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Carbohydrate Polymers 41 (2000) 339–349

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Thermally reversible acid-induced gelation of low-methoxy pectin

P.M. Gilsenan, R.K. Richardson, E.R. Morris*

Cranfield University, Silsoe College, Silsoe, Bedford MK45 4DT, UK

Received 18 May 1999; accepted 21 June 1999

Abstract

Gelation of low-methoxy pectin (DE 31.1) on cooling under acidic conditions in the absence of Ca^{2+} has been investigated by rheological measurements under low-amplitude oscillatory shear. The mechanical spectra obtained after 60 min at 5°C showed a progressive increase in solid-like response (increasing G'; decreasing tan δ; increasing frequency-dependence of η^*) as the pH was reduced from 4.0 to 1.6, with formation of a critically crosslinked network at ~pH 3.0 (for a polymer concentration of 3.0 wt%). By extrapolation from X-ray fibre diffraction analysis of pectic acid, it is suggested that crosslinking occurs by association of three-fold helices. At pH values between ~3.5 and ~2.5 there is no detectable thermal hysteresis between the sol–gel transition on cooling and gel–sol transition on heating, and both are accompanied by a sigmoidal change in optical rotation (attributed to formation and melting of three-fold order). Substantial hysteresis is, however, observed at lower and higher pH, and is attributed to extensive aggregation as electrostatic repulsion is suppressed (below ~pH 2.5) and slow formation of intermolecular hydrogen bonds by protonated carboxyl groups (above ~pH 3.5), respectively. The transition enthalpy from DSC heating scans has a maximum value of $\Delta H \approx 11$ J/g at ~pH 3.0, but decreases sharply at lower and higher pH, with accompanying loss of a detectable transition in optical rotation. It is suggested that the chain conformation in solution at low pH is predominantly three-fold with, therefore, little conformational change on adoption of the ordered, intermolecular structure, whereas at high pH the solution conformation is predominantly two-fold, with only limited conversion to the three-fold (acid) form on cooling.

Keywords: Conformation; Gelation; Rheology; Pectin

1. Introduction

In traditional use of pectin as a gelling agent (see, for example, Christensen, 1986; Rolin, 1993), gelation is induced by cooling in the presence of high concentrations of co-solute at acid pH (typically ~65 wt% sucrose at ~pH 3.0). Formation of an acceptable gel by this mechanism requires most of the (1 → 4 linked) α-D-galacturonic acid residues of the polymer backbone to be present in the (naturally occurring) methyl ester form, with the rate of gelation on cooling increasing with increasing ester content. The degree of esterification (DE) of commercial "high-methoxy" pectins (expressed conventionally as a percentage of total galacturonic acid content) ranges from ~58 for "extra slow set" pectin to ~76 for "ultra rapid set". Pectins of lower ester content, however, can be produced by mild hydrolysis, and gelled with Ca^{2+} (normally for applications where a low sugar content is required). The DE of commercial "low-methoxy" pectins is typically in the range ~30–40, with the rate of gelation now increasing as DE is lowered (i.e. with samples of lower ester content interacting more effectively with Ca^{2+}).

The α-D-galacturonate sequences of pectin are almost the exact mirror image of the α-L-guluronate regions in alginate (differing only in the orientation of O(3)). As might therefore be expected, the two materials show some direct parallels in their interaction with Ca^{2+}. In particular, the amount of bound Ca^{2+} resistant to displacement by increasing concentrations of monovalent cations reaches a final plateau value equivalent to half the total stoichiometric requirement of polyguluronate in alginate samples (Morris, Rees, Thom & Boyd, 1978) or of (fully de-esterified) polygalacturonate from pectin (Morris, Powell, Gidley & Rees, 1982), consistent with a particularly stable dimeric "egg-box" structure (Grant, Morris, Rees, Smith & Thom, 1973) in which the participating sequences adopt a two-fold zig-zag conformation, with chelation of calcium ions to carboxyl groups along the inner (but not the outer) faces of both chains (Morris et al., 1982). Also, the Ca^{2+}-induced gelation of poly-D-galacturonate is accompanied by large changes in circular dichroism (CD), which are almost exactly equal and opposite to those observed for poly-L-guluronate

* Corresponding author. Present address: Department of Food Science and Technology, University College Cork, Cork, Ireland.

E-mail address: ed.morris@ucc.ie (E.R. Morris)

0144-8617/00/$ - see front matter
PII: S0144-8617(99)00119-8

sequences in alginate (as would be expected for near mirror-image structures subjected to the same perturbation).

Similar changes in CD are observed (Powell, Morris, Gidley & Rees, 1982) when partially esterified pectins are gelled with Ca^{2+} at neutral pH. The magnitude of the changes decreases with increasing ester content (i.e. as the proportion of unesterified sequences available for Ca^{2+}-chelation is decreased), but in all cases the effect is to reduce the overall intensity of the CD spectrum, whereas conventional gelation of high-methoxy pectin, at high levels of co-solute and low pH, is accompanied by a small increase in CD (Morris, Gidley, Murray, Powell & Rees, 1980). However, although some applications of low-methoxy pectins involve gelation at neutral pH, many others (e.g. in fruit-based products) require some acidity.

In an extension of the CD approach to Ca^{2+}-induced gelation of low-methoxy pectin under acidic conditions, Gidley, Morris, Murray, Powell and Rees (1980) found that as the pH was lowered the spectral changes accompanying gel formation gradually shifted from a decrease in intensity, as observed under neutral conditions, to an overall increase, as seen for conventional high-methoxy pectin gels, with virtually no net change in the centre of the range (at ~pH 3). It was also found that inhibition of gelation by 8 M urea (which abolishes gel formation by high-methoxy pectin at high solids and low pH but has little effect on Ca^{2+}-gelation of low-methoxy pectin under neutral conditions) became progressively more evident as pH was reduced; conversely, poly-D-galacturonate blocks (which cause large reductions in calcium pectinate gel strength at neutral pH, by competitive inhibition of association through dimeric junctions, but have no effect on gelation of high-methoxy pectin under acidic conditions) were found to become progressively less effective. The overall conclusion was that two types of intermolecular association are involved: (i) interchain chelation (egg-box binding) of calcium ions, and (ii) non-ionic associations analogous to those in conventional high-methoxy pectin gels, with the second mechanism becoming progressively more significant as the pH is decreased.

In the present work we have explored the non-ionic interactions further, by investigation of the gelation of low-methoxy pectin under acidic conditions, but in the absence of Ca^{2+} (or cosolute).

2. Materials and methods

The pectin used was a research sample (X4907) prepared in the free acid form by the Copenhagen Pectin Division of Hercules, and kindly supplied with the following analytical information: content of pure pectin = 96.2%, of which 85.2% is galacturonate with DE 31.1, giving an effective formula weight per carboxyl group of 319. The Ca^{2+} content is 0.80 mg/g, which is ~1.3% of stoichiometric equivalence to the carboxyl groups of the polymer. (100% stoichiometric = 20 g Ca^{2+} per 319 g pectin = 62.7 mg/g.) The polymer concentration used in most of the investigations was 3.0 wt%. The natural pH of the pectin sample at this concentration is ~3.2; solutions of higher or lower pH were obtained by addition of sodium bicarbonate or hydrochloric acid (AnalaR grade from BDH), respectively. In all cases the starting solution was prepared at a concentration ~10% higher than the required value and was then diluted to the final concentration after adjustment of pH. Distilled deionised water was used throughout.

Small-deformation measurements of storage modulus (G'), loss modulus (G'') and complex dynamic viscosity ($\eta^* = (G'^2 + G''^2)^{1/2}/\omega$, where ω is the frequency in rad/s) were made using cone-and-plate geometry (50 mm diameter; 0.05 rad cone angle) on a sensitive prototype rheometer designed and constructed by one of us (R.K.R.). To circumvent problems of thermal expansion/contraction during heating and cooling, the cone was truncated over 45% of its diameter, giving a gap of 0.5 mm between the flat surfaces of the two elements, but keeping strain constant at a fixed, maximum, value across the outer portion (which constitutes 80% of the total area). Temperature was controlled by a Haake circulating water bath and measured with a thermocouple attached to the stationary element. Samples were loaded onto the rheometer in the solution state at 85°C and their periphery was coated with light silicone oil to minimise evaporation. They were then cooled to 5°C, held for 100 min, and re-heated to 85°C. The heating and cooling scans were made at 1°C/min, with measurements of G' and G'' at 10 rad/s and 2% strain. During the last 40 min of the holding period at 5°C (i.e. 60 min after completion of cooling) a mechanical spectrum (frequency-dependence of G', G'' and η^*) was recorded (at 2% strain).

Optical rotation was measured at 436 nm on a Perkin–Elmer 241 polarimeter, using jacketed cells of pathlength 1 or 10 cm, as appropriate. Temperature was again controlled by a Haake circulating water bath, and measured using a thermocouple in the neck of the cell, but out of the light path. The results are reported as specific rotation, $[\alpha]$, which is conventionally defined as:

$$[\alpha] = 100\alpha/lc \quad (1)$$

where α is optical rotation in degrees, l is pathlength in dm, and c is concentration in g/100 ml. The factor of 100 can be eliminated by expressing α in mdeg and l in cm.

Differential scanning calorimetry (DSC) measurements were made on a Setaram microcalorimeter, using a scan rate of 0.1°C/min and sample mass of ~0.8 g.

3. Results

3.1. Rheology

The rheological studies were made using a fixed pectin

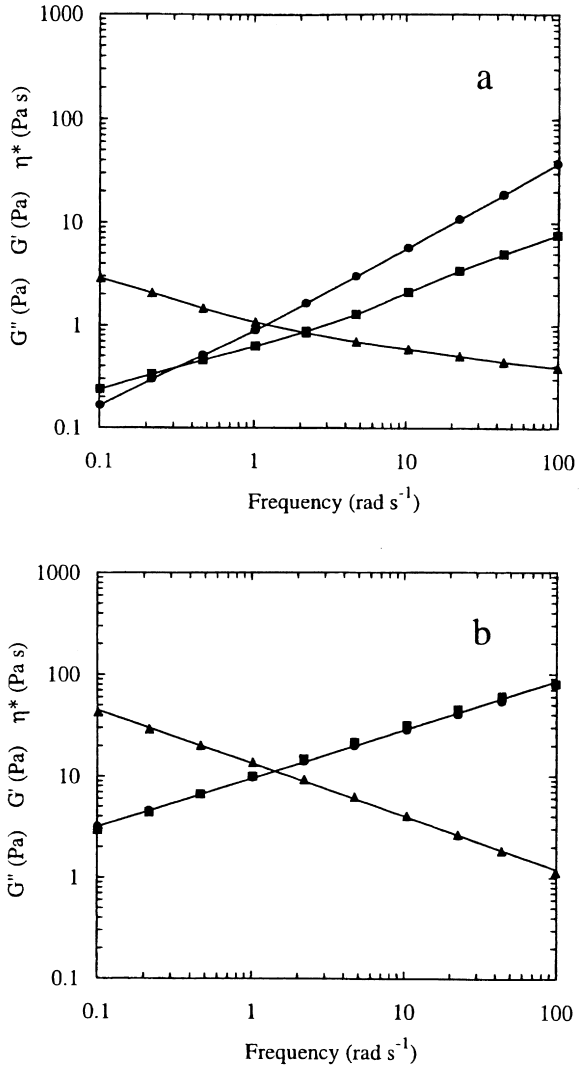

Fig. 1. Mechanical spectra (2% strain; recorded after 60 min at 5°C), showing the frequency-dependence of G′ (■), G″ (●) and η^* (▲) for 3.0 wt% pectin at: (a) pH 4.0; and (b) pH 3.0.

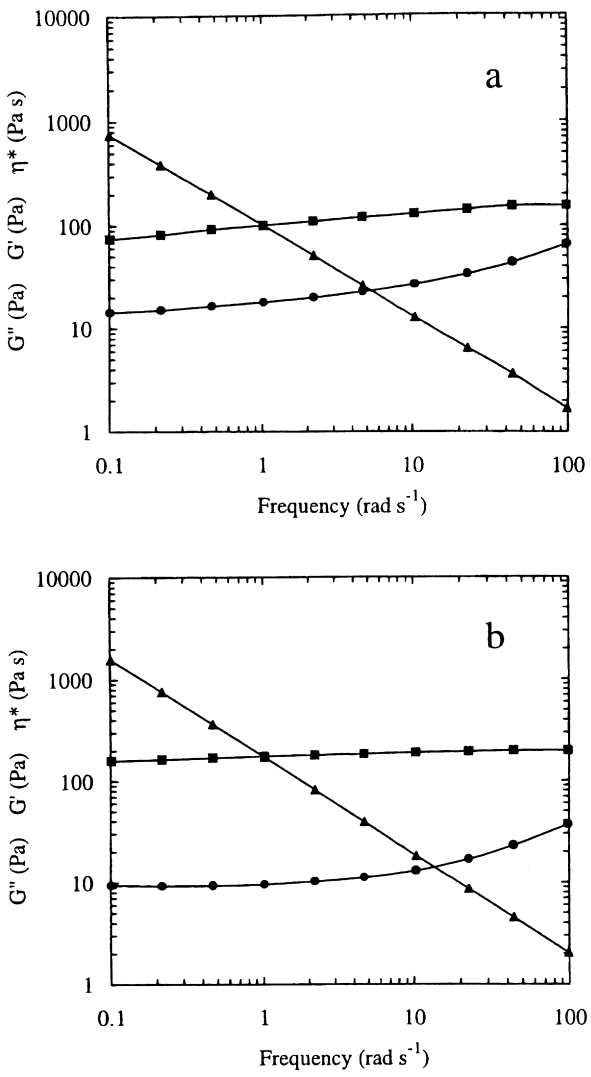

Fig. 2. Mechanical spectra (2% strain; recorded after 60 min at 5°C), showing the frequency-dependence of G′ (■), G″ (●) and η^* (▲) for 3.0 wt% pectin at: (a) pH 2.0; and (b) pH 1.6.

concentration of 3.0 wt%, at pH values of 4.0, 3.5, 3.0, 2.5, 2.0 and 1.6. Figs. 1 and 2 show illustrative mechanical spectra recorded after 60 min at 5°C. At pH 4.0 (Fig. 1(a)) the mechanical response is predominantly liquid-like, with $G'' > G'$ over most of the accessible frequency range (0.1–100 rad/s). The spectrum obtained at pH 3.0 (Fig. 1(b)) is typical (Durrand, Delsanti, Adam & Luck, 1987; te Nijenhuis & Winter, 1989) of a gelling system where the degree of crosslinking is just sufficient to give a continuous network; log G' and log G'' both vary linearly with log ω over the entire frequency range, with the same slope for both moduli. The response at pH 2.0 is typically gel-like (Fig. 2(a)), with $G' \gg G''$ and little frequency-dependence in either modulus. On further reduction in pH to 1.6 (Fig. 2(b)), the gel-like character becomes more pronounced, with greater separation of G' and G'' and even less variation with frequency.

As shown in Fig. 3(a), there is a monotonic increase in G' (at 10 rad/s) as the pH is lowered (by about two orders of magnitude between pH 4.0 and pH 1.6), with an accompanying monotonic decrease in tan δ (the ratio of G''/G'). As would be anticipated from the mechanical spectrum shown in Fig. 1(b), the value of tan δ passes through 1.0 ($G' = G''$) at pH 3.0, the point of critical crosslinking. The progressive change from solution-like to gel-like response is further illustrated by the pH-dependence of the average slope of log η^* vs log ω across the frequency range studied. As shown in Fig. 3(b), the slope at pH 1.6 is close to the theoretical maximum value of -1.0 for a perfectly elastic (Hookean) network, but as the pH is raised there is a steep and essentially linear decrease in slope towards the minimum value of 0.0 anticipated for a perfect (Newtonian) liquid. Thus by normal critieria of biopolymer network rheology (e.g. Ross-Murphy, 1984) the mechanical spectra obtained for 3.0 wt% low-methoxy pectin (DE 31.1) after 60 min at 5°C show a smooth progression from solution-like

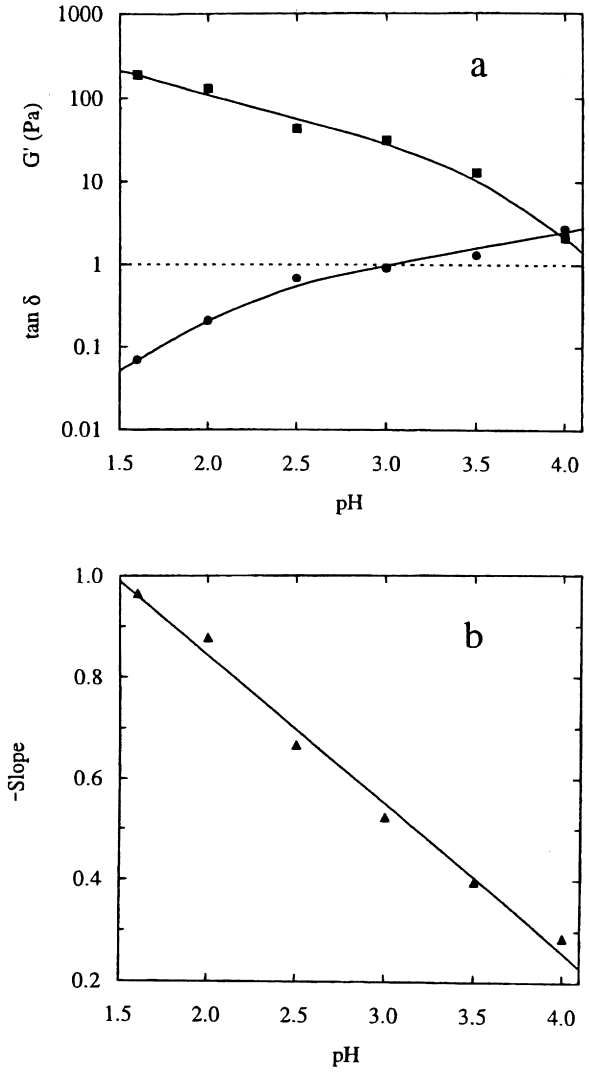

Fig. 3. Effect of pH on: (a) G′ (■) and tan δ (●), recorded at 10 rad/s and 2% strain; and (b) the (negative) slope of log η^* vs log ω for 3.0 wt% pectin after 100 min at 5°C.

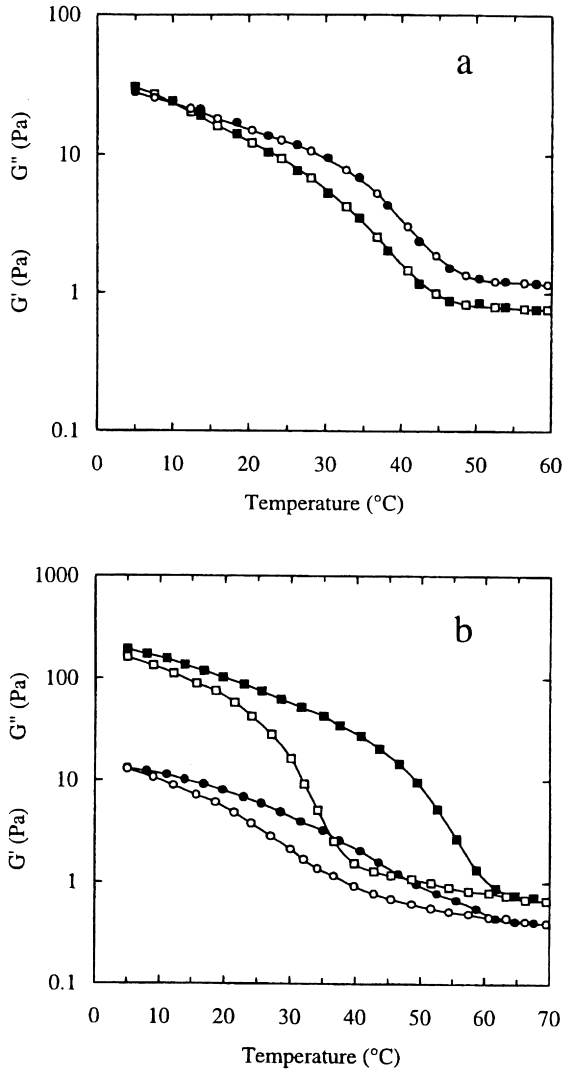

Fig. 4. Temperature-dependence of G′ (squares) and G″ (circles), recorded at 10 rad/s and 2% strain during cooling (open symbols) and heating (filled symbols) at 1°C/min, for 3.0 wt% pectin at: (a) pH 3.0; and (b) pH 1.6. Samples were held for 100 min at 5°C between the end of the cooling scan and start of the heating scan.

response at pH 4.0 to typical gel-like properties at pH 2.0 and below, with critical crosslinking at ~pH 3.0.

The underlying structures and processes, however, appear somewhat more complex. Fig. 4(a) shows the formation and melting of the critically crosslinked network at pH 3.0, as characterised by the temperature dependence of G' and G'' on cooling and heating. Both moduli show a sigmoidal transition across the approximate temperature range 10–45°C, with no detectable thermal hysteresis between the cooling and heating traces. The cooling and heating scans obtained at pH 2.5 and 3.5 were also virtually independent of the direction of temperature change. At pH 1.6, however, melting occurred at substantially higher temperature than gel formation on cooling (Fig. 4(b)). Similar thermal hysteresis was also observed at pH 2.0. Visual inspection of the gels formed at these low pH values showed obvious turbidity, which cleared on heating through the temperature range of the gel–sol transition, whereas the samples prepared at pH 2.5 and above remained clear at all temperatures. It seems reasonable to conclude, therefore, that as found for other polysaccharide systems (Morris & Norton, 1983), the hysteresis is associated with aggregation.

Massive thermal hysteresis was also observed (Fig. 5) at pH 4.0, but appears to originate in an entirely different way. As illustrated in Fig. 4, the values of G' and G'' recorded at the end of the cooling scan for samples in the pH range 1.6–3.5 were virtually identical to those found at the beginning of the heating scan, after 100 min at 5°C. At pH 4.0, however, there is a large increase in G″ and (particularly) G' during this holding period (Fig. 5), indicating that the hysteresis is now due to slow kinetics of structural change on cooling, rather than to enhancement of thermodynamic stability by aggregation.

As mentioned in Section 2, the pectin sample used has a

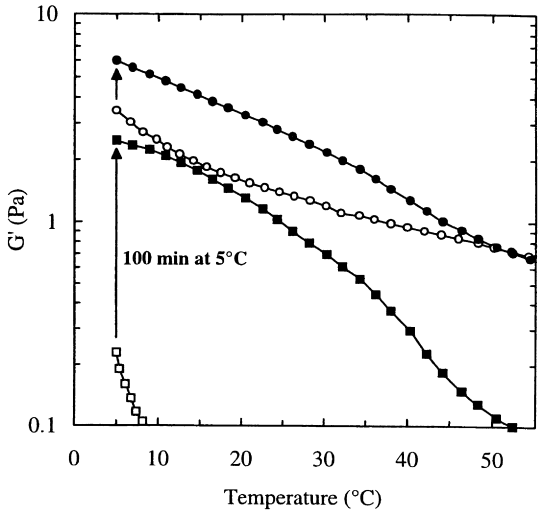

Fig. 5. Temperature-dependence of G′ (squares) and G″ (circles), recorded at 10 rad/s and 2% strain during cooling (open symbols) and heating (filled symbols) at 1°C/min, for 3.0 wt% pectin at pH 4.0. The sample was held for 100 min at 5°C between the end of the cooling scan and start of the heating scan.

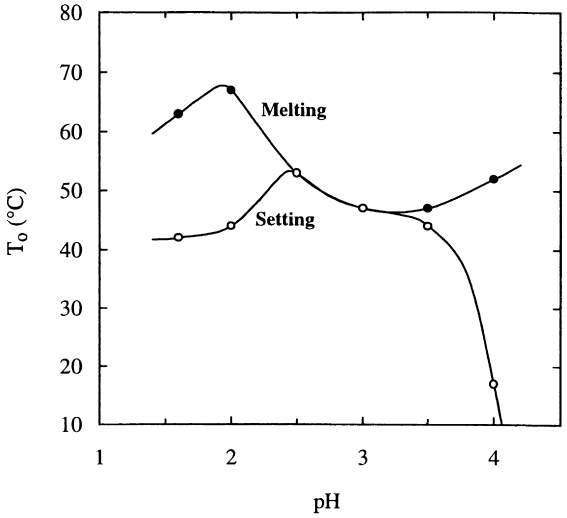

Fig. 6. Effect of pH on the temperature (T_0) at the onset of gelation on cooling (○) and completion of gel melting on heating (●) for 3.0 wt% pectin; cooling and heating was at 1°C/min.

small, but detectable, content of Ca^{2+} (~1.3% of stoichiometric equivalence to the carboxyl groups of the polymer), raising the possibility that the slow development of structure during holding at 5°C might be due to formation of a calcium pectinate network. This possibility was tested by repeating the measurements at pH 4 for a solution incorporating a large excess of calcium sequestrant (100 mM EDTA). The cooling and heating traces obtained were close to those shown in Fig. 5 for the solution with no sequestrant. In particular, the increase in moduli during the holding period at 5°C was virtually identical for both samples, which indicates clearly that it does not arise from Ca^{2+}-induced association.

As shown in Fig. 4, the changes in moduli on the low-temperature side of the sol–gel and gel–sol transitions are smooth and progressive, with no quantifiable end-point. The temperature (T_0) at the onset of the steep increase in moduli on cooling and on completion of the corresponding reduction on heating, however, is much more clearly defined, and can be estimated to within about ±1–2°C. Fig. 6 shows the variation of T_0 on cooling and heating across the range of pH values studied. The most noticeable features are the massive thermal hysteresis at pH 4.0 and sharp increase in melting temperature between pH 2.5 and pH 2.0 which, as discussed above, can be attributed to slow kinetics of structure formation on cooling and enhancement of thermal stability by aggregation, respectively. There is also, however, a maximum in T_0 values on cooling at ~pH 2.5, whose possible origin will be discussed later.

3.2. Differential scanning calorimetry

Fig. 7 shows DSC heating scans (0.1°C/min) recorded for the same samples as in the rheological studies described above (3.0 wt% pectin at pH 1.6–4.0). In all cases there is a well-defined endotherm, but with substantial variation in position and size. As shown in Fig. 8(a), the transition mid-point temperature (T_m) increases sharply between pH 2.5 and pH 2.0, paralleling the increase in melting temperature observed rheologically (Fig. 6). The most striking feature, however, is a sharp maximum in transition enthalpy (ΔH) at ~pH 3.0 (Fig. 8(b)).

The effect of polymer concentration (1.5, 3.0 and 4.5 wt%) on the position and size of the DSC endotherm at this pH is shown in Fig. 9. The transition enthalpy increases in direct proportion to concentration (Fig. 10(a)), with a small accompanying rise in transition temperature, as characterised by either the temperature of maximum heat

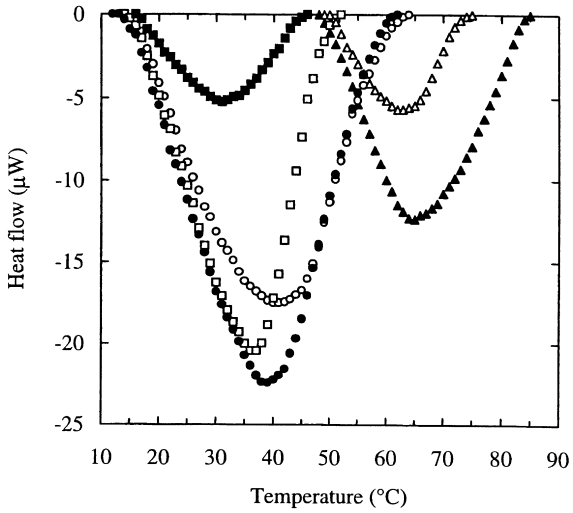

Fig. 7. DSC heating scans (0.1°C/min after 100 min at 5°C) for 3.0 wt% pectin at pH values of 1.6 (△), 2.0 (▲), 2.5 (○), 3.0 (●), 3.5 (□) and 4.0 (■). The heat-flow values are scaled to 1.0 g of solution.

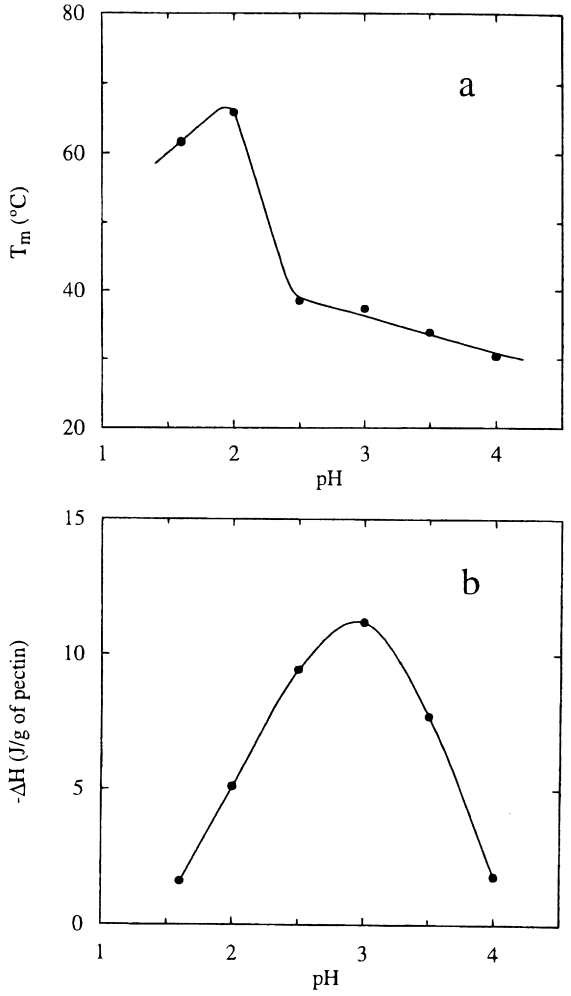

Fig. 8. Effect of pH on: (a) transition mid-point temperature (T_m); and (b) transition enthalpy (ΔH) from the DSC heating scans shown in Fig. 7 (for 3.0 wt% pectin held for 100 min at 5°C before heating).

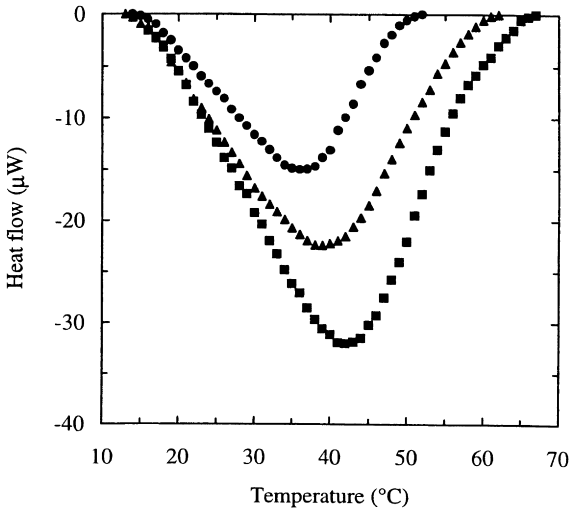

Fig. 9. DSC heating scans (0.1°C/min after 100 min at 5°C) for pectin at pH 3.0, recorded for concentrations of 1.5 (●), 3.0 (▲) and 4.5 (■) wt%. The heat-flow values are scaled to 1.0 g of solution.

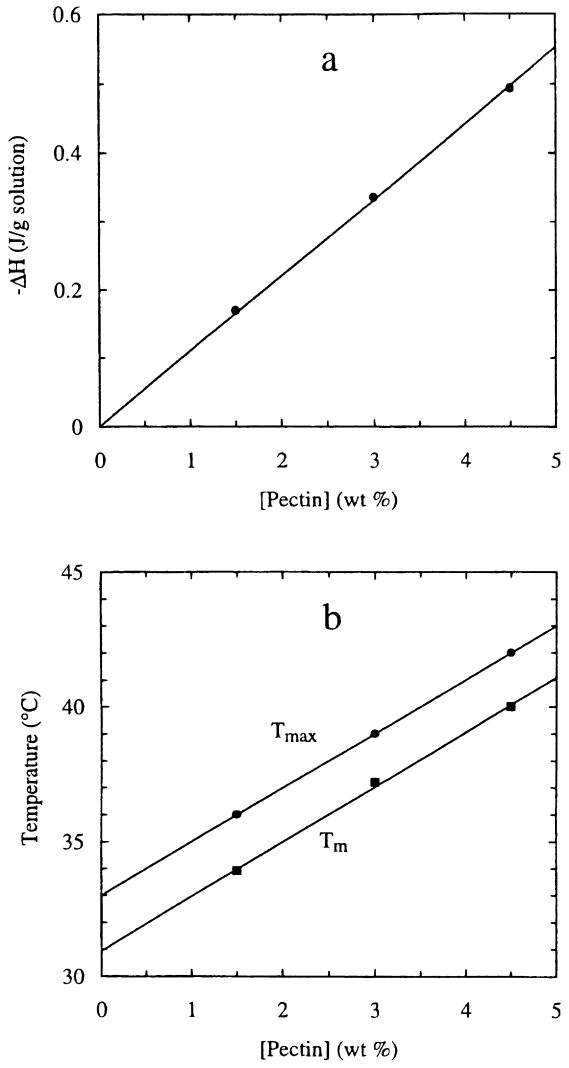

Fig. 10. Concentration-dependence of: (a) the transition enthalpy (ΔH); and (b) the temperature at the peak maximum (T_{max}; ●) and transition mid-point (T_m; ■) from the DSC heating scans shown in Fig. 9 (pH 3.0).

flow (T_{max}) or the mid-point temperature (T_m), both of which increase by ~6°C as the pectin concentration is raised from 1.5 to 4.5 wt% (Fig. 10(b)). Interpretation of the significance of the DSC results, and in particular of the maximum in ΔH at pH 3.0 (Fig. 8(b)), is deferred to Section 4.

3.3. Optical rotation

The changes in macromolecular organisation underlying the thermal transitions seen by rheology (Figs. 4 and 5) and DSC (Figs. 7 and 9) were investigated further by optical rotation, which is well established as a sensitive index of changes in polysaccharide chain conformation (Rees, 1970; Rees, Morris, Thom & Madden, 1982; Morris, 1994). Fig. 11 shows the effect of pH on the optical rotation values obtained for 0.4 wt% pectin on cooling and heating across the approximate temperature range 5–85°C. The temperature of the circulating water bath was adjusted manually and

measurements were recorded after the polarimeter readings had reached stable values at each temperature. As shown in Fig. 11(a), the temperature-dependence of specific rotation at pH 7.0 and pH 2.0 is essentially linear. At intermediate pH values (3.0 and 3.5), however, there is clear evidence (Fig. 11(b)) of a conformational transition which, as illustrated for the pH 3.0 sample, occurs over the same temperature range as the gelation and melting processes observed rheologically (Fig. 4(a)). As found in the rheological studies (Fig. 6) the changes in optical rotation in this pH range show no detectable hysteresis between cooling and heating.

Similar temperature-dependent changes in optical rotation at pH 3.0 were observed (Fig. 12) at higher pectin concentration (4.5 wt%), and span essentially the same temperature range as the DSC endotherm obtained (Fig. 9)

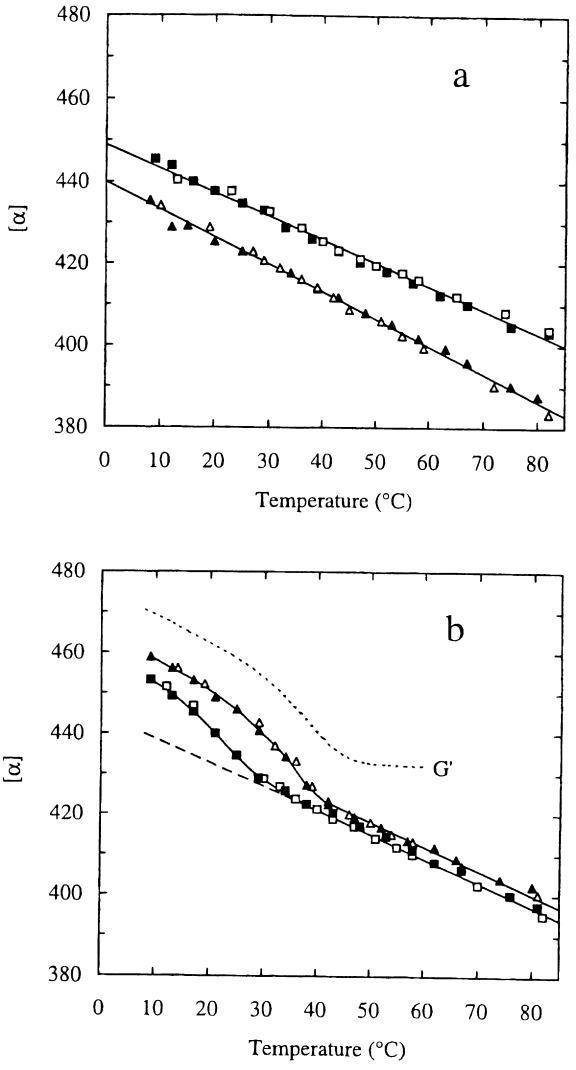

Fig. 11. Temperature-dependence of specific rotation, [α], (436 nm; 10 cm pathlength) recorded during cooling (open symbols) and heating (filled symbols) for 0.4 wt% pectin at pH values of (a) 2.0 (triangles) and 7.0 (squares), and (b) 3.0 (triangles) and 3.5 (squares); the temperature-course of rheological change at pH 3.0 (G′ curve from Fig. 4(a)) is also shown (···).

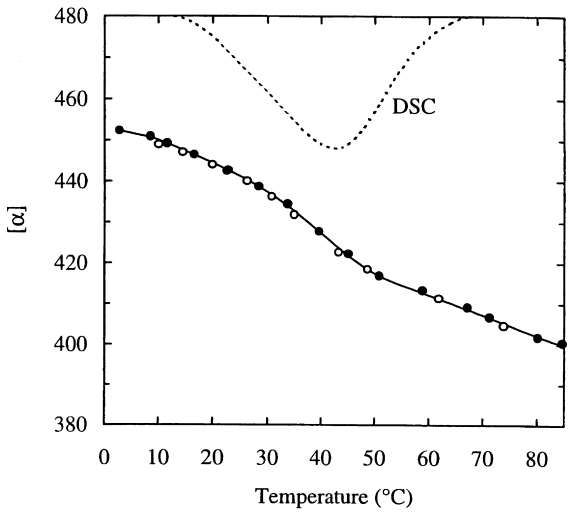

Fig. 12. Temperature-dependence of specific rotation (436 nm; 1 cm pathlength) on cooling (○) and heating (●) for 4.5 wt% pectin at pH 3.0. The DSC heating scan (Fig. 9) for the same sample is also shown (···).

for the same sample, indicating that the chiroptical and thermal transitions have a common molecular origin.

4. Discussion

Despite the structural similarities between (1 → 4)-linked poly-α-D-galacturonate and poly-α-L-guluronate outlined in Section 1, the change in configuration at C(3) has some profound effects on conformational preferences. X-ray fibre diffraction studies have shown that polyguluronic acid, in the solid state, has a buckled two-fold conformation (Atkins, Nieduszynski, Mackie, Parker & Smolko, 1973) which persists in all salt forms so far studied (Mackie, 1971; Mackie, Perez, Rizzo, Taravel & Vignon, 1983). It was this structure that inspired the egg-box model for binding of Ca^{2+} by alginate under hydrated conditions (Grant et al., 1973). The solid-state structures derived by fibre-diffraction studies of pectin, by contrast, are invariably three-fold (Walkinshaw & Arnott, 1981a,b), albeit with substantial differences in packing in response to the presence or absence of methyl ester substituents and to conversion between acid and salt forms (including Ca^{2+}). Conformational energy calculations for isolated poly-D-galacturonate chains (Sathayanarayana & Rao 1973), however, suggest marginally greater stability in the two-fold conformation required for egg-box binding. Indeed one line of experimental evidence in support of the egg-box model (Morris et al., 1982) is that when calcium pectate gels are dried to solid films there is a massive change in CD, consistent with conversion from two-fold to three-fold geometry, whereas no such change is seen for calcium alginate, where, as discussed above, the two-fold structure is retained on drying. A change in conformation from 2_1 to 3_1 between the hydrated and dried states of calcium poly-D-galacturonate,

but not of calcium poly-L-guluronate, has been confirmed more recently by ^{13}C NMR (Jarvis & Apperley, 1995).

A likely contributing factor to the difference in solid-state packing is that the O(3) hydroxyl groups of polygalacturonate participate in a concerted array of intermolecular hydrogen bonds with carboxyl groups on adjacent (anti-parallel) three-fold helices (Walkinshaw & Arnott, 1981a), whereas an analogous hydrogen-bonding scheme would not be possible for three-fold polyguluronate chains because of the difference in configuration at C(3).

On the basis of evidence from potentiometry, viscometry, isothermal calorimetry and chiroptical measurements, a change in conformation from an extended (two-fold) structure to a more compact (three-fold) form has also been proposed for polygalacturonate in dilute solution, in response to reduction in pH at fixed temperature (Ravanat & Rinaudo, 1980; Cesàro, Ciana, Delben, Manzini & Paoletti, 1982). This proposal is entirely consistent with the results of the present investigation, which indicate that the same transition can be induced by changes in temperature at fixed values of pH.

The description of chain conformation in solution as two-fold or three-fold should not, of course, be interpreted as implying rigid, fixed geometry of the type found in the solid state and in the junction zones of polysaccharide gels, but rather as the state of minimum free energy around which the local conformation fluctuates. At pH values where most of the carboxyl groups are ionised, the chain will be stiffened and extended by intramolecular electrostatic repulsion, giving local conformations close to the extended two-fold structure. Reduction in charge-density by lowering pH will allow the chain to adopt a more compact arrangement close to the three-fold structure, perhaps stabilised by some intramolecular hydrogen-bonding between O(2) of one residue and the carboxyl group on the next residue (towards the reducing end), as found in the solid state (Walkinshaw & Arnott, 1981a). Our present results indicate that the three-fold arrangement can be disrupted by heating, displacing the average conformation towards the two-fold structure, with re-establishment of the more compact chain geometry on cooling.

However, since the transitions observed in the present work are associated with formation and melting of network structure, they must obviously also involve intermolecular association of three-fold chains into larger assemblies. This concept of both intramolecular and intermolecular ordering provides a useful starting point for detailed discussion of the experimental results. First, it seems reasonable to conclude from previous investigations of polysaccharide gelation (Rees et al., 1982), and from fundamental analysis of saccharide optical activity (Stevens & Sathyanarayana, 1987; Stevens, Sathyanarayana & Morris, 1989), that the changes in optical rotation (Figs. 11 and 12) can be attributed almost entirely to changes in chain conformation (i.e. to intramolecular ordering). Conversely, the changes in rheology (Figs. 1–6) can be attributed almost entirely to intermolecular association. Both levels of organisation might, however, be expected to contribute to the thermal changes characterised by DSC (Figs. 7–10).

The appearance of a detectable optical rotation transition in the centre of the pH range (Fig. 11(b)) but not at higher or lower pH (Fig. 11(a)), and the associated maximum in ΔH from calorimetry (Fig. 8(b)), can now be explained very simply. At high pH, there is little conversion to the three-fold acid form; at low pH, there is little need for conversion, since the chain conformation in the solution state will already be predominantly three-fold. The changes in modulus on cooling and heating at pH 4.0 (Fig. 5) indicate some conversion to the three-fold ordered structure, but the solution-like rheology at low temperature (Fig. 1(a)) suggests that the extent of ordering is very low, with the associated change in optical rotation therefore being too small to be detected.

At the bottom end of the pH range studied (below ~pH 2.5), the gelling transition must arise predominantly from association of pre-existing three-fold structure. As discussed previously, the intermolecular junctions formed at these low pH values appear to consist of large aggregates; indeed light-scattering studies (Sawayama, Kawabata, Nakahara & Kamata, 1988) have shown substantial aggregation of pectin, even in dilute solution, as the pH is reduced below ~2.5. As found for other aggregating polysaccharides (Morris & Norton, 1983), final loss of network structure on heating occurs at a temperature substantially higher than the temperature of initial gel formation on cooling (Fig. 4(b)). Comparison of the DSC endotherms (Fig. 7) obtained at pH 2.0 and pH 1.6 with the temperature (T_0) at completion of gel-melting for the same samples (Fig. 6), however, shows that the thermal changes continue for ~15°C after loss of network cohesion. A possible interpretation is that disaggregation (i.e. loss of intermolecular order) occurs during the first part of the thermal transition, but that, under these very acidic conditions, a further increase in temperature is required for destabilisation (melting) of the individual three-fold helices.

At intermediate pH values (between ~2.5 and ~3.5), the chiroptical, rheological and thermal changes occur over approximately the same temperature range (Figs. 11(b) and 12), indicating that intramolecular and intermolecular ordering also occur together, and, as would be anticipated for an acid-induced process, the transition temperature from all three investigative techniques increases (Figs. 6, 8(a) and 11(b)) as the pH is lowered. The absence of any significant thermal hysteresis in this pH range (Figs. 6, 11(b) and 12) argues against the formation of large, aggregated junctions. The minimum requirement for development of network structure (Fig. 3) would be association of individual three-fold helices into intermolecular dimers, but the absence of hysteresis would not necessarily preclude somewhat larger assemblies such as trimers or tetramers.

As shown in Fig. 8(b), the transition enthalpy (ΔH) passes

through a maximum at ~pH 3.0. The pK_a of pectin, like those of other polyelectrolytes (Milas & Rinaudo, 1997), varies with degree of ionisation, but extrapolates to a minimum value (pK_0) of ~3.3 in the limit of complete protonation (Ravanat & Rinaudo, 1980; Rinaudo, 1996). Thus at the pH of maximum enthalpy change (~3.0) somewhat less than half of the carboxyl groups will be ionised. A maximum in ΔH at about the same degree of ionisation has been observed previously in a study of the heat of dissociation of the carboxyl groups of pectic acid in dilute solution (Cesàro et al., 1982). The experiments were carried out by isothermal calorimetry, using dilution with water to displace the equilibrium between the protonated and ionised forms. Comparison with calculated values for simple dissociation, and with experimental results for galacturonic acid monomer, showed a large excess change in enthalpy for the polymer, which, as discussed above, was attributed to conversion from a compact (hydrogen-bonded) conformation in the protonated form to a more extended structure in the ionised state.

The reported value of (endothermic) ΔH for the transition from the acid to the ionised form was ~500 cal/equiv, and the accompanying change in entropy was estimated at ~1.6 cal/equiv K. Converting from calories to joules (1 cal = 4.186 J) and substituting the equivalent weight of 222.8 reported by Cesàro et al. (1982) for the sample studied gives $\Delta H \approx 9.4$ J/g and $\Delta S \approx 0.030$ J/g K, which can be compared with the corresponding values for the thermally induced transition observed in the present work. The maximum enthalpy change at pH 3.0 (Fig. 8(b)) is ~11 J/g, and the corresponding value of T_m (Fig. 8(a)) is ~37°C (~310 K), from which the transition enthalpy (ΔS) can be derived by the following standard relationships:

$$\Delta G = \Delta H - T\Delta S = 0 \text{ at } T_m \quad (2)$$

i.e.

$$\Delta S = \Delta H / T_m \quad (3)$$

giving $\Delta S \approx 0.038$ J/g K. In view of the different approaches used, and the likely experimental errors in both studies, the standard of agreement between the values of ΔH (9.4 and 11.0 J/g) and ΔS (0.030 and 0.038 J/g K) obtained in the two investigations is remarkably good, and gives further assurance that the transitions observed in the present work on changing temperature at fixed values of pH have the same molecular origin as those observed previously on changing degree of ionisation (pH) at fixed temperature.

Finally, we offer a speculative interpretation of the complex changes in setting and melting temperatures observed (Fig. 6) on varying pH. The expected consequence of converting carboxyl groups from the ionised to the protonated form would be to facilitate intermolecular association and, ultimately, extensive aggregation, by suppressing electrostatic repulsion between chains. As discussed above, the progressive rise in setting temperature between ~pH 3.5 and ~pH 2.5, and the sharp increase in melting temperature between ~pH 2.5 and ~pH 2.0, can both be explained in this way. Protonation/ionisation of carboxyl groups may however have other effects on the stability of intermolecular association.

X-ray diffraction analysis (Walkinshaw & Arnott, 1981a) suggests that the carboxyl groups of pectic acid are involved in three types of hydrogen bonding. Two of these have been mentioned above: an intramolecular bond to O(2) of an adjacent residue along the same chain, and an intermolecular bond to O(3) on a neighbouring (antiparallel) helix. The third also involves association with a neighbouring antiparallel chain, but by hydrogen bonding between carboxyl groups on both. Obviously, this type of bonding can occur only when at least one of the participating carboxyl groups is protonated, and therefore capable of acting as the hydrogen-bond donor.

The involvement of an intermolecular bond that requires protonation of carboxyl groups might explain the slow kinetics of structure formation (Fig. 5) at pH 4.0, where the degree of protonation in the solution state will be low. We suggest, tentatively, that the normal equilibrium between ionised and protonated carboxyl groups may be displaced by progressive incorporation (effectively site-binding) of hydrogen ions within the ordered structure, to allow the hydrogen bond to form. As would be expected from this interpretation, the solutions prepared at pH 4.0 showed a progressive increase in pH (to ~4.5) over the period of rheological change, whereas no such increase was observed at pH values below ~3.5, where there is already substantial protonation in the solution state. Neutral solutions, however, showed an even greater variation in pH, from ~7.0 to ~8.1 over a period of 24 h.

Although protonation of a proportion of the carboxyl groups may be an important factor in stabilising intermolecular association of pectin chains, hydrogen bonds formed with ionised carboxyl groups as receptors will be stronger than those involving groups that carry only a partial negative charge (hydroxyl or protonated carboxyl), because the electrostatic attraction will be greater. This concept may explain why the aggregates formed at pH 2.0 are more thermally stable (Figs. 6 and 7) than at pH 1.6 (where the proportion of residual ionisation will be lower) and why the setting temperature (Fig. 6) passes through a maximum at ~pH 2.5, which may reflect the optimum balance between (i) facilitation of intermolecular association by suppression of charge and (ii) loss of enthalpic stability from hydrogen-bonding to ionised carboxyl groups, as the pH is reduced.

5. Conclusions

In summary, we conclude that, as suggested in previous

studies (e.g. Ravanat & Rinaudo, 1980), the preferred conformation of pectin in the solution state at high temperature and high pH (i.e. well above pK_0) is highly extended, with local geometry close to that in the two-fold ordered structure. Reduction in temperature and/or pH can promote a (reversible) conformational transition to the more compact three-fold structure. The minimum interpretation of our experimental results would be that gelation occurs by dimerisation of (antiparallel) three-fold helices, with more extensive aggregation at very low pH where the chains become essentially uncharged. Protonation of carboxyl groups appears to promote conformational ordering and association by two different mechanisms: (i) suppression of electrostatic repulsion, and (ii) allowing the carboxyl groups to act as hydrogen-bond donors. An opposing consideration, however, is that hydrogen bonds formed with carboxyl groups as receptors may be weakened by protonation (because of loss of the additional electrostatic contribution to their stability), with optimum interaction therefore requiring some residual ionisation.

Acknowledgements

This work was carried out as part of an industrial-academic LINK project, "Behaviour of Biopolymer Mixtures in Structuring Food Products". We thank the participating companies (Unilever, Nestlé, Cerestar, Hercules and SKW Biosystems), and the UK Ministry of Agriculture, Fisheries and Food, for financial support. We also thank Professor A.H. Clark and Dr M.J. Gidley (Unilever Research, Bedford), Professor M. Rinaudo (CNRS, Grenoble) and Dr W. Mackie (University of Leeds) for helpful discussions.

References

Atkins, E. D. T., Nieuduszynski, I. A., Mackie, W., Parker, K. D., & Smolko, E. E. (1973). Structural components of alginic acid. II. The crystalline structure of poly-α-L-guluronic acid. Results of X-ray diffraction and polarized infrared studies. *Biopolymers, 12*, 1879–1887.

Cesàro, A., Ciana, A., Delben, F., Manzini, G., & Paoletti, S. (1982). Physicochemical properties of pectic acid. I. Thermodynamic evidence of a pH-induced conformational transition in aqueous solution. *Biopolymers, 21*, 431–449.

Christensen, S. H. (1986). Pectins. In M. Glicksman (Ed.), *Food hydrocolloids*, 3. (pp. 205–230). Boca Raton: CRC Press.

Durrand, D., Delsanti, M., Adam, M., & Luck, J. M. (1987). Frequency dependence of viscoelastic properties of branched polymers near gelation threshold. *Europhysics Letters, 3*, 297–301.

Gidley, M. J., Morris, E. R., Murray, E. J., Powell, D. A., & Rees, D. A. (1980). Evidence for two mechanisms of interchain association in calcium pectate gels. *International Journal of Biological Macromolecules, 2*, 332–334.

Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., & Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Letters, 32*, 195–198.

Jarvis, M. C., & Apperley, D. C. (1995). Chain conformation in concentrated pectic gels: evidence from ^{13}C NMR. *Carbohydrate Research, 275*, 131–145.

Mackie, W. (1971). Conformations of crystalline alginic acids and their salts. *Biochemistry Journal, 125*, 89p.

Mackie, W., Perez, S., Rizzo, R., Taravel, F., & Vignon, M. (1983). Aspects of the conformation of polyguluronate in the solid state and in solution. *International Journal of Biological Macromolecules, 5*, 329–341.

Milas, M., & Rinaudo, M. (1997). On the electrostatic interactions of ionic polysaccharides in solution. *Current Trends in Polymer Science, 2*, 47–67.

Morris, E. R. (1994). Chiroptical methods. In S. B. Ross-Murphy (Ed.), *Physical techniques for the study of food biopolymers*, (pp. 15–64). London: Blackie.

Morris, E. R., & Norton, I. T. (1983). Polysaccharide aggregation in solutions and gels. In E. Wyn-Jones & J. Gormally (Eds.), *Aggregation processes in solution*, (pp. 549–593). Amsterdam: Elsevier.

Morris, E. R., Gidley, M. J., Murray, E. J., Powell, D. A., & Rees, D. A. (1980). Characterization of pectin gelation under conditions of low water activity by circular dichroism, competitive inhibition and mechanical properties. *International Journal of Biological Macromolecules, 2*, 327–330.

Morris, E. R., Powell, D. A., Gidley, M. J., & Rees, D. A. (1982). Conformations and interactions of pectins. I. Polymorphism between gel and solid states of calcium polygalacturonate. *Journal of Molecular Biology, 155*, 507–516.

Morris, E. R., Rees, D. A., Thom, D., & Boyd, J. (1978). Chiroptical and stoichiometric evidence of a specific, primary dimerisation process in alginate gelation. *Carbohydrate Research, 66*, 145–154.

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

Ravanat, G., & Rinaudo, M. (1980). Investigation on oligo- and polygalacturonic acid by potentiometry and circular dichroism. *Biopolymers, 19*, 2209–2222.

Rees, D. A. (1970). Conformational analysis of polysaccharides. Part V. The characterization of linkage conformations (chain conformations) by optical rotation at a single wavelength. Evidence for distortion of cyclohexa-amylose in aqueous solution. Optical rotation and the amylose conformation. *Journal of the Chemical Society B*, 877–884.

Rees, D. A., Morris, E. R., Thom, D., & Madden, J. K. (1982). Shapes and interactions of carbohydrate chains. In G. O. Aspinall (Ed.), *The Polysaccharides*, 1, (pp. 195–290). New York: Academic Press.

Rinaudo, M. (1996). Physicochemical properties of pectin in solution and gel states. In J. Visser & A. G. J. Voragen (Eds.), *Pectins and pectinases*, (pp. 21–33). Amsterdam: Elsevier.

Rolin, C. (1993). Pectin. In R. L. Whistler & J. N. BeMiller (Eds.), *Industrial gums: polysaccharides and their derivatives*, 3. (pp. 257–293). San Diego: Academic Press.

Ross-Murphy, S. B. (1984). Rheological methods. In H. W. -S. Chan (Ed.), *Biophysical methods in food research*, (pp. 195–290). *Critical reports on applied chemistry*. London: SCI.

Sathyanarayana, B. K., & Rao, V. S. R. (1973). Conformation studies of pectic acid. *Current Science, 42*, 773–775.

Sawayama, S., Kawabata, A., Nakahara, H., & Kamata, T. (1988). A light scattering study on the effects of pH on pectin aggregation in aqueous solution. *Food Hydrocolloids, 2*, 31–37.

Stevens, E. S., & Sathyanarayana, B. K. (1987). A semiempirical theory of the optical activity of saccharides. *Carbohydrate Research, 166*, 181–193.

Stevens, E. S., Sathyanarayana, B. K., & Morris, E. R. (1989). Optical rotatory dispersion of saccharides: testing a theory. *Journal of Physical Chemistry, 93*, 3434–3436.

te Nijenhuis, K., & Winter, H. H. (1989). Mechanical properties at the gel

point of a crystallizing poly(vinyl chloride) solution. *Macromolecules*, *22*, 411–414.

Walkinshaw, M. D., & Arnott, S. (1981). Conformations and interactions of pectins. I. X-ray diffraction analyses of sodium pectate in neutral and acidified forms. *Journal of Molecular Biology*, *153*, 1055–1073.

Walkinshaw, M. D., & Arnott, S. (1981). Conformations and interactions of pectins. II. Models for junction zones in pectinic acid and calcium pectate gels. *Journal of Molecular Biology*, *153*, 1075–1085.