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DETERMINATION OF THE DEGREE OF ESTERIFICATION AND THE DISTRIBUTION OF METHYLATED AND FREE CARBOXYL GROUPS IN PECTINS BY ^{1}H-N.M.R. SPECTROSCOPY

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ABSTRACT

The 400-MHz ^{1}H-n.m.r. spectra of pectins with various degrees of esterification (d.e.), as obtained by methylation and subsequent partial saponification, have been measured. The d.e. was obtained from the intensity of the signal for H-5, which shifted downfield (~3 p.p.m.) on esterification. Sequence-dependent shielding of H-5 and H-1 made it possible to determine the fractions of the four possible diads and the four galacturonate (G)-centred triads. The diad and triad frequencies obtained from the relevant peak-area ratios indicated that the action of alkali leads to pectic molecules in which free and methyl-esterified carboxylic acids are randomly distributed.

INTRODUCTION

Pectins are ionic polysaccharides extracted from plant cell walls, which consist of (1→4)-linked α-D-galacturonic (G) with a few (1→2)-linked α-L-rhamnose residues in the main chain, and some galactose and arabinose as side chains[1]. In Nature, the uronate residues occur partially esterified. The composition and the degree of esterification (d.e.) depend upon the species from which the pectin is isolated. The methyl ester is easily released in alkaline and acidic media, and commercial pectin preparations have a d.e. in the range 0–75%. Low-methoxyl pectins form gels in the presence of Ca^{2+} ions in neutral solutions by auto-cooperative binding of divalent cations between chains, leading to stable junctions as in alginate[2]. Pectins of higher ester content can form gels in an acidic medium on addition of sucrose[2].

Recently, the synergistic gelation of alginates and high-methoxyl pectin in the absence of sugar at low pH was reported[3]. Besides the content of L-guluronate residues in alginate, their sequence was correlated markedly with the gelling effect. Blocks of at least four contiguous guluronate residues were necessary for gelation to occur[3]. Most probably the sequence of methyl ester groups in pectin plays an equally important role. A model has been put forward which suggests that blocks

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of contiguous, esterified units interact with guluronate blocks in alginate to form strong junctions in the synergistic gel[4].

Although d.e. values can be determined by physico-chemical methods[5], it is more difficult to determine the distribution of methoxyl and free carboxyl groups along the pectin chains. Some knowledge about this distribution has been obtained indirectly by enzymic methods[6] and by studies of calcium binding which is strongly cooperative in pectins in which the carboxyl groups are arranged in blocks along the polymer chain[7].

N.m.r. spectroscopy is now a useful method for characterizing the composition and sequence of units in polysaccharides[8,9]. ^{13}C-N.m.r. spectroscopy has previously been used to determine d.e. values[10], and the resonances of C-1 of the minor constituents galactose and arabinose and C-6 of rhamnose have been discerned[11].

The present study demonstrates the feasibility of using high-frequency ^{1}H-n.m.r. spectroscopy in monitoring the sequence of units in pectins.

EXPERIMENTAL

Materials. — A commercial apple pectin of Danish origin with d.e. of 61% was almost completely esterified with methanolic sulphuric acid[12], then partially de-esterified with aqueous NaOH in order to obtain pectins with different d.e. It has long been assumed that this leads to a random distribution of the carboxyl groups over the macromolecule[13].

The molecular weight of some samples was reduced by sonication in an ice-cooled glass tube using a probe type sonicator (MSE Ultrasound disintegrator Mk 2) at 20 kHz. In order to avoid uncontrolled heating of the samples, the sonication was carried out intermittently for 1.5 h effective time at a power level of 60 W.

The only n.m.r. signal seen from neutral sugars, at 1.26 p.p.m., arises from L-rhamnose and its intensity corresponded to a content of 2%, which also was verified by chemical analysis[14].

Esterification[12]. — A sample (5 g) of freeze-dried apple pectin was mixed with methanolic 2M H_2SO_4 (200 mL) at 4°. After storage for 4 days at 4°, the methanolic sulphuric acid was replaced. After an additional 5 days at 4°, the pectin was collected and washed twice with methanol, then with methanol–water (3:1) (ion free). The pectin was moistened with dimethyl ether, stored for 2 h at 60°, and then kept in a vacuum desiccator.

The d.e. values of a series of pectin samples were determined by an acid/base titrimetric method[15].

De-esterification. — Samples of esterified pectin (250 mg, d.e. 98%) were each dissolved in 30 mL of distilled water. The pH was adjusted to the selected value, in the range 8–12, by the addition of 0.1M NaOH and kept at this value by titration with 0.01M NaOH. The de-esterification was stopped by adjusting the pH to 5.5 by titration with 0.1M HCl. The temperature was 25°.

N.m.r. spectroscopy. — The samples were dissolved in D_2O in 5-mm tubes at pD 5 (20 mg/mL). The deuterium resonance was used as field-frequency lock, and chemical shifts were expressed relative to the signal of internal sodium 3-(trimethylsilyl)propionate-d_4. Sodium triethylene tetra-amino hexa-acetate (25 mg/mL) was added to prevent traces of divalent cations from interacting with the pectin. 400-MHz ^{1}H-n.m.r. spectra were recorded with a Bruker WM-400 spectrometer, using 16k data points and a spectral width of 2400 Hz. In order to reduce the HDO peak, a 180°–*t*–90° pulse sequence (t ~2–3 s) was employed with a recycling time of 7 s. The spectra were recorded at 90° in order to shift the HDO peak from the region for anomeric protons, to diminish viscosity, and, thereby, improve the spectral resolution. Nevertheless, the spectra showed a rather small sequential splitting and they were reconstructed by computer simulations from which the relative peak areas were obtained. A computer program was written to superimpose the proper number of Lorentzian lines for which the positions were known from the spectra. An intelligent guess of the line widths was made and the program then iterated all line widths within reasonable limits until the best least-squares fits of the peak heights were found.

RESULTS AND DISCUSSION

Fig. 1 shows the region of H-1 and H-5 resonances in the ^{1}H-n.m.r. spectra of a series of pectin samples with d.e. values within the range 100% to 7.9%. The main peaks in the last spectrum therefore correspond to those reported for galacturonic oligomers[16], with that for H-1 at low field, that for H-4 at high field, and that for H-5 in between. As the d.e. increased, the H-5 resonance of the galacturonate residues became split, decreased, and was almost absent at d.e. ~100%. Evidently, this resonance shifts downfield upon esterification and, in the spectrum of the fully esterified pectin, the resonance of H-5 is much closer to that of H-1. Partial protonation of the carboxyl groups, as obtained by decreasing pD, also shifted downfield the three overlapping lines at the position of the H-5 resonances in the charged residues (data not shown). Therefore, these lines must arise from H-5. The remainder of the spectrum was almost unchanged, apart from small splittings (<0.1 p.p.m.). These findings suggest that the sugar residues exist in the chair conformation regardless of esterification, sequence, or pD, and that the splittings must be associated with the sequence of sugar units.

Quantitatively, the values of d.e. are related to the intensities (I) of the line patterns A and B (Fig. 2) by the relationship:

$$\text{D.e. (\%)} = \frac{I_A - I_B}{I_A + I_B} \times 100, \qquad (I)$$

where B denotes the three lines from H-5 of charged units discussed above, and A

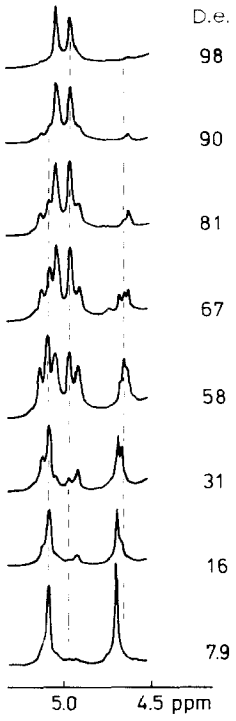

Fig. 1. 400-MHz ^{1}H-n.m.r. spectra of pectins with various d.e. values.

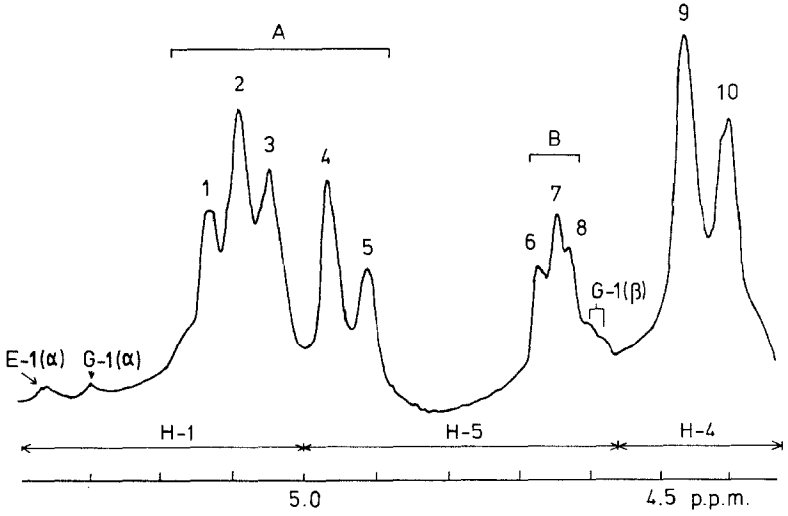

Fig. 2. The lower-field region in the 400-MHz ^{1}H-n.m.r. spectrum of a pectin with d.e. ~56%. Peaks 1–5 are due to the diads EG(GE), GG + GE(EG), EE, EE, and EG, respectively, peaks 6–8 are due to the triads, GGG, EGG + GGE, and EGE, respectively, and peaks 9 and 10 are due to the monads E and G, respectively. The lines denoted by E-1(α), G-1(α), and G-1(β) are due to H-1 at the reducing end groups of esterified and unesterified α-anomer and unesterified β-anomer, respectively.

TABLE I

D.E. OF PECTINS DE-ESTERIFIED FOR TIMES *t* AT pH 11, ESTIMATED BY N.M.R. SPECTROSCOPY AND THE TITRIMETRIC METHOD[15]

t *(min)*	*D.e.*	
	N.m.r. method	*Titrimetric method*
0	98	92
3	55	59
9	51	45
26	33	29
34	24	31

stands for the five lines at lower field, including those of H-1 and H-5 of esterified units. The numerator in equation *1* is the sum of the intensities of the resonances of H-1 and H-5 in esterified units, and the denominator is the total of the intensities of these resonances. Five pectin preparations obtained by de-esterification at pH 11 for different times were analyzed for methoxyl content by this n.m.r. method, and the results agreed reasonably with the d.e. values determined titrimetrically (Table I).

Concerning the five low-field peaks, the three signals at the lowest field are provisionally assigned to H-1 and the other two to H-5 of esterified units. A splitting of ~0.05 p.p.m. was found within these two groups of lines, showing that the chemical shifts depend on whether the nearest neighbour is esterified or not. The relative areas of the two H-5 resonances of residues E (esterified galacturonate residues) point towards a random distribution of ester groups. The left-hand line preponderates at high d.e. values and must arise from EE diads, whereas the right-hand line must represent H-5 in an E residue having a neighbouring unesterified unit (G), *i.e.*, the diad EG. The latter peak preponderates at low d.e.

In order to interpret the low-field resonances including the patterns of H-1 and the pair of H-5 lines from esterified units, their relative intensities were plotted against d.e. With the interpretation denoted in Fig. 2, the results support a random distribution of ester groups as illustrated by the solid lines in Fig. 3. H-1 from GG resonates at the position of that in the fully charged polymer, whereas the signal due to EE, the dominant diad at high d.e. values, appears at a slightly higher field. The H-1 lines from EG and GE, respectively, which are typical for alternating structures, are equally intense for long chains and cannot be discriminated in our spectra. However, one of these lines coincides with that from GG and the other gives rise to the low-field signal at 5.15 p.p.m. The signals from H-5 of charged residues exhibit a different signal pattern displaying three lines (6–8, Fig. 2) which means that this resonance is sensitive to the nature of both neighbouring units, thus providing the key for directly measuring the G-centred triad frequences.

The identification of the symmetric triads, GGG and EGE, is given by the

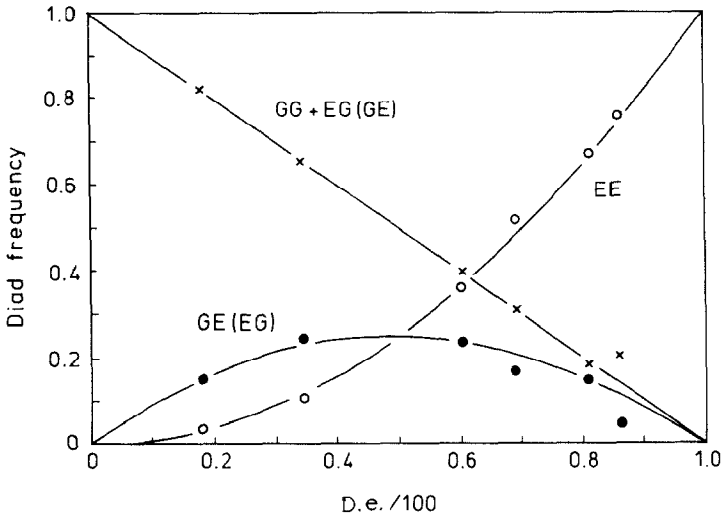

Fig. 3. Experimental probabilities (x, ○, and ●) for diad frequencies in pectins, compared to calculated (solid lines) Bernoullian diad probabilities.

lines in the bottom and top spectrum in Fig. 1, respectively. De-esterification initially introduces single charged units which are identified by EGE triads. As the reaction proceeds, blocks of contiguous charged units are formed and, besides GGG, the termination of these blocks also gives rise to the asymmetric triads GGE and EGG. The probabilities of the latter triads have a maximum at d.e./100 = 1/3 in a random distribution (d.e./100 is the probability of finding an esterified unit), which fits in with the variation in intensity of the intermediate line showing a maximum at around d.e. = 30–40% (Figs. 1 and 4). The relative areas of the peaks for these triads multiplied by the fraction of unesterified units (1 − d.e./100) correspond to the triad probabilities. Although the accuracy of the results is not high, the experimental points fit the calculated Bernoullian triad distribution reasonably well (Fig. 4). The latter is shown as solid lines marked by GGG, EGG + GGE, and EGE with fractional values given by $F_{GGG} = (1 - \text{d.e./100})^3$, $F_{GGE} = F_{EGG} = (1 - \text{d.e./100})^2 \cdot \text{d.e./100}$, $F_{EGE} = (\text{d.e./100})^2 \cdot (1 - \text{d.e./100})$.

The experimental diad and triad fractions represent values giving the best fit in reconstructed spectra of H-1 and H-5 by taking the following relationships into account:

$$F_G = F_{GG} + F_{GE} = 1 - \text{d.e./100} \tag{2}$$

$$F_{GG} = F_{GGG} + F_{EGG} \tag{3}$$

$$F_{GE} = F_{GGE} + F_{EGE}. \tag{4}$$

Corresponding expressions for esterified units, E, are given by interchanging G and E. Since the chains are fairly long (d.p. > 25), it is assumed that

$$\left.\begin{aligned} F_{GE} &= F_{EG} \\ F_{GGE} &= F_{EGG} \end{aligned}\right\} \quad (5)$$

According to the assignments (Table II, Fig. 2), this means that the intensities of lines are interrelated in different ways independently of any statistics. It can be inferred easily that signals 1,5 and 3,4 are pairs of equally intense lines as is seen clearly in Fig. 1. The intensity of line 2, composed of the diads GG + GE(EG), matches the total intensity of the G-centred triads, *i.e.*, the lines 6–8.

As shown in Fig. 2, the H-4 signal is also split into a pair of components and the ratio of their intensities suggests that they contain information about monad frequencies only. Esterification causes a downfield shift of 0.06 p.p.m. However, the line-shape is distorted by the solvent HDO peak, and, therefore, the H-4 resonances has not been used for quantitative analysis.

As shown in Fig.2, there are also small resonances due to H-1 of the reducing end-groups which give information on the molecular weight. They have been intensified in the spectrum from this particular sample which had been degraded by ultrasonication. Even if the spectrum still was not sufficiently resolved, approximate J values in addition to chemical shifts could be estimated. For unesterified

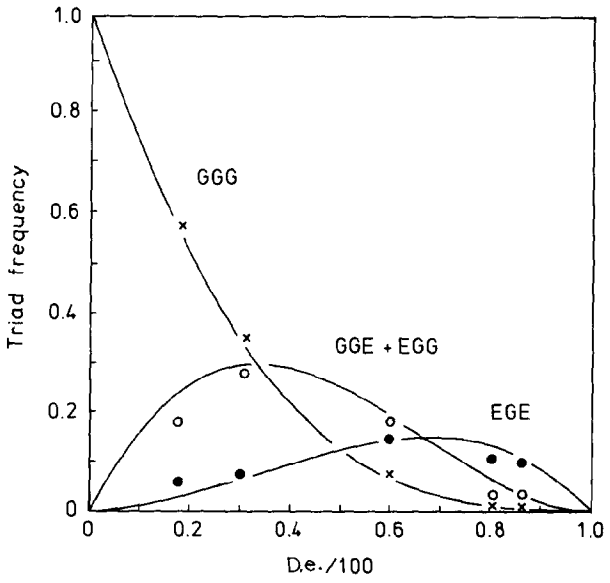

Fig. 4. Experimental probabilities (x, O, and ●) for unesterified galacturonate (G)-centred triads (points), compared to calculated (solid lines) Bernoullian triad probabilities.

TABLE II

CHEMICAL SHIFTS[a] OF PROTON RESONANCES FOR SOLUTIONS OF PECTIN IN D_2O AT 90° (pD 5)

Residue[b]	*Sequence*	*Proton*				
		H-1$_{red.\ end}$		*H-1*$_{int}$	*H-5*$_{int}$	*H-4*$_{int}$
		(α)	(β)			
E	EE	5.36[c]		5.05	4.96	4.47
	EG			5.1[d]	4.91	
G	GG	5.30[c]	4.59	5.10		4.40
	GE			5.15[d]		
	GGG				4.67	
	EGG				4.64	
	GGE				4.64	
	EGE				4.61	

[a]In p.p.m. downfield from the resonance of internal sodium 3-(trimethylsilyl)propionate-d_4 for the resonances of the left-hand residue in the diads, and of the intermediate residue in the triads. [b]G, D-galacturonate; E, esterified D-galacturonate. [c,d]Assignments may have to be interchanged.

units, they resemble those of the corresponding oligomers[16], namely, 5.3 and 4.59 p.p.m. for the α and β anomers, respectively, with $J_{1,2}$ ~7.5 Hz for the latter. The α anomer displays two resonances with chemical shifts depending on the presence or absence of esterification. The provisional assignments are given in Table II and Fig. 2. H-1 of the β anomer resonates close to that of the G-centred triad line pattern, and only one split signal ($J_{1,2}$ ~7.5 Hz) can be seen. A downfield shift upon esterification, as suggested for the α anomer, would produce a signal masked by the H-5 peaks in this spectrum.

Work on the sequence of sugar units of pectin de-esterified by other methods is in progress.

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