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Calcium gelation of pectic polysaccharides isolated from unripe tomato fruit

Alistair J. MacDougall *, Paul W. Needs, Neil M. Rigby, Stephen G. Ring

Department of Biochemistry, Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, UK

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Abstract

Cell-wall material was prepared from unripe tomato fruit, and a pectic polysaccharide extracted with cyclohexanediaminetetraacetic acid. The structure of the purified pectic polysaccharide was examined by sugar and methylation analysis, and was typical of a rhamnogalacturonan from the primary cell wall. The physicochemical properties of the isolated polysaccharide were characterised by viscometry and size-exclusion chromatography. The polysaccharide was polydisperse, but of large molecular size as indicated by an intrinsic viscosity of 810 mL g^{-1}. At concentrations above ~0.2–0.6% w/w, coil entanglement was observed as an increase in the dependency of viscosity on concentration. For these concentrated solutions, clear elastic gels were formed on addition of calcium ions. At concentrations in the range 0.6–2.8% w/w the shear modulus of the gel showed a $c^{1.9}$ dependence on concentration. The modulus of the gel increased linearly with absolute temperature in a rubberlike way, enabling an estimate of cross-link density to be made.

Keywords: Pectic polysaccharide; Gelation; Calcium; Tomato; Ripening

1. Introduction

The relationship between the molecular structure of primary cell walls of plants and the physical properties of plant tissues is a topic of considerable interest, particularly for those whose aim is to control the texture of fruit and vegetables. Unlike synthetic

* Corresponding author.

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cellular solids, in which this relationship between composition and physical behaviour is relatively well understood [1], there is as yet no clear description for assemblies of plant cells, with the exception of wood. Although this is partly due to the fluid-filled nature of plant tissues, where turgor plays a significant role [2], much of the difficulty comes from our poor understanding of plant cell-wall architecture. Whilst the fine structure of the polysaccharides of the primary cell wall is steadily being elucidated [3,4], the nature of their association, and the extent to which crystallisation, hydrogen bonding, covalent linkage, ionic bonding, and physical entanglement play a role in determining the physical properties of the cell wall, remains unclear.

In order to understand at least part of the overall picture we have concentrated on the ionic bonding of pectic polysaccharides. These polysaccharides are a major component of the primary cell wall and comprise the middle lamella. They thus contribute to the mechanical properties of the cell wall and influence cell adhesion. They are a heterogeneous grouping with significant structural variation [4–6]. The main structural features include a backbone of (1 → 4)-linked α-D-galacturonic acid which is interrupted by (1 → 2)-linked L-rhamnopyranosyl residues. A fraction of these rhamnosyl residues are 4-substituted by arabinose- and galactose-containing side-chains. The uronic acid in the main chain may be partially esterified with methanol, and in some pectic polysaccharides the galacturonic acid is partially acetylated.

The properties of gels formed by pectic polysaccharides in the presence of calcium ions, and the mechanism of gelation, have been the subject of numerous studies [7–10]. The relevance of this work to cell-wall structure has been hard to establish, as the preparations used were extracted under degradative conditions, or from tissues such as citrus pith which have a different physiological role. Pectic polysaccharides with a high degree of methyl esterification (dm) can be induced to gel by lowering the pH and the addition of a low molecular weight carbohydrate such as sucrose which promotes chain association. Pectic polysaccharides extracted from citrus pith, which have a high dm but also contain blocks of contiguous unesterified residues [11], can gel through addition of calcium ions. For pectic polysaccharides with a more random distribution of unesterified residues [12] the dm must fall below ~ 40% before gelation is observed on addition of calcium ions [10]. Studies of chain association for polygalacturonic acid have led to the 'egg-box' model [8]. It has generally been assumed that the pectic polysaccharides isolated from cell walls with chelating agents form gels through ionic association in vivo. Ion-binding studies of the isolated polysaccharide question this assumption [13], with uncertainty over the nature of the ionic bonds (if any) formed during the association of pectic polysaccharides in the cell wall. The best evidence currently available for the presence of ionic associations involving contiguous unesterified regions in the plant cell wall comes from the binding of antibodies that recognise low-dm regions of pectic polysaccharides [14,15], and from a recent study using ^{13}C NMR spectroscopy, in which some of the features associated with gelation in isolated pectic polysaccharides were also found in spectra of cell-wall material [16].

There is a need for more information on the relationship between the molecular structure of cell-wall polysaccharides and their physicochemical properties. In this paper we describe the method of isolation, structure, and gelation properties of the chelator-extractable pectic polysaccharide from unripe tomato fruit. In this tissue, cyclohexanedi-

aminetetraacetic acid (CDTA) readily causes cell separation, and the ultimate aim of this work is to see if the physical properties of cell–cell adhesion can be explained on the basis of gelation of this polysaccharide.

2. Experimental

Plant material.—Pericarp tissue was taken from greenhouse-grown tomatoes (var Rutgers) at the mature green stage, and frozen in liquid N_2, without removal of the cuticle. Material collected over several seasons, and stored at −40 °C, was combined to prepare cell-wall material.

Preparation of cell-wall material.—Approximately 7.5 kg of tomato pericarp was extracted with buffer-saturated phenol, prepared by equilibration with 0.5 M Tris HCl (pH 8.1). The plant tissue was homogenised with the phenol layer (1:4 w/w) at 0 °C. The cellular debris was collected by filtration through muslin, then washed with water, and the phenol extraction repeated four times. The pH was adjusted to 4.5 with AcOH and the cell-wall preparation stored at −20 °C. Batches containing ca. 10 g dry weight of material were ball-milled with water in a 1-L container for 4 h at 40 rpm, collected by centrifugation at 20,000 *g* for 30 min, and washed twice with water to give a purified cell-wall material. An aliquot of the material solubilised during ball-milling was retained and concentrated by rotary evaporation.

Assessment of residual enzyme activities in the cell-wall material.—Polygalacturonase was assayed by adding a 1 M NaCl extract (200 μL) of a 1.5% w/w suspension of cell-wall material to 0.1% w/w polygalacturonic acid in NaOAc (800 μL) (25 mM, pH 4.5). Activity was assessed from the appearance of reducing groups [17]. β-D-Galactosidase was assayed by adding *p*-nitrophenyl β-D-galactopyranoside (3 mM, 1 mL) in NaOAc (50 mM, pH 4.5) to 0.5 g of a 3% w/w slurry of cell-wall material. To develop the assay, buffer was added (1:1 $Na_2B_4O_7$–KCl, 200 mM, 2 mL, pH 9.8), the sample centrifuged, and the absorbance of the supernatant solution measured at 400 nm.

Preparation of the CDTA extract.—In subsequent steps the extract was separated from the undissolved solids by centrifugation at 4000 *g*. The cell-wall material (50 g) was first extracted by stirring for 24 h in a solution of NaCl (9 L, 100 mM) which was buffered with NaOAc (50 mM, pH 4.5) and contained NaOBz (0.1 w/v) as a bacteriostat, and was then washed with 100 mM NaCl. CDTA extraction was carried out for 24 h in Na-CDTA (9 L, 50 mM, pH 6.8). The cell-wall material was then washed once with CDTA (15 mM, pH 6.9) and twice with water, and the extract and washings were combined. Sodium azide was added at 0.02% w/v, and the extracts were concentrated with an Amicon CH2 ultrafiltration system incorporating an H1P10-20 fibre cartridge (Amicon Division, W.R. Grace & Co.-Conn, Beverly, MA). The NaCl extract was reduced to 550 mL and the CDTA extract to 3 L (ca. 1.5 mg mL^{-1}).

Purification of CDTA-extracted material.—CDTA and azide were removed from the CDTA-extracted material by diafiltration of 600-mL batches with distilled water (7.5 L) at a rate of 6–10 mL min^{-1}, whilst standing the sample on ice. Residual cations were removed from the retentate by stirring for 20 min over Dowex AG 50-X8 (H^+) and the

solutions were concentrated by rotary evaporation. The potassium salt of the pectic polysaccharide was prepared by adjusting a solution of the acidic polymer to pH 6.5 with 1 M KOH [stored over Dowex AG 50-X8 (H^+) to prevent carbonate accumulation]. The ammonium salt was formed by adjusting the pH to 6.5 with dilute NH_3.

Analysis of the composition of the purified CDTA extract.—Residual CDTA was measured as its tetrapropyl ester by GC in an adaptation of the method of Wanke and Eberle [18] using ethylenediaminetetracetic acid (EDTA) as an internal standard. Thus a solution of the polysaccharide (5 mL, 1.5 mg mL^{-1}) containing EDTA (3.5 mM, 50 μL) in a screw-cap glass test-tube (40 mL) was lyophilised and dried over P_2O_5. A 1:9 mixture of dry PrOH and AcCl was added (5 mL), and the tube was heated at 100 °C for 1 h. The sample was filtered through glass-fibre paper and the filtrate evaporated to dryness under a stream of Ar at 40 °C. The derivatised sample was redissolved in EtOAc (1 mL) and 2 μL of the solution was analysed on a BPX 5 capillary GC column (SGE, Melbourne, Australia). The column was 15 m in length with an internal diameter of 0.32 mm and a coating of 0.5 μm. The injector was in splitless mode with helium as carrier gas at 80-kPa column-head pressure, and detection by flame ionisation. The temperature profile was 1 min at 160 °C, followed by an increase to 280 °C at 8 °C per min, and a further 4 min at this temperature. The injector and detector temperatures were 250 and 280 °C, respectively.

Carbohydrate analysis.—Neutral sugars were released by Seaman hydrolysis [19] and reduced after neutralisation with ammonia [20]. The resulting alditols were acetylated and then analysed by GC [21].

Methylation analysis of the CDTA-extracted pectic polysaccharide.—The CDTA-extracted pectic polysaccharide was methylated by the sequential, sodium hydroxide mediated method of Needs and Selvendran [22]. Carboxyl reduction of the methylated material used $LiEt_3BD$ in a modification of the method of York et al. [23]. Thus the dry methylated material was treated with a solution of $LiEt_3BD$ in tetrahydrofuran (1 M, 1 mL) for 3 h at room temperature. The reaction was quenched by the dropwise addition of acetic acid until effervescence ceased. Dichloromethane (3 mL) was added and the solution was washed with water (3 × 3 mL). The methylated, carboxyl-reduced material was recovered by evaporation at 40 °C in a stream of Ar. Hydrolysis, reduction, and acetylation of the sample were performed as described [24] (using 1-methylimidazole-promoted acetylation).

Degree of methyl esterification (dm) and degree of acetylation (da).—Methanol released after saponification of samples was determined as described previously [25]. Acetic acid released under the same saponification conditions was determined by HPLC on an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The column was eluted with H_2SO_4 (4 mM) at 0.6 mL min^{-1} with UV detection at 210 nm.

General methods.—Soluble protein was measured by the Lowry method [26]. Phenolic compounds released by treatment with 1 M NaOH for 2 h at 25 °C were extracted with EtOAc after adjustment of the sample to pH 2 with HCl. The sample was dried down and redissolved in 20% v/v MeOH containing CF_3CO_2H (1 mM), and chromatographed on an ODS2 column with a gradient of 20–60% v/v MeOH containing CF_3CO_2H (1 mM throughout), and a UV detector at 290 nm. Sodium was measured by flame photometry.

Size-exclusion chromatography.—This was performed at room temperature on a 1-m × 1.6-cm diameter column of Sepharose CL-2B linked to a refractive index detector. The column was eluted at ca. 15 mL h^{-1} with 0.1 M NH_4Cl. The column was calibrated with Shodex pullulan standards [27]. The pectic polysaccharide (ca. 2 mg) in 0.1 M NH_4Cl (2 mL) was applied to the column, and fractions collected were analysed for uronic acid content without hydrolysis, and for hexose content by the anthrone method [28] with galactose as standard. To obtain a value for neutral hexose content, a correction was made for the galacturonic acid present in each fraction.

Viscometry.—Measurements of intrinsic viscosity, $[\eta]$, and specific viscosity as a function of concentration of aqueous solutions of pectic polysaccharide in 0.1 M NH_4Cl were carried out using Ubbelohde suspended level viscometers at 25 °C. In principle for a polyelectrolyte solution, isoionic dilution should make an allowance for the contribution of the polyelectrolyte to the ionic strength of the solution [29]. For a highly methyl-esterified pectic polysaccharide such as in the present study, we calculated that this allowance was small and therefore preferred simple dilution with 0.1 M NH_4Cl. A typical efflux time for water was 100 s, and no kinetic energy correction was made.

Formation of gels and measurement of gel stiffness.—Cylindrical gels of 7.5 mm diameter and 5–8 mm length were formed by weighing 300–600 mg of a concentrated solution of the pectic polysaccharide into straight-sided 2-mL polythene centrifuge tubes. The appropriate amount of $CaCl_2$ in approximately 15 μL was added in a thin layer on the surface, and the gel was left to equilibrate at 4 °C for 24–48 h. Gels were removed from their casts by making a small cut through the conical base to the centrifuge tube and pushing the gel out with air pressure from a syringe. The shear modulus, G′, of gels formed from the pectic polysaccharide was obtained from measurement of the velocity of a shear wave (frequency 200 Hz) through the gel using a Rank Brothers' Pulse Shearometer as described [30].

Temperature dependence of gel stiffness.—Cylindrical gels of 12 mm diameter and 10 mm length were formed in a 5-mL disposable syringe. After measuring the initial stiffness, gels were replaced in the syringe and heated in boiling water for 3 min. The gel was placed between the shear plates and changes in G′ were recorded over a 6-min period. After reheating and handling the gel as before, a temperature decay curve was obtained by placing a thermistor in the centre of the gel. The relationship between G′ and temperature was obtained by comparison of the two data sets.

3. Results

Preparation of cell-wall material.—Procedures used for the isolation and purification of cell-wall material should aim to minimise physical and chemical modification, remove cytoplasmic contaminants, including proteins and low molecular weight species, and extract or inhibit cell-wall enzymes. We chose the buffered phenol method of Huber [31,32] to avoid dehydration steps (e.g., ethanol and acetone treatment and freeze-drying) which are known to lead to irreversible precipitation of carbohydrate polymers. This extractant has been reported to be good for solubilising protein and inactivating cell-wall enzymes [31]. We also found it to be effective: after a single extraction neither

polygalacturonase nor β-D-galactosidase activity could be detected; the degree of methyl esterification of the purified cell wall (Table 1) was the same as that of whole freeze-dried fruit powder (72%). Phenol–CH_3COOH–H_2O (2:1:1 PAW) was avoided as an extractant because repeating the extraction with this solution three times led to a cell-wall material virtually devoid of calcium (0.02 $\mu g\ mg^{-1}$ dry weight) and caused a large amount (9%) of the crude cell-wall material to be solubilised during ball-milling. Subsequent treatment with CDTA extracted relatively little (1.5%) of the cell-wall material, suggesting that most of the calcium-bound pectic polysaccharide had already been removed from the cell wall. With buffered phenol, less than 6% of the cell wall was solubilised during ball-milling, and the yield of CDTA-extracted material was much greater (Table 1). These results confirm those reported by Huber [32].

The monosaccharide composition of the purified cell-wall material from tomato was similar to that obtained previously, and is typical of the primary cell wall of the parenchymatous tissue of many fruits and vegetables. Glucose in solubilised polymeric material (Table 1) is most probably due to starch. Preliminary extraction of the cell-wall material with dilute salt solution yielded very little material ($\sim 0.4\%$ w/w), whereas subsequent extraction with CDTA yielded a fraction comprising 7% w/w of the isolated cell-wall material. The CDTA was used under conditions where it would be a powerful chelating agent for calcium and other divalent metal ions, so we infer that the extraction of this polysaccharide fraction occurred as a result of the disruption of ionic associations. The monosaccharide composition of this fraction was typical of that of pectic polysaccharides, with galactose, arabinose, and galacturonic acid being major components with a smaller amount of rhamnose being present. The dm of the uronic acid was 68%. The carbohydrate content of this fraction was $\sim 85\%$ w/w with the residue being accounted for as inorganic salts, acetyl ester (4.6%), and a small amount of soluble protein (1.3%). The CDTA content of this fraction was less than 1% w/w. In conclusion, procedures used for the isolation of the cell-wall material and extraction of the pectic polysaccharide resulted in a preparation substantially free from contamination.

Methylation analysis of CDTA extract.—Linkage analysis data for the CDTA extract are shown in Table 2. The recovery of galacturonic acid was low, which is usual for chelator-extracted pectic polysaccharides [33]. The ratio of branch points to terminal residues was close to unity, which, together with the absence of small amounts of 'unusual' linkage types, suggested little undermethylation of neutral sugars. The recovery of rhamnosyl residues was higher than in the sugar analysis; rhamnose is thought to be lost in the latter procedure as the hydrolysis-resistant aldobiouronic acid GalA-(1 → 4)-Rha [24]. Thus the molar ratio of galacturonosyl to rhamnosyl residues is in the region of (50–80):1, and a little over one-third of the rhamnosyl residues are branched. These data are again typical of a chelator-extracted pectic polysaccharide and are in broad agreement with the analysis of CDTA-extracted material from unripe tomato cell wall (prepared from a phenol–CH_3COOH–H_2O- and Me_2SO-extracted acetone-insoluble residue) by Seymour et al. [34]. The apparent ratio of arabinose to galactose from linkage analysis was higher than from sugar analysis; this was also reported by Seymour et al. [34]. We have found this to be common; analyses of a pectic polysaccharide from lupin behaved similarly (Needs et al. [35]). Seymour et al. [34] speculated that this might be due to selective loss, on dialysis, of galactan side-chains released by β-elimination

Table 1
Yield and composition of fractions prepared from 7.5 kg of unripe tomato pericarp

	Yield (g)	Yield (%) [a]	'Anhydro sugars' (μg mg^{-1} dry weight)								Dm (%)	Total 'anhydro sugars' (μg mg^{-1} dry wt.)
			Gal	Ara	Rha	Glc	Xyl	Fuc	Man	U.A.		
Ball-mill solubilised	3.4	6	94	24	4	92	75	< 1	14	616	–	919
Cell-wall material	55	–	125	32	4	385	28	< 1	39	220	67	833
100 mM NaCl, 50 mM NaOAc (pH 4.5)	0.2 [b]	0.4	10	2	< 1	20	< 1	< 1	1	60	–	93
50 mM Na-CDTA (pH 6.8) [c]	3.9	7	101	29	7	5	1	1	< 1	710	68	854

	Degree of acetylation (%) [d]	Saponifiable phenolics (μg mg^{-1} dry weight)	Soluble protein (μg mg^{-1} dry weight)	Sodium (μg mg^{-1} dry weight)	Total weight accounted for [e] (μg mg^{-1} dry weight)
Additional features of CDTA extract	4.6	< 0.1	13	60	980

[a] Expressed as a % of the yield of cell-wall material.
[b] Yield of carbohydrate.
[c] Analysed after diafiltration and before ion-exchange treatment.
[d] Expressed as a % of the galacturonic acid residues.
[e] Includes the weights of methyl and acetyl groups, protein, and sodium.

Table 2
Glycosyl-linkage composition of CDTA-solubilised material

Linkage	mol %	Linkage	mol %
T-Ara*f*	1.6	T-Gal*p*	1.8
T-Ara*p*	0.8	3-Gal*p*	2.9
2-Ara*f*	0.3	4-Gal*p*	25.9
5-Ara*f*	31.6	6-Gal*p*	1.4
		3,4-Gal*p*	1.1
2-Rha*p*	8.4	2,4-Gal*p*	1.1
2,4-Rha*p*	2.2	4,6-Gal*p*	2.6
		3,6-Gal*p*	1.3
T-Xyl*p*	0.5		
		4-Gal*p*A	12.0
4-Glc*p*	3.0		
4,6-Glc*p*	1.4		

during methylation. We have shown that this is not the case for the pectic polysaccharide from lupin and that other factors, such as the choice of base during methylation and the nature of the carboxyl-reducing agent, can also affect this ratio [35]. Apart from the 2,4-substituted rhamnosyl residues only a very small proportion of the neutral residues were branched. This, and the nature of the branched residues, suggested that the majority of the pectic side-chains were linear (1 → 5)-linked arabinans and (1 → 4)-linked galactans each of approximate average dp 11, together with a small proportion of Type 1 arabinogalactan [36] containing 2,4- and 3,4-substituted galactosyl residues. The 3-, 6-, and 3,6-substituted galactosyl residues probably constituted a small proportion of Type 2 arabinogalactan [36]; this may have been pectic in nature, but, as 1.3% by weight of the CDTA extract was protein, it is more likely that it was part of a contaminating arabinogalactan protein [37].

Physicochemical studies.—The size and polydispersity of the pectic polysaccharide in 0.1 M aq NH_4Cl was examined. At this salt concentration the molecular dimensions of pectic polysaccharides show only a weak dependence on ionic strength. The measured intrinsic viscosity ($[\eta] = 810$ mL g^{-1}) was comparable to that of another chelator-extractable pectic polysaccharide from apple (760 mL g^{-1}), and indicates that extraction under these mild conditions gives relatively large macromolecules, which are minimally degraded [13]. A rather broad peak was obtained by size-exclusion chromatography (Fig. 1), indicating that the material was highly polydisperse. The elution profile of neutral sugars and uronic acids was comparable, with no obvious separation into a neutral sugar-rich or a uronic acid-rich fraction on the basis of molecular size.

The solution behaviour was further characterised by examining the dependence of the specific viscosity on concentration. For dilute solutions the observed viscosity is primarily related to the size and number of macromolecules in solution. As concentration increases, the polymer coils start to overlap and become entangled, with the viscosity showing a more marked dependence on concentration. In Fig. 2 is shown the dependence of viscosity on concentration, for an aqueous solution of the pectic polysaccharide in the range 0.065–1.5% w/w. At low concentration, < 0.16% w/w,

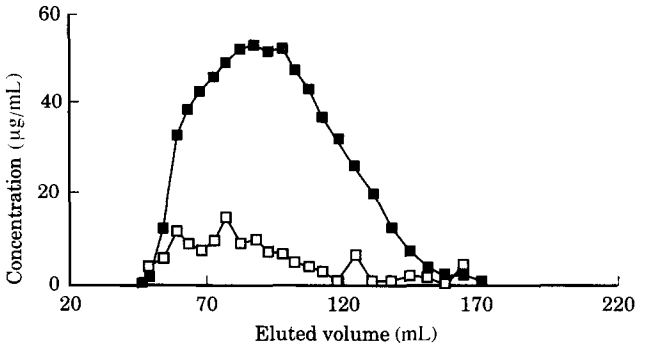

Fig. 1. Chromatography on Sepharose CL-2B of CDTA-extracted pectic polysaccharide. Elution was with 0.1 M NH_4Cl at 16.6 mL h^{-1}: ■, galacturonic acid; □, neutral hexose sugars.

the specific viscosity shows a $c^{1.5}$ dependence on concentration, changing to a $c^{3.3}$ dependence at concentrations above ~ 0.6% w/w. The higher dependence is characteristic of entangled polymer solutions, where dependencies in the range c^{4-5} can be found [38]. We therefore attribute this change in viscous behaviour to chain entanglement. The results are comparable with those of an earlier study of the solution behaviour of citrus pectic polysaccharides [29]. The latter showed that the viscous behaviour of pectic polysaccharides differing in molecular size and degree of esterification could be usefully compared if specific viscosity was plotted as a function of the dimensionless parameter $c[\eta]$, to give a single master curve. The transition from dilute to concentrated solution behaviour occurred over the range of $c[\eta]$ of 0.7–8. In the present study the transition occurs within these bounds over a slightly narrower range, ~ 1.3–5.0. A concentrated,

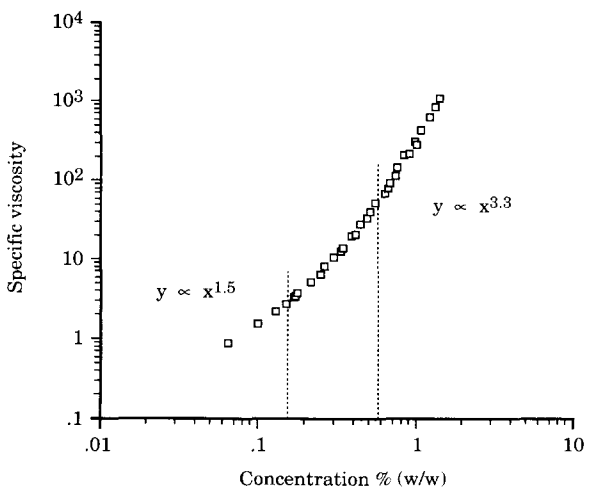

Fig. 2. Double logarithmic plot of specific viscosity versus concentration for CDTA-extracted pectic polysaccharide at 25 °C in 0.1 M NH_4Cl.

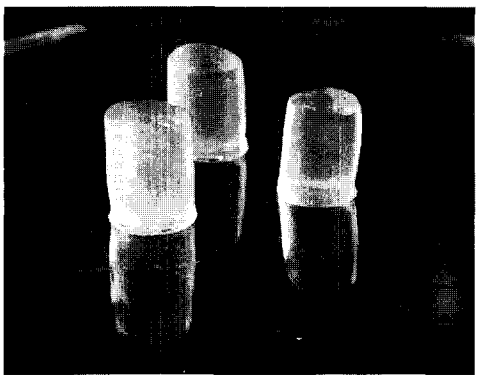

Fig. 3. Pieces of gel (7.5 mm diameter) formed by the addition of $CaCl_2$ (final concentration 35 mM) to a 2% w/w solution of CDTA-extracted pectic polysaccharide.

entangled polymer solution can be viewed as a temporary network; if a mechanism exists to cross-link this entanglement network a more permanent gel network is obtained.

Gel formation.—For the tomato pectic polysaccharide, at polymer concentrations above ~ 0.2% w/w (i.e., at concentrations greater than the coil overlap concentration) gelation could be induced by the addition of $CaCl_2$. In this study, gels were formed without heating to avoid the possibility of polymer degradation. The length of time required for equilibration was determined by drawing a 2% w/w solution of pectic polysaccharide into a 1-mL disposable syringe, and adding 1 M $CaCl_2$ (30 μL) to one end (overall concentration 50 mM at equilibrium). After 24 h the level of calcium in the segment 5–10 mm from the end to which calcium had been applied was 92% of that in the terminal 5 mm. We also found no evidence that there was a difference in the shear modulus of upper and lower halves of gels cast in Eppendorf tubes as described above. Gels formed in this way were clear elastic solids (Fig. 3) which recovered from small static deformations on removal of the applied stress. The gel clarity suggests the presence of a three-dimensional molecular network. This is in contrast to gels formed from the substantial aggregation of polymer chains, for example, the amylose gel, the network strands of which consist of assemblies of chains of sufficient size to lead to gel opacity [39].

Gels formed as described did not show syneresis in the presence of excess of $CaCl_2$ and could not be pelleted by centrifugation at 45,000 g. To determine the polymer composition of the aqueous phase, pieces of gel formed by the addition of $CaCl_2$ (final concentration 10 mM) to a solution of the pectic polysaccharide at 0.7% w/w were soaked in distilled water containing 0.1% w/v sodium azide for 4 days. At the end of this time 28% of the original material had leached into the surrounding liquid. This suggests that a significant proportion of the pectic polysaccharide is not involved in gel formation. However, the profile on gel filtration (Fig. 4) was very similar to that of the starting material, and there was no difference in the degree of methyl esterification. Unless the loss is due to the extended soaking time, these results suggest that the pattern

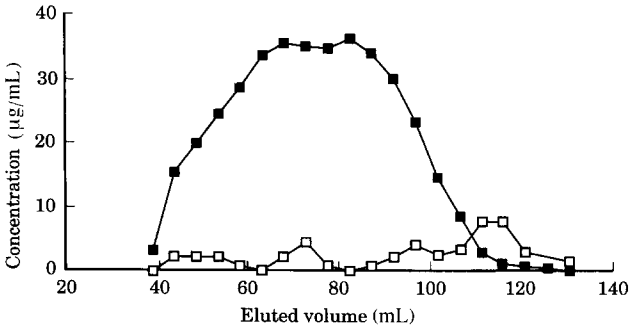

Fig. 4. Chromatography on Sepharose CL-2B of material released from segments of gel stored in distilled water for 4 days. Gels were initially formed by the addition of $CaCl_2$ (final concentration 10 mM) to a 0.7% w/w solution of CDTA-extracted pectic polysaccharide. Elution was with 0.1 M NH_4Cl at 12.1 mL h^{-1}: ■, galacturonic acid; □, neutral hexose sugars.

of methyl esterification in part of the extracted material is not conducive to gel formation.

Gel properties.—Gel stiffness increased with increasing concentration of calcium ions in gels prepared from a single concentration of pectic polysaccharide (Fig. 5). At the highest concentration of $CaCl_2$ tested (where the concentration of calcium ions, on the basis of charge, was 150% of the unesterified carboxyl groups) G′ was still found to be increasing. The dependence of G′ on polymer concentration was therefore studied with calcium ions at 250% of the concentration of carboxyl groups (also calculated on the basis of charge). The shear modulus, G′, of gels formed from solutions of pectic polysaccharide varying from 0.6 to 3% w/w ranged from 250 to 6000 N m^{-2} with a dependence on concentration of $c^{1.9-2.0}$ (Fig. 6). Similar concentration dependencies have been observed for other 'molecular network' gels such as gelatin [40]. In contrast

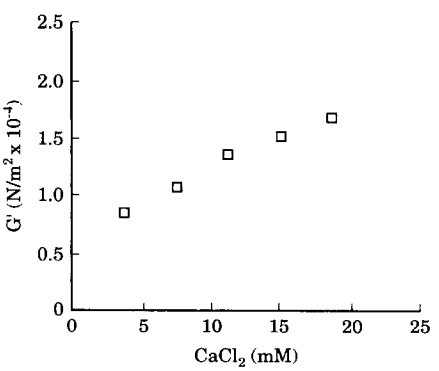

Fig. 5. Dependence of the shear modulus G′ on the concentration of $CaCl_2$ in gels formed by a 2.3% w/w solution of CDTA-extracted pectic polysaccharide. $CaCl_2$ was added to give concentrations of calcium ions equivalent (on the basis of charge) to 30%, 60%, 90%, 120%, and 150% of the level of unesterified carboxyl groups.

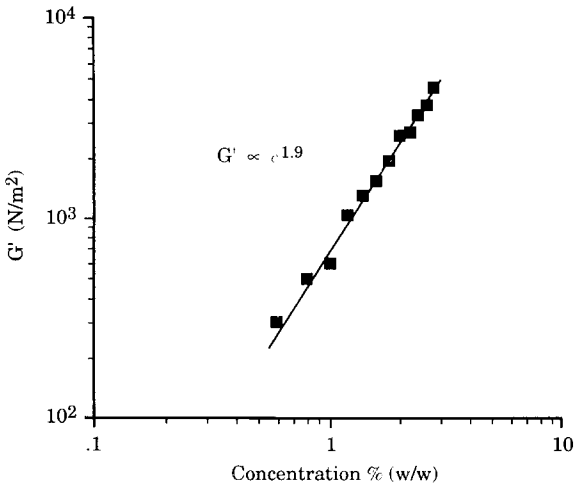

Fig. 6. Double logarithmic plot of shear modulus G′ versus concentration of CDTA-extracted pectic polysaccharide. The amount of $CaCl_2$ in the gels was adjusted to give a calcium ion concentration equivalent (on the basis of charge) to 250% of the unesterified carboxyl groups.

to gelatin, the gel formed by this pectic polysaccharide could not be melted by heating to 120 °C. On prolonged heating (over 20 min) there was some evidence of dissolution although this could have arisen as a result of polymer degradation through a β-elimination mechanism. The physical associations in a calcium-mediated gel of pectic polysaccharide are thus relatively heat-stable. The temperature dependence of gel stiffness was tested on a gel formed from a 1.5% w/w solution of the polysaccharide in the presence of 12 mM $CaCl_2$. Fig. 7 shows an increase in stiffness with increasing temperature, and indicates that there is a direct proportionality between gel stiffness and absolute temperature within the range studied.

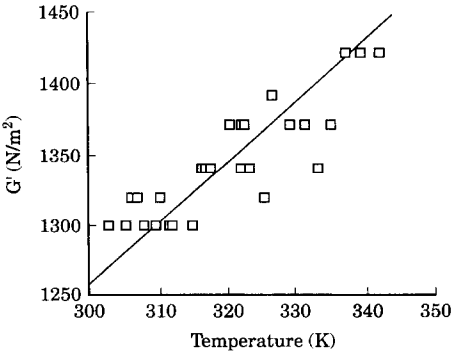

Fig. 7. Plot of shear modulus G′ versus temperature for a gel formed by the addition of $CaCl_2$ (final concentration 11 mM) to a 1.4% w/w solution of CDTA-extracted pectic polysaccharide. The line of best fit was weighted to ensure that it passed through the origin.

4. Discussion

One of the aims of cell-wall research is to identify the aspects of molecular behaviour of cell-wall polysaccharides that influence such wall properties as mechanical behaviour, swelling, and porosity, which, in turn, influence the mechanical properties and texture of plant tissues. For the cell-wall pectic polysaccharides there is a need to identify the molecular mechanisms involved in their association in the cell wall. We have established that a chelator-extracted cell-wall pectic polysaccharide forms elastic gels on addition of calcium ions. As gelation is reversed by adding the chelating agent CDTA, cross-links involving ionic interactions are indicated. In order to relate these interactions to molecular structure it is useful to determine the number of cross-links in these systems. For cross-linked synthetic polymers a suitable starting point is the theory of rubber elasticity [41]. In this theory the mechanism of energy storage is entropic, arising as a result of a constraint on the number of accessible configurations of the polymer as the material is stretched. In its simplest form it leads to the following expression relating shear modulus, G′, to the chain molecular weight between cross-links, M_c:

$$G' = cRT/M_c$$

where c is the concentration of polymer in the network, and R and T have their usual meanings. For synthetic polymer networks there is discussion on how the real network differs from the ideal network of theory. With a polysaccharide as the network polymer, additional factors need to be considered. Firstly, many polysaccharides are relatively stiff and as a result the chain length between cross-links should be relatively high in order for the network to match the requirement of theory that the network strands adopt a flexible conformation equivalent to that of a random coil. Secondly, with a polyelectrolyte network there is the probability of long-range electrostatic interactions which change as the material deforms. Both of these factors can lead to an energetic contribution to gel elasticity, which will diminish as temperature is raised. In the current study the elasticity of the pectic polysaccharide gel showed sufficient temperature dependence (Fig. 7) to justify the use of rubber theory in an attempt to understand the molecular structure of the gel. The main point of interest was the determination of an average molecular weight between cross-links. In this calculation, contributions to gel elasticity from restricted mobility of polymer chains, electrostatic interactions, or the involvement of extended regions of the polymer backbone in cross-link formation will all lead to an underestimate of the distance between cross-links. Using our data for a 2% w/w gel with a modulus of 2000 N m^{-2} leads to a value of M_c of $\sim$ 25 000 with 130 or so residues between cross-links, suggesting that relatively few cross-links between pectic polysaccharides are required to explain the elasticity of gels formed with calcium ions.

Although it is possible for pectic polysaccharides to form ionic cross-links involving calcium when adopting a 3_1 configuration, the predominant association is thought to be between 2_1 helices [9,42], with a stable cross-link being formed by the complexing of several calcium ions with a stretch of 14 contiguous unesterified residues on each of two polymer chains. As discussed above, high-dm pectic polysaccharides extracted from citrus contain blocks of unesterified residues which permit gelation with calcium ions.

However, where two molecules in the 2_1 helical configuration associate, full de-esterification of the section of the molecules forming the associated region is not required. On each polysaccharide molecule, alternate galacturonosyl residues face in the correct direction to form the complex, and the substitution of the intervening residues will not prevent cross-link formation. Interestingly, a pectic polysaccharide containing alternating esterified and unesterified galacturonosyl residues has been isolated from cotton suspension culture [12]. The precise nature of the associated regions will be addressed in a subsequent publication.

The data we have presented are for the physical properties of a dilute gel formed by random association of the molecules from a CDTA-extracted pectic polysaccharide. In the cell wall this polysaccharide is at a higher concentration, and there is a suggestion that pectic polysaccharides may be specifically orientated [43]. Both these differences, and the possible association of the polysaccharide with other cell-wall polymers, suggest that caution is required in extrapolating the properties we have observed back to the cell wall. However, on the basis that the material we have extracted is primarily from the middle lamella region and that gelation of this material is primarily responsible for cell–cell adhesion in unripe tomato, we would expect a close relationship between the physical properties of this gel and conditions leading to cell separation in vivo.

5. Conclusions

A high-methoxy pectic polysaccharide extracted from the cell-wall material of unripe tomato fruit with CDTA formed clear elastic gels on addition of calcium ions. The structure of the pectic polysaccharide was typical of rhamnogalacturonans isolated from the primary cell wall of dicotyledons. Gel formation in the presence of calcium ions occurred in aqueous solutions at concentrations where the polymer chains were entangled. At temperatures less than 120 °C gel formation could not be reversed by heating. The elastic behaviour of the gel enabled an estimate to be obtained of cross-link density, with an estimate of one cross-link for every 130 monomer residues for a 2% w/w aqueous gel at room temperature.

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