Eur Biophys J (1998) 27: 127–136

ARTICLE

Laurent Catoire · Renée Goldberg · Monique Pierron
Claudine Morvan · Catherine Hervé du Penhoat

An efficient procedure for studying pectin structure which combines limited depolymerization and ^{13}C NMR

Received: 10 July 1997 / Accepted: 12 November 1997

Abstract A protocol for partial thermally-induced depolymerization of differently methoxylated pectin samples is described. The resulting macromolecules have been fully characterized with various complementary techniques, such as size exclusion chromatography (SEC), potentiometry, viscometry and ^{13}C NMR. Optimum conditions afford samples at 50–80% yield with weight-average molecular weights in the 4 to 20 kDa range. The major fraction of these polysaccharides adopts the random-coil conformation and such samples are suitable for ^{13}C NMR structural studies at room temperature. The methoxyl distributions of two apple pectin samples with a degree of esterification (DE) between 54 and 74% and a citrus pectin (DE, 72%) were shown to be random in nature, whereas that of a lightly methoxylated apple pectin (DE 39%) was partially blockwise. The carbon relaxation parameters of the depolymerized pectins attain asymptotic values for $M_W > 4$ kDa. The M_W values estimated from intrinsic viscosity data with the Mark-Houwink relationship reported for native pectins are in good agreement with those obtained by either end-group analysis (NMR) or SEC. Thus, all the physicochemical data indicate that the secondary structure of the isolated chains of depolymerized pectin is closely related to that of the parent polymers. Finally, pectinmethylesterase activity towards the depolymerized pectins was similar to that of the untreated samples.

Key words Pectins · ^{13}C NMR · Relaxation data · Thermally-induced depolymerization · Pectinmethylesterase

L. Catoire · C. Hervé du Penhoat (✉)
URA CNRS 1679, Département de Chimie,
Ecole Normale Supérieure,
F-75231 Paris Cedex 05, France

R. Goldberg · M. Pierron
Laboratoire d'enzymologie en milieu structuré,
Institut Jacques Monod,
2 Place Jussieu, F-75251 Paris, France

C. Morvan
SCUEOR CNRS URA 203, Faculté des Sciences,
Université de Rouen,
F-76821 Mont-Saint-Aignan Cedex, France

Introduction

Pectin, one of the main structural polysaccharides of plant cell walls, is composed of a (1→4)-linked α-*D*-galacturonan backbone with regions containing alternating units of →4)-α-*D*-Gal*A* (1→ and →2)-α*L*-Rha*p* (1→. The homogalacturonan parts of the polymer are referred to as "smooth" regions while the rhamnose-rich zones are called "hairy" regions as the latter sugars carry neutral oligosaccharide side-chains frequently composed of D-Gal*p* and L-Ara*f* sugars (Fig. 1). A considerable proportion of the galacturonic acid residues of the backbone is methyl-esterified. In vivo, pectin is considered to be a key molecule in the loosening of cell walls and it is involved in the growth, development, and senescence of all higher plants. Commercially, pectin has found widespread use in both the food processing and pharmaceutical industries owing to its gelling capacity. Although much work has been devoted to

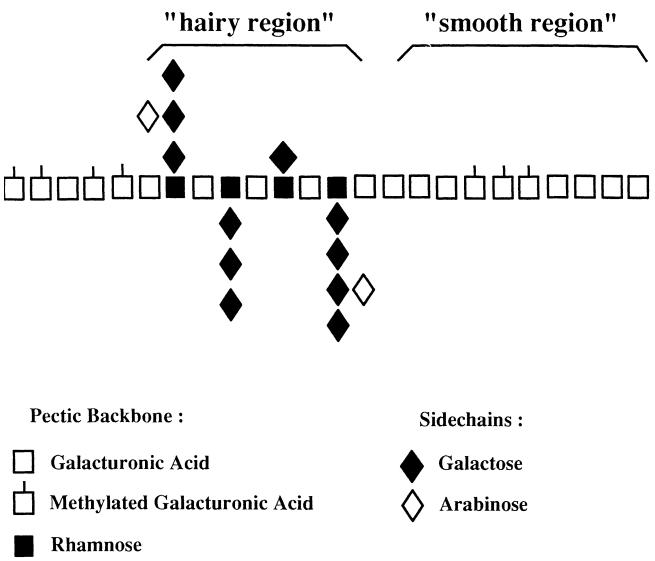

Fig. 1 Schematic drawing of pectin (rhamnogalacturonan I) showing the smooth and hairy regions

characterizing pectin at both the molecular and macroscopic levels, a clear picture of its three-dimensional network has not yet emerged (Cros et al. 1996).

Although NMR spectroscopy is normally the method of choice for probing the primary or secondary structure of polysaccharides, in the case of pectin, ^{1}H and ^{13}C NMR spectra recorded at room temperature have very low signal-to-noise ratios owing to the reduced molecular mobility of aggregated species. It has been recently shown that ^{1}H (Andersen et al. 1995; Grasdalen et al. 1996) and ^{13}C NMR spectroscopy (Westerlund et al. 1991) are well-suited to probing the primary structure of pectins when spectra are recorded at high temperatures (85–95°C) which favor a random-coil conformation. However, under such extreme conditions, the secondary structure is not necessarily related either to the conformation recognized in vivo by cell wall enzymes or to the geometry required for gel formation.

In the present work we set out to develop a procedure for obtaining pectin samples suitable for NMR spectroscopy at room temperature. Basically, this approach requires an efficient depolymerization technique which would produce shorter chains of unaltered primary structure which are less prone to aggregation. At the same time, a complete description of the depolymerized polysaccharide would be needed to demonstrate that it could successfully adopt the secondary structure corresponding to that of dilute solutions of native pectin. The following information was required to validate the protocol: 1/ the primary structure of the pectin samples both before and after depolymerization, 2/ the average length of the depolymerized chains, 3/ the "visible" fraction (random-coil conformation) of the depolymerized pectin, 4/ PME activity towards the depolymerized substrates and finally 5/ the efficiency of the protocol with differently methoxylated pectins. The first two points could be established with various complementary techniques, such as size exclusion chromatography, potentiometry, viscometry and ^{13}C NMR while the third and fourth points would rely on ^{13}C NMR and kinetic measurements of PME activity, respectively.

Methods

Pectin samples

Three apple pectin samples with varying DE (PN-1, Apple Pectin Classic AU 201-74.4%; PN-2, Apple Pectin Classic AU 602-53.6%; and PN-3, Apple Pectin Classic AU 701-39.4%) and a citrus pectin sample (PN-4, Citrus Pektin Classic CU 301-72.8%) were graciously supplied by Herbstreith & Fox KG (Neuenbürg). The fully deesterified citrus pectin sample (PN-5) was a commercial product (Sigma).

Depolymerization procedure

0.400 g of methoxylated pectin, PN-1 and 10 mL of pure water (18 megohm, Sigma) were heated at 85°C for 30 min while rotating the mixture on a rotary evaporator at atmospheric pressure. Mechanical stirring was occasionally necessary to pulverize clotted lumps. The resulting gel was then placed in a vacuum-sealed tube (19 cm long, 1.6 cm in diameter) and heated at 155°C for 2 h. Vigorous boiling of the opaque mixture was observed for a certain period and then the gel became still and translucent with simultaneous formation of a small amount (<5%) of colored precipitate. Upon cooling the solution was filtered over a cotton plug and evaporated under reduced pressure to afford a raw product, PH-1 (85–90%). This residue was dialyzed against distilled water in a membrane (Sophyc, cutoff 10 kDa; Spectra/Por, cellulose ester from Bioblock Scientific for cutoffs at 50 or 300 kDa) for 24 h and then evaporated under reduced pressure to yield the depolymerized product, PD-1 (~70% yield for the membrane with a cutoff of 10,000).

In the case of PN-1, PN-3 and PN-4, this procedure was modified as follows: the temperature was increased from 135°C to 153°C in four 15-min steps of 4–5°C. After a total reaction time of 1 h the sealed tube was cooled and workup was performed as described above.

Viscometry

Viscosities were measured with a Cannon-Manning semi-micro viscometer at 25±0.1°C over a range of concentrations (PN 0.1 to 0.4 g/dL; PD 0.2 to 5.0 g/dL) on pectin solutions in 2.5 mM NaCl at acidic pH (PN – pH 2.8–3.0; PD – pH 4–5). The intrinsic viscosities $[\eta]$ of the samples were determined by the double Huggins and Kraemer extrapolation (Garnier et al. 1993) using the Mark-Houwink relationship established by Garnier et al. (1993) and these data have been given in Table 1.

Size exclusion chromatography

Size exclusion chromatography was performed in 0.2 M NaCl on a column of Sephacryl S100 (Pharmacia, 5–100 kDa, 300 mm×26 mm, 75 ml h^{-1}). The eluted components were detected by measuring absorbance at 214 nm. Fractions were combined and MHDP colorimetric estimation of galacturonic acids performed (Blumenkrantz and Asboe-Hansen 1973). When needed, polymers were dialysed and freeze-dried. Elution profiles are described by the absorbance at 214 nm as a function of Kav where Kav is equal to (Ve–Vo)/(Vi–Vo) and Vo, Vi and Ve are respectively, the void volume, the inclusion volume of the gel and the retention time of the pectin sample.

Potentiometry

The degree of esterification was estimated potentiometrically as previously reported (Goldberg et al. 1986). Briefly, samples were dialysed, protonated with Amberlite IR 120H, and neutralized under nitrogen flux with sodium hy-

Table 1 Weight-average molecular weights, M_w, of native (PN), heated (PH) and dialyzed (PD) pectin samples estimated from intrinsic viscosity [η] along with the mass equivalent (MM'eq) obtained by potentiometry. The degree of esterification (DE) indicated by the supplier is also given

PN Sample (DE)	[η] of PN	M_w (kDa) of PN*	MM'eq of PN	Cutoff of Dialysis Membrane in kDa	Yield of PD	[η] of PD	M_w (kDa) of PD* from [η]	M_w (kDa) of PD from ^{13}C NMR	MM'eq of PD
1 (74.4)	5.67	188	200	10	70	0.27	3.7	4	215
				50	56	0.30	4.3	6.6	
				300	15–20	0.86	17	>20	
2 (53.6)	4.71	148	200	10	55	0.12	1.3	2	170
				50	45	0.18	2.2	2	
3 (39.4)	4.86	154	190	10	45 (65)**	0.12 (0.27)	1.3 (3.7)	2 (4)	200
				50	30	0.10	1.1	2.9	
4 (72.8)	4.78	151	180	10	60	0.39	6.1	20	235
				50	50	0.90	17.6	>20	

The average yield after the heating is around 85–90%. Samples **1–3** were apple pectin while **4** was citrus pectin. * Calculated using the Mark-Houwink relation reported by Garnier et al ($[\eta] = 4.36\ 10^{-4}\ M_w^{0.78}$). ** Obtained under milder conditions (see experimental section)

droxide. After saponification (4 h, 4 °C) the new negative charges were estimated as above. The degree of esterification was calculated as the number of new charges over the total amount of galacturonic acid.

NMR spectroscopy

The majority of the ^{13}C NMR measurements were conducted at 100.6 MHz on 4% (w/v) aqueous solutions (D_2O) of pectin at 21°C on a Bruker DRX 400 spectrometer. Chemical shifts were referenced to the C1 signal of the internal standard (93.85 and 100.00 ppm for sucrose and α-MeGlc*p*, respectively). For comparative purposes spectra of PN-1 were also acquired at 85°C.

Quantitative analysis. The "visible" fraction (non-aggregated species) of the pectin samples, PN, PH, and PD, were determined by comparison of the integrated signals of the methine carbons in gated decoupled ^{13}C spectra with those of an internal standard (either sucrose or α-MeGlc*p*). A prescan delay of 5 times the longest T_1 was used.

Carbon relaxation measurements. Spin-lattice relaxation times were measured with the inversion-recovery sequence. The recycle time was greater than $10 \times T_1$ and data were collected for 10-tau values which varied from 6 ms to $2 \times T_1$. The integrals of the peaks were fitted to a three-parameter exponential function using the spectrometer system software. Experiments were run at least twice. Spin-spin relaxation times were obtained by measurement of the half-height linewidth rather than the spin-echo technique. An inhomogeneity contribution was established from the natural line width of a reference sample of acetone (<0.4 Hz). Nuclear Overhauser enhancements (nOe) were determined by comparison of the integrals of gated decoupled and fully decoupled spectra. A prescan delay of $7 \times T_1$ was used.

Enzymic demethylation

Biological material. Cell walls were isolated from the upper 2.5 cm of hypocotyl tissues of 3-day-old seedlings of mung bean (Vigna radiata (L.) Wilzeck) according to a procedure previously described (Goldberg et al. 1986).

Enzymatic assays. PME activity was measured titrimetrically by following the increase in free carboxyl groups. The carboxyl groups released by PME from pectin in the presence of 150 mM NaCl were titrated with 10 mM NaOH under nitrogen, the pH being maintained at 7.60 with an automatic titrator (TTT 80, Radiometer).

Results and discussion

Intact pectin

The weight-average molecular weights, M_W, of the native pectin samples PN-1 to PN-4, which are given in Table 1, were found to be between 150 and 200 kDa from viscosity measurements using the Mark-Houwink relationship reported by Garnier et al. (1993). It has been demonstrated through combined viscosity and light-scattering measurements of GPC fractions that commercial pectins of the same origin were polydisperse with a wide distribution of molecular weights (Berth and Lexow 1991). In the present study, no attempt was made to purify the commercial pectin samples but size exclusion chromatography corroborated a M_W value of greater than 100 kDa as all of these samples were eluted in the void volume of a Sephacryl S100 column (-curve labeled with filled squares in Fig. 2). The mass equivalent, MM'eq, established for PN-1 to PN-4 by potentiometry (200 ± 30) indicated that galacturonic acid residues were the main components of the pectin samples.

A ^{13}C NMR spectrum was recorded for PN-1 under the conditions recommended by Westerlund et al. (4% w/v;

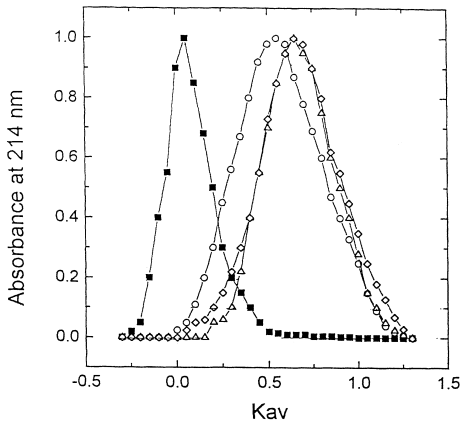

Fig. 2 Elution profiles of size exclusion chromatography of apple pectin samples on a Sephacryl S100 column: ■ native pectins, ○ PD-1 (DE, 74.4%), △ PD-2 (DE, 53.6%), ◇ PD-3 (DE, 39.4%). Absorbance at 214 nm has been plotted as a function of Kav

85°C). The DE (74%) and the proportion of side-chains (β-galactan, 7%; α-ara*f* residues, 3–4%) determined from these data (Hervé du Penhoat et al. 1987) confirmed that this pectin was highly methylated and contained only a small amount of neutral sugars. A quantitative inverse-gated experiment at 85°C was conducted in the presence of a known amount of α-MeGlc*p*. Comparison of the methine carbon integrals showed that only about 35% of the sample was detected under these conditions. Loss of intensity in NMR spectra of pectin due to the presence of aggregated species (junction zones) which exhibit very reduced molecular mobility has been reported previously by many authors (Rinaudo and Ravanat 1980; Keenan et al. 1985; Goldberg et al. 1989). However, as the DE established for the pectin sample from chemical analysis (74%) was identical to the one detected for the visible proportion, it could be assumed that the fraction of random-coil conformer detected by NMR was representative of the macromolecule as a whole.

Depolymerized pectin

Depolymerization method. Various protocols have been recommended for depolymerizing polysaccharides, including ultrasonication (Szu et al. 1986; Chun and Park 1994), treatment with base (Aspinall 1982), partial acid hydrolysis (Powell et al. 1982), and heating at high temperature (> 150°C) for a short time (Gidley and Bociek 1988). The depolymerization had to be very limited in order to produce a fairly long average sequence which would be able to adopt the secondary structure of the native macromolecule. It has been recently shown (Catoire et al. 1997) that the secondary structure of the random-coil form of sodium polygalacturonate consists of a two-fold helical segment of roughly 30 residues of galacturonic acid. Partial acid-catalyzed hydrolysis, which generally leads to fairly short segments (DP 25, Powell et al. 1982), did not seem appropriate. In contrast, ultrasonication would not necessarily be expected to reduce the M_W sufficiently as the M_W of polysaccharides depolymerized with this technique tends towards a finite value of 50 kDa (Szu et al. 1986). Thermally-induced depolymerization has been used recently by Oviatt and Brant (1994) in a detailed study of aqueous xanthan. In the case of pectin, this approach would be expected to lead to β-elimination of deesterified residues and afford depolymerized products without modification of the polysaccharide primary structure and this was an obvious requirement for evaluating the methoxyl group distribution.

The scheme for the thermally-induced depolymerization of native apple pectin with a DE of 74%, PN-1, is given in Fig. 3. Heating PN-1 in a sealed tube at 155°C for 2 h produced a colloidal mixture which was separated into a small amount (< 5%) of a brownish solid residue and an aqueous solution of PH-1. It was verified in the ^{13}C spectrum of PH-1 prior to evaporation of the solvent (spectrum not given), that the thermal treatment did not produce significant amounts low-molecular weight compounds such as furfurals which have been described in procedures in which polysaccharides are heated at extreme temperatures (Montreuil et al. 1981). Upon evaporation of the solvent PH-1 was recovered in 85–90% yield and its ^{13}C spectrum at 21°C is given in Fig. 4A. In the anomeric region, signals (~ 10%) of reducing sugars (94.11 and 97.86 ppm for the α and β residues, respectively) are readily identified along with those of the non-reducing sugars of the depolymerized pectin (102.13 ppm). The excellent signal-to-noise (S/N) ratio of PH-1 allowed identification of the signals of β-galactan side-chains (< 10%; C1 106.06, C4 79.37, C5 76.21, C3 75.08, C2 73.58, and C6 62.45).

Dialysis against a membrane with a molecular weight cutoff of 10 kDa affords PD-1 in 70% yield and the signals of the reducing anomeric carbons were considerably attenuated in the corresponding ^{13}C spectrum (Fig. 4B). It could be concluded from the low intensity of anomeric signals for reducing sugars that the degree of polymerization

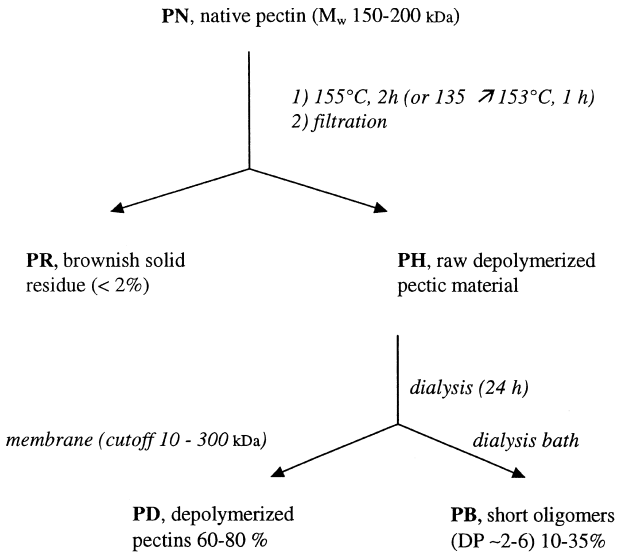

Fig. 3 Thermal depolymerization procedure

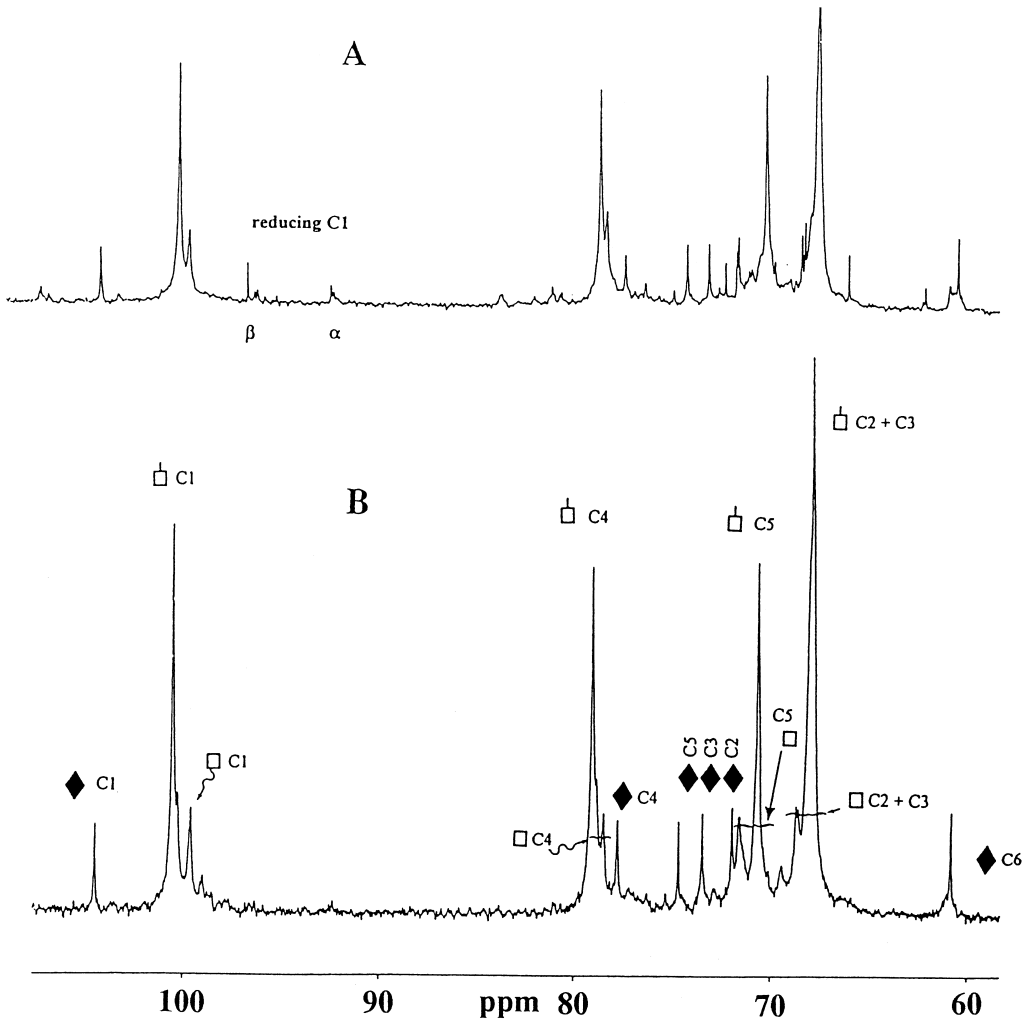

Fig. 4A,B Partial 100.6 MHz ^{13}C NMR spectra of depolymerized pectins (DE 74.4%): **A**, before dialysis (PH-1) and **B**, after dialysis against a membrane with a cutoff of 10 kDa (PD-1). Assignments for the signals of the ring carbons of the esterified (⍈) and unesterified (□) galacturonic acid and galactose (◆) residues have been indicated

(DP) was greater than 20 (i.e. $M_W \geq 4$ kDa). The average value of M_W evaluated for PD-1 from the intrinsic viscosity was 3.7 kDa (Table 1). Comparison of the PD-1 Kav value (0.50±0.05) obtained from the elution profile of PD-1 on a Sephacryl S100 column (curve labelled with open circles in Fig. 2) with that of an authentic dextran sample (0.30±0.05 for a M_W of 40 kDa) corroborated that M_W was considerably lower than 40 kDa. Dialysis of PH-1 against membranes with cutoffs of 10, 50 and 300 leads to samples showing a considerable range of $[\eta]$ values (corresponding to a spread in M_W values of roughly 3.7–17 kDa), indicating a polydisperse mixture. In the case of the membrane with a cutoff of 10 kDa, evaporation of the dialysis bath afforded about 8% of a mixture of oligomers with a DE (12%) and an average DP of about 2 as determined by a fully-decoupled ^{13}C spectrum.

Generally speaking, when the DE of the apple pectins decreased both the yield of dialyzed pectin and the weight-average molecular weight determined from viscosity measurements were lower, Table 1. The Kav profiles of PD-2 and PD-3 on a Sephacryl S100 column (curves indicated with open triangles and diamonds, respectively in Fig. 2) confirmed this tendency. Thermal treatment of the citrus pectin sample afforded a pectin with a M_W of 6 kDa. Finally, the MM'eq values of the various PD pectin samples in Table 1 confirmed that galacturonic acid was the major component.

In the case of the pectin sample with the lowest DE (PN-3 39.4%), when thermally-induced depolymerization was conducted under milder conditions (data in parentheses) both the yield of PD-3 and the value estimated for M_W from $[\eta]$ increased notably (more than twofold). Moreover, the average DP (~ 5) of the oligomers recovered upon evaporation of the dialysis bath, PB-3, was also higher. This latter PD-3 sample gave the lowest Kav (0.40±0.05, curve not shown) detected for the various PD polymers. The integral of the reducing sugar signals in the ^{13}C spectrum of this PD-3 sample was less than 5% of that of the non-reducing residues, pointing to a M_W greater than 4 kDa. The increase in chain length upon conducting depolymerization under milder conditions proved to be a general tendency as such treatment of PN-1 and PN-4 afforded PD-1 and PD-4 with M_W values estimated from $[\eta]$ of 20 and 21.2 kDa, respectively.

The DE, relative intensity of the β-galactan anomeric signal, and "visible fraction" for the various PD samples

Table 2 Quantitative analysis of pectin samples by ^{13}C NMR at 294 K in the presence of an internal standard. The visible fraction (%), the corresponding DE, the percentage of side-chain, and the triad distribution are indicated

Pectin sample Membrane cutoff in kDa (DE %*)	Pectin Composition			Triad Distribution		
	DE %	Visible fraction %	Side-chain %	UEU % (δ in ppm)	EEU+UEE % (δ in ppm)	EEE % (δ in ppm)
PH-**1** (74.4)	71 (65–70)	53 (44)	10 (9–10)	0	45 (172.38)	55 (172.34)
PB-**1** (74.4)	≈12		0			
PD-**1** 10 (74.4)	72	70	5	0	40 (172.41)	60 (172.34)
PD-**1** 50 (74.4)	68	69	6	15 (172.52)	35 (172.42)	50 (172.34)
PD-**1** 300 (74.4)	64	60	8	10	35	55
PD-**2** 10 (53.6)	48	76	6	25 (172.46)	45 (172.40)	30 (172.34)
PD-**2** 50 (53.6)	48	67	5	25 (172.48)	45 (172.42)	30 (172.36)
PD-**3** 10 (39.4)	37	51	3.5	40 (172.46)	35 (172.40)	25 (172.34)
	38**	64**	3**	35** (172.44)	40** (172.41)	25** (172.36)
PD-**3** 50 (39.4)	43	50	6	25 (172.53)	45 (172.43)	30 (172.34)
PD-**4** 10 (72.8)	69	56	4	10 (172.53)	35 (172.43)	55 (172.33)
PD-**4** 50 (72.8)	71	42	7	15 (172.59)	35 (172.45)	50 (172.34)
5 (0)	0	~100	0			

Samples **1–3** were apple pectin, **4** was citrus pectin, and **5** was sodium polygalacturonate (Sigma). *DE according to the supplier. **Obtained under milder conditions (see experimental section)

are collected in Table 2. On the whole, the DE values of the PD pectins determined by ^{13}C NMR are only slightly higher (~ 5%) than the values obtained for the PN precursors by chemical analysis. This tendency was accompanied by detection of a slightly lower (10–15%) DE for the oligomers recovered from the dialysis bath (PB, ~ 10–25% yield). All of the samples contained roughly 6–7% of $\rightarrow$4)βGalp(1$\rightarrow$ residues but the signals of the labile α-Ara*f* residues observed in the PN-1 sample were absent, suggesting that these moieties were degraded by the thermal treatment. The "visible fraction" was estimated from quantitative inverse-gated experiments, which were conducted in the presence of a known amount of sucrose. It is well-known that the formation of aggregated species is increased in the presence of sucrose owing to a decrease in the activity of water (Ross-Murphy and Shatwell 1993). Recently, a mechanism has been proposed for the cross-linking of high methoxyl pectins in the presence of sucrose under acidic conditions (pH < 3.5; Rinaudo 1996). However, at pH values in the 4–5 range this mechanism would be much less efficient and therefore sucrose was chosen as an internal standard as its NMR characteristics (chemical shifts, relaxation parameters etc.) were very favorable. The goal of these quantitative analyses was to show that a major fraction of the PD pectins could be detected for samples with a wide range of DE. In all cases where dialysis was performed against the membrane with a cutoff of 10 kDa, at least 50% of the pectin sample was observed. It is interesting to note that PH-1, which was a mixture of fairly short (average DP of 2) galacturonan oligomers of low methoxyl content and the highly esterified pectin PD-1, contained more junction zones (47%) than PD-1 alone (30%) pointing to the importance of short unesterified galacturonan segments in the formation of aggregated species. Finally, the citrus pectin PD-4 presented a similar junction zone population to that of PD-3 in spite of a much larger DE (72.8% as opposed to 39.4%).

Methoxyl distribution. In a study of chemically-modified pectin samples with DE varying from 0 to 100%, Westerlund et al. (1991) showed that the carbon chemical shifts of C6 are influenced by next-neighbour effects of both the preceding and following residues, allowing identification of triad structures such as U<u>E</u>U, E<u>E</u>E, U<u>U</u>U etc. (where U and E stand for unesterified and esterified sugars respectively and the underscore denotes the observed residue). A similar effect was observed in the spectra recorded at 21°C in the present study where the esterified C6 groups resonated between 172.34 (E<u>E</u>E) and 172.59 ppm (U<u>E</u>U) and presented fairly sharp peaks, Fig. 5. Very broad signals were detected for the unesterified groups between 175 and 177 (U<u>U</u>U of sodium polygalacturonate) ppm. At 85°C, the more significant variations in the chemical shifts of the methine carbons in going from a totally esterified pectin to a totally unesterified pectin (C1 102.9 to 102.5 ppm; C4 81.5 to 81.0 ppm) were less pronounced than at 21°C (C1 102.13 to 100.5 ppm; C4 80.74 to 79.5 ppm). This latter signal (C4 of U<u>U</u>U) was, however, of limited use as it was masked by the C4 resonance of the β-galactan side-chains.

Inspection of the triad populations in Table 2 showed that the methoxyl group distribution did not change markedly with the membrane cutoff. The Bernoullian distribution of triads has been calculated for theoretical pectins of varying DE and the relative populations of the <u>E</u>-triads are represented by the curves in Fig. 6. As regards the more highly methoxylated sample PD-1, the E<u>E</u>E (51–58%) population corresponded to a random methoxyl distribution. At the low methoxyl end, the E<u>E</u>E population (25%) of PD-3 was too high for a random distribution (15%).

Comparison of the EEE population of the apple (51% for PD-1; membrane cutoff of 50 kDa) and citrus (50% for

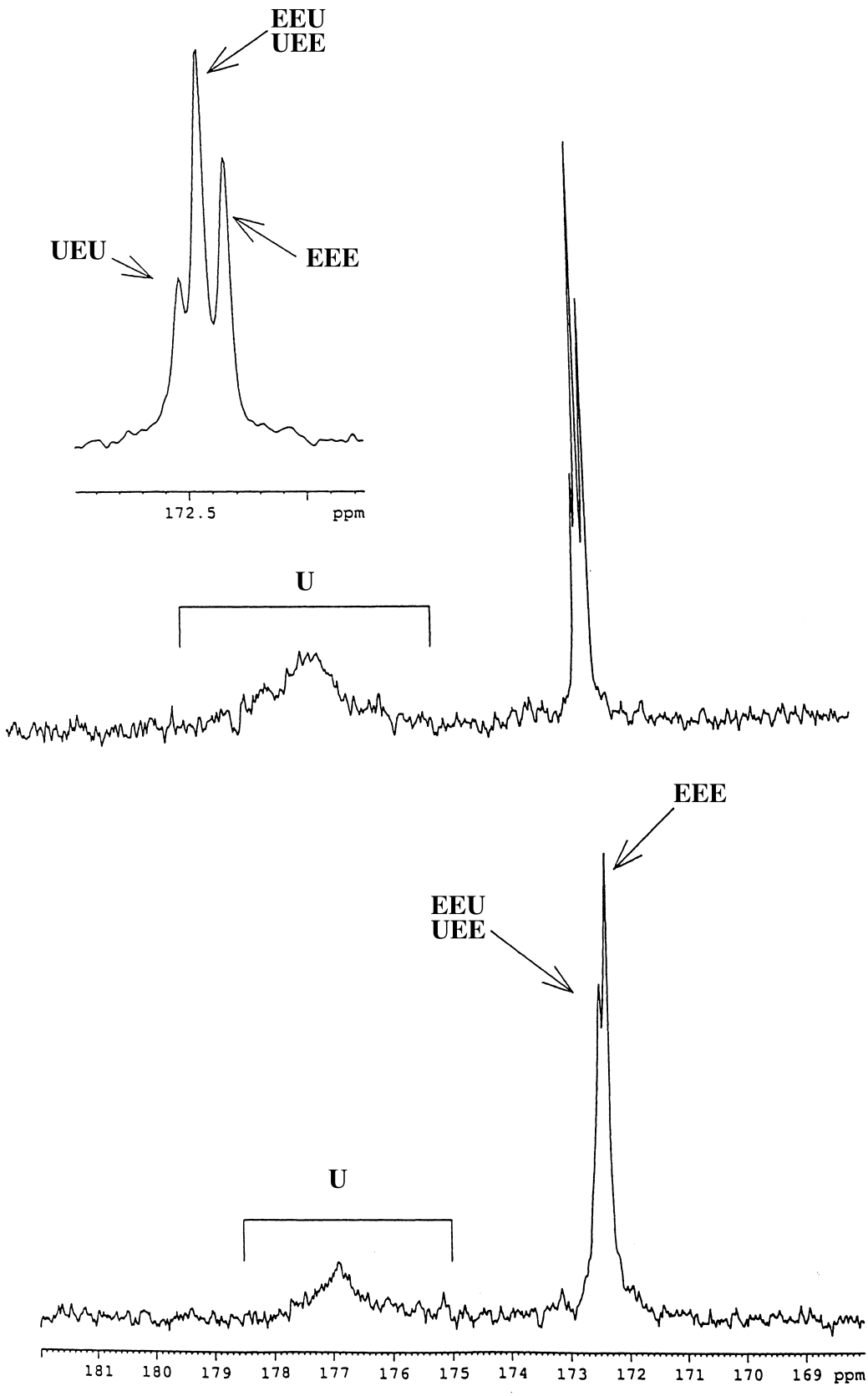

Fig. 5 Carboxyl carbon region of 100.6 MHz ^{13}C NMR spectra of depolymerized pectins (DE 72%, below; DE 48%, above). Assignments for the triads with an esterified central residue have been indicated

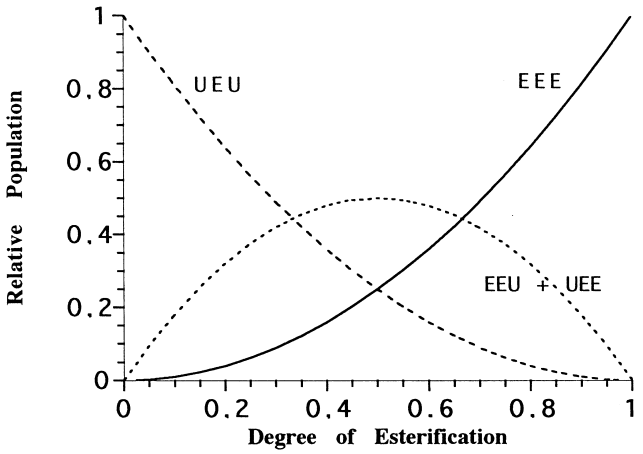

Fig. 6 Plots of the relative populations of the triads with an esterified central residue (E<u>E</u>E, E<u>E</u>U + U<u>E</u>E, and U<u>E</u>U) as a function of DE corresponding to a Bernoullian distribution

PD-4, membrane cutoff of 50 kDa) pectins with the most similar DE values indicates that the nature of the methoxyl distribution in these samples is analogous. In the aforementioned study of chemically-modified pectin (Westerlund et al. 1991), native papaya (DE 70%) and tobacco pectins (DE 20%) were also examined and a random distribution of the methoxyl groups was postulated based on ^{13}C NMR spectra. This is in agreement with the conclusions of some previous reports on fruit pectins (Kohn and Furda 1967; Anger and Dongowski 1985).

Relaxation data. In order to obtain a detailed picture of molecular dynamics which can in turn be related to the size of the macromolecule, it is necessary to simulate multifield relaxation data (Dais 1995; Catoire et al. 1997). The 100.6 MHz carbon relaxation parameters, T_1, T_2 and nOe factor, of PD-1 which was dialyzed against membranes with cutoffs of 10, 50 and 300 kDa are collected in Table 3. Inspection of these data shows that the relaxation parameters change in going from the sample prepared with a membrane with a cutoff of 10 kDa to the one with a cutoff of 50 kDa. In contrast, comparison of the data sets obtained for dialysis against the membranes with cutoffs of 50 and 300 kDa indicated that the relaxation parameters of PD-1 had attained their asymptotic values for the former sample. As a general rule, for polymers of relatively low molecular weights (<10 kDa), the T_1 increases while the T_2 and the heteronuclear nOe decrease with increasing molecular weight, reflecting a progressive increase in the overall tumbling time. In parallel, the contribution of segmental motion to dipolar relaxation increases at the expense of that of overall tumbling. In high molecular-weight polysaccharides the contribution of overall motion becomes negligeable and relaxation parameters attain their asymptotic values. This latter behavior, which has been reported for oligomers of varying DP (15–50 residues; Benesi and Brant 1985; Dais 1995), is well suited to the determination of a dynamic model based on carbon relaxation data. It is noteworthy that the viscosity measurements on the PD samples were adequately described by the Mark-Houwink relationship established for much larger pectins (Garnier et al. 1993), also suggesting that the solution behavior of the shorter chains is closely related to that of the parent compounds.

PME activity

PME in their natural state, i.e. still bound to cell walls, were then assayed in parallel using native or treated pectins as substrates in order to check whether or not the treatment had induced conformational modifications. We have previously reported that although at least 3 different PME isoforms could be solubilized from cell wall fragments, only one of them (whose pI was around 8.5) was involved in the deesterification of exogenous pectin occurring when isolated cell walls were incubated with commercial citrus pectin (Bordenave and Goldberg 1994). Table 4 shows the kinetic parameters of bound PME activity acting on pectins differing in their methylation degrees. Results obtained with crude and treated pectins were very close except in the case of PD-3 which had a very low esterification degree (39.4%, results not indicated in Table 4). Indeed, it was not possible to measure accurately a deesterification rate for the PD-3 sample obtained under the more drastic conditions ($M_W \sim 2$ kDa) whatever the concentration.

Table 3 ^{13}C Relaxation parameters (T_1, T_2 and nOe factor η) of depolymerized apple pectin PD-1 (DE 74%) dialyzed against membranes with cutoffs of >10, >50 and >300 kDa, respectively

Membrane cutoff	Relaxation parameter	C1 (E)	C1 (U)	C2 & C3	C4	C5
>10	T_1 in ms	252 ± 5	252 ± 7	285 ± 6	250 ± 14	285 ± 11
	η	0.63 ± 0.09	0.62 ± 0.08	0.44 ± 0.01	0.56 ± 0.12	0.45 ± 0.17
	T_2 in ms	56 ± 1			37 ± 2	
>50	T_1 in ms	280 ± 9	293 ± 23	352 ± 7	307 ± 8	364 ± 9
	η	0.42 ± 0.07	0.45 ± 0.06	0.37 ± 0.02	0.41 ± 0.02	0.36 ± 0.04
	T_2 in ms	19 ± 1			17 ± 2	17 ± 2
>300	T_1 in ms	295 ± 24	272 ± 15	343 ± 24	279 ± 18	342 ± 16
	η	0.44 ± 0.02	0.43 ± 0.01	0.30 ± 0.09	0.44 ± 0.02	0.49 ± 0.12
	T_2 in ms	18 ± 2				17 ± 1

Table 4 The Michaelis constant (K_m), maximum rate (V_{max}) and the V_{max}/K_m ratio (which represents the catalytic efficiency of the enzyme) for kinetic measurements of PME activity (*Vigna radiata*) performed at pH 7.6 in NaCl 150 mM are given for various pectin samples

Pectin sample	DE %	K_m mM	V_{max} µeq min^{-1} mg $parois^{-1}$	V_{max}/K_m
Native pectins				
Apple pectins				
PN-1	74.4	1.61, 1.91	0.16, 0.19	0.10
PN-2	53.6	0.35, 0.25	0.19, 0.18	0.62
PN-3	39.4	0.18, 0.14	0.23, 0.21	1.37
Citrus pectin				
PN-4	72.8	1.07, 1.27	0.21, 0.23	0.19
Treated Pectins*				
Apple pectins				
PD-1	71	1.49, 1.64	0.18, 0.17	0.11
PD-2	50	0.50, 0.48	0.21, 0.21	0.43
PD-3	37	0.40; 0.21	0.24; 0.20	0.73
Citrus pectin				
PD-4	69	0.98, 1.40	0.13, 0.16	0.12

* The PD fractions were obtained with a membrane cutoff of 10 kDa and milder conditions were used in the thermal treatment of PD-3

When the PD-3 sample was prepared under milder conditions (M_W ~ 4 kDa), the PME activity was similar to that of the untreated pectin, PN-3 (Table 4). These data suggest that the PME cannot act on short oligouronides since it only worked on galacturonan whose DP was around or higher than 20. The lower the DE, the higher the affinity and the catalytic efficiency (V_{max}/K_m). In contrast, the V_{max} appears to be rather independent of the DE of the substrate. It also appeared that the $\log(1/K_m)$ was linearly related to log (% free carboxyl groups in the substrate) with a slope of 2.8. Similarly, some years ago, Versteeg had also observed that orange pectinesterases have strongly increased affinity for substrates with a lower DE (Versteeg 1979). These data underline the fact that free carboxyl groups in the vicinity of the ester linkage increase the affinity of the enzyme for its substrate. This suggests that the fixation of the enzyme, which is known to occur at anionic sites, might induce conformational modifications. According to this scheme, and taking into account the distribution demonstrated by the data in Table 4, it appears that suitable conformations require the presence of free carboxyl groups at a given distance from the ester bond. In any case, the sample with a DE of 74.4 will provide a useful tool for the study of the action pattern of the different PME isoforms since it can be used even after being treated. It will then become possible to determine the distribution of the carboxyl groups generated by enzymatic hydrolysis using ^{13}C NMR spectroscopy.

General conclusions

A general procedure for obtaining pectin samples amenable to NMR analysis at room temperature has been established. Thermal treatment results in a ten-fold decrease in weight-average molecular weight leading to polymers which preferentially adopt the random-coil conformation in dilute solution with a concommitant decrease in the population of aggregated species. However, the secondary structure of the partially-depolymerized chains has been shown to be closely related to that of the native polymers based on viscosity measurements, carbon relaxation data, and PME activity. Work is under way to determine the secondary structure of chains of highly methoxylated pectins through simulation of the multi-field ^{13}C relaxation data reported here. This thermally-induced partial depolymerization will also be used in future work to establish the action pattern of different isoforms of PME from *Vigna radiata* (Bordenave and Goldberg 1994).

Acknowledgements This work was supported by a fellowship for L. C. from the French Ministère de la Recherche et de l'Enseignement Supérieure. The authors would like to thank Laetitia Pommereul for skillful technical assistance.

References

Andersen AK, Larsen B, Grasdalen H (1995) Sequential structure by ^1H NMR as a direct assay for pectinesterase activity. Carbohydr Res 273:93–98

Anger H, Berth G (1986) Gel permeation chromatography and the Mark-Houwink relation for pectins with different degrees of esterification. Carbohydr Polym 6:193–202

Aspinall GO (1982) In: The Polysaccharides, vol 1. Academic Press, London, pp 36–104

Anger H, Dongowski G (1985) Verteilung der freien Carboxylgruppen in nativen Pektinen aus Obst and Gemüse. Nahrung 29: 397–404

Axelos MAV, Thibault JF, Lefebvre J (1989) Structure of citrus pectins and viscosimetry study of their solution properties. Int J Biol Macromol 11:186–191

Benesi AJ, Brant D (1985) Trends in molecular motion for a series of glucose oligomers and the corresponding polymer Pullulan as measured by carbon-13 NMR relaxation. Macromolecules 18: 1109–1116

Bordenave M, Goldberg R (1994) Immobilized and free apoplastic pectinmethylesterases in mung bean hypocotyl. Plant Physiol 106:1151–1156

Catoire L, Derouet C, Redon AM, Goldberg R, Hervé du Penhoat C (1997) An NMR study of the dynamic single-stranded conformation of sodium pectate. Carbohydr Res 300:19–29

Chun MS, Park OO (1994) On the intrinsic viscosity of anionic and nonionic rodlike polysaccharide solutions. Macromol Chem Phys 195:701–711

Cros S, Garnier C, Axelos MAV, Imberty A, Perez S (1996) Solution conformations of pectin polysaccharides: determination of chain characteristics by small angle neutron scattering, viscometry, and molecular modeling. Biopolymers 39:339–352

Garnier C, Axelos MAV, Thibault JF (1993) Phase diagrams of pectin-calcium systems: Influence of pH, ionic strength, and temperature on the gelation of pectins with different degrees of methylation. Carbohydr Res 240:219–232

Gidley MJ, Bociek SM (1988) ^{13}C CP/MAS NMR studies of amylose inclusion complexes, cyclodextrins, and the amorphous phase of starch granules: relationships between glycosidic linkage conformation and solid-State ^{13}C chemical shifts. J Am Chem Soc 110:3820–3829

Goldberg R, Morvan C, Roland JC (1986) Composition, properties and localization of pectins in young and mature cell walls of the mung bean hypocotyl. Plant Cell Physiol 27:417–429

Goldberg R, Morvan C, Hervé du Penhoat C, Michon V (1989) Structure and properties of acidic polysaccharides from mung bean hypocotyls. Plant Cell Physiol 30:163–173

Grasdalen H, Andersen AK, Larsen B (1996) NMR spectroscopy studies of the action pattern of tomato pectinesterase: generation of block structure in pectin by a multiple-attack mechanism. Carbohydr Res 289:105–114

Hervé du Penhoat C, Michon V, Goldberg R (1987) Development of arabinans and galactans during the maturation of hypocotyl cells of mung bean (Vigna radiata Wilzech). Carbohydr Res 166:31–42

Keenan MHJ, Belton PS, Matthew JA, Howson SJ (1985) A ^{13}C study of sugar-beet pectin. Carbohydr Res 138:168–170

Kohn R, Furda I (1967) Calcium ion activity in solutions of calcium pectinate. Collect Czech Chem Commun 32:1925–37

Montreuil J, Spik G, Fournet B, Tollier MT (1981) In: Glucides. Analyse des constituants alimentaires. APRIA, Paris, pp 85–143

Oviatt HW, Brant DA (1994) Viscoelastic behavior of thermally treated aqueous xanthan solutions in the semidilute concentration regime. Macromolecules 27:2402–2408

Powell DA, Morris ER, Gidley MJ, Rees DA (1982) Conformations and interactions of pectins. I. Influence of residue sequence on chain association in calcium pectate gels. J Mol Biol 155: 517–531

Rinaudo M (1996) Physicochemical properties of pectins in solution and gel states. In: Visser J, Voragen AGJ (eds) Pectins and Pectinases, Progress in Biotechniology. Elsevier 14:21–33

Rinaudo M, Ravanat G (1980) NMR investigation on oligo- and poly(galacturonic acid)s; gel formation in the presence of Ca^{2+} counterions. Makromol Chem 181:1059–1070

Ross-Murphy SB, Shatwell KP (1993) Polysaccharide strong and weak gels. Biorheology 30:217–227

Szu SC, Zon G, Schneerson R, Robbins JB (1986) Ultrasonic irradiation of bacterial polysaccharides. Characterization of the depolymerized products and some applications of the process. Carbohyr Res 152:7

Versteeg C (1979) Pectinesterases from orange fruit – their purification, general characteristics, and juice cloud destablizing properties. Center for Agriculatural Publishing and Documentation, PhD Thesis, Wageningen, pp 109

Westerlund E, Åman P, Andersson RE, Andersson R (1991) Investigation of the distribution of methyl ester groups in pectin by high-field ^{13}C NMR. Carbohydr Polym 14:179–187