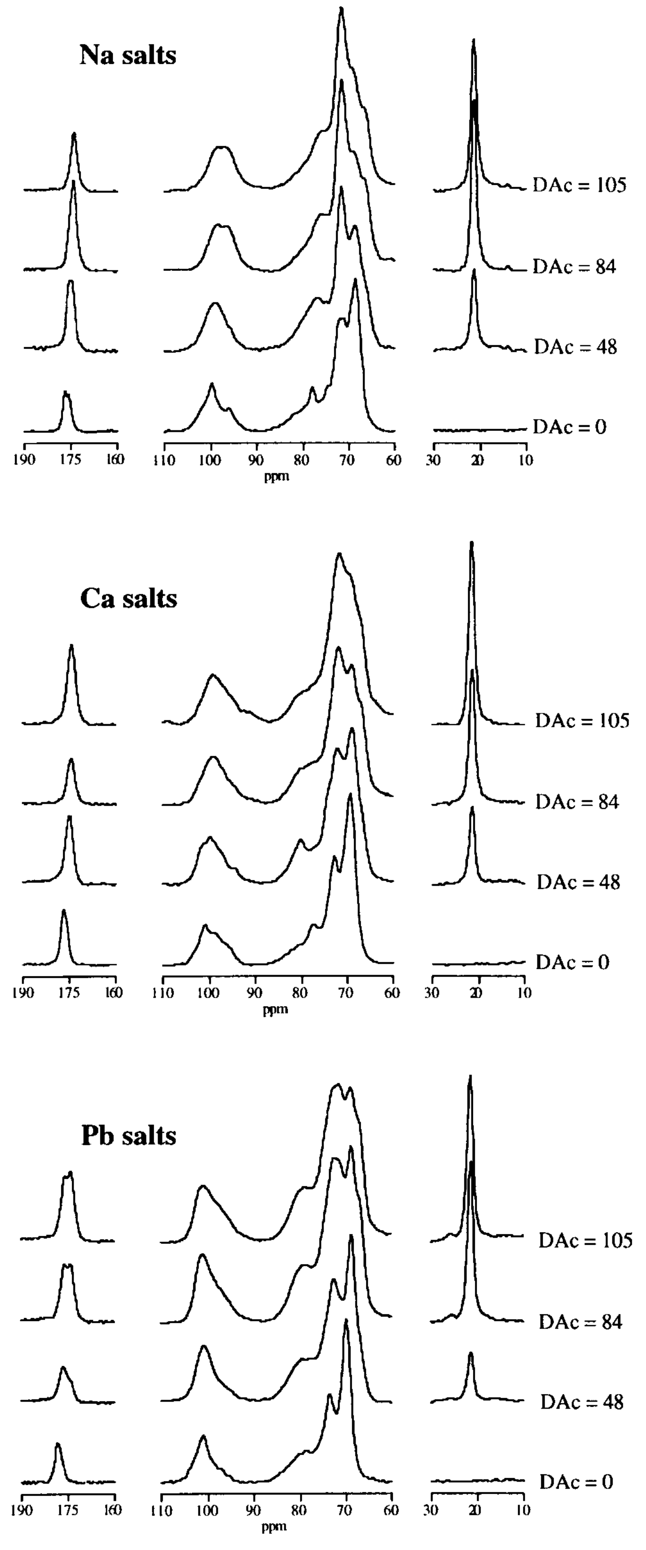

Fig. 4. ^{13}C CP-MAS NMR spectra of Na^+, Ca^{2+} and Pb^{2+} salts of partially acetylated homogalacturonans.

could be distinguished. Acetylation occurs on the secondary hydroxyls on C-2 and C-3, the signals of which consequently changed with the DAc. However these signals also varied with the salt form of the homogalacturonans, with Ca^{2+} salts being intermediate between the Na and Pb^{2+} salts. The C-1 and C-4 signals became less clearly defined with increasing DAcs, suggesting a wider range of conformations as acetylation introduced more irregularities in the chain, preventing orderly aggregation. For the Na salts two C-1 signals seemed to be superimposed.

4. Conclusion

The homogeneous distribution of the acetyl groups and the presence of monoacetylated galacturonic acid residues might explain some differences from earlier results where pectins were acetylated in suspension. Blockwise distribution of acetylated galacturonic acid residues might explain precipitation at higher DAcs with Ca^{2+} and Zn^{2+} obtained by Schweiger (1964), although the size of the homogalacturonans (degree of polymerisation of 70–100, Thibault et al., 1993) might also play a role. Similarly, Kohn and Malovikova (1978) observed the plateau for binding of Ca^{2+} by acetylated pectic acid at higher DAc values, which could be expected if, as stated by Rexova-Benkova, Mrackova, Luknar and Kohn (1977), the acetylation occurred preferentially as a double substitution for pectins suspended in formamide, as less galacturonic residues would be modified for the same overall DAc.

Acetylation, like methylation, decreased affinity of pectins and homogalacturonans for cations. However there were differences in the effects of these two forms of substitution, e.g. a greater effect of methylation on calcium binding. Contrary to methylation, acetylation should not influence the charge density: Kouwijzer, Schols and Pérez (1996) calculated that fully acetylated homogalacturonan helices would have stable conformations with lengths per residue of 0.414 or 0.445 nm, comparable to those obtained for non-acetylated pectates (0.43 nm) (Walkinshaw & Arnott, 1981a,b). Effects of acetylation are thus linked to modification of conformation and complexation, and underline the deviation of binding of divalent metal cations by pectins from the condensation theory. Presence of an anticooperative or cooperative character can be interpreted as prevalence of electrostatic repulsion or of facilitated binding of the divalent cations for two spatially close polyelectrolyte chains. For pectins and divalent cations, this appears to involve complex interactions between at least three parameters: charge density of the pectin (DM), ionic strength of the solution and strength of the interaction between the cation and the pectin. Presence of acetyl groups could lower the strength of binding of the cation to individual galacturonic acid residues or to hinder adoption by the polymer of binding-favourable conformations.

The ^{13}C CP-MAS NMR spectra of the partially methylated homogalacturonans showed that the H and Me forms adopted conformations which were similar and different from that of the Na salt. These results are in agreement with Jarvis and Apperley (1995), but in contrast with the conclusions of Walkinshaw and Arnott (1981a,b), who found roughly the same structure for Na salts and the H form of pectins (obtained by reacidification of sodium pectate). There appeared to be two different conformations in the sodium homogalacturonan sample. As solid-state NMR does not rely on the existence of a long-range order, it commonly detects more conformations than X-ray diffraction. The conformations of Ca and Pb salts were different: the difference in affinity is not due to a better or worse fit in a static egg box. Apparently a more limited number of conformations were present in the Pb salts, and these conformations were more similar to those of the H and Me forms. It might be that Pb^{2+} somehow was better able to interact with pectin in its 3_1 conformation, binding effectively without long range cooperativity. This would fit with the stronger interaction observed also at high DMs. CP-MAS NMR spectra of acetylated homogalacturonans show influence of the co-ion on conformation.

Binding of ions to polycarboxylates such as pectins is not due to a simple electrostatic condensation (Mattai & Kwak, 1986). It is also not due to an interaction only between the carboxyl and the ions as acetylation of the secondary hydroxyls of the galacturonic acid has a strong effect. Different conformations, i.e. different binding mechanisms are in play for weakly bound Ca^{2+} and strongly bound Pb^{2+}. In particular, the stronger binding of Pb^{2+} occurs in the absence of the 2_1 conformation, and is thus due to better binding by individual galacturonic acid moieties or easier insertion in another conformation.

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