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# Gelation of high-methoxy pectin by enzymic de-esterification in the presence of calcium ions: a preliminary evaluation

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ABSTRACT

Cohesive gels have been obtained by de-esterification of 1.0 wt % high-methoxy citrus pectin (degree of esterification ≈ 68%) in the presence of $Ca^{2+}$ cations, using a commercial preparation (NovoShape) of fungal methyl esterase cloned from *Aspergillus aculeatus*. A convenient rate of network formation (gelation within ~30 min) was achieved at an enzyme concentration of 0.2 PEU/g pectin. At a $Ca^{2+}$-concentration of 40 mM and incubation temperature of 20 °C, severe syneresis (>7% of sample mass) was observed, but release of fluid decreased with decreasing concentration of $Ca^{2+}$ and increasing temperature of incubation, becoming undetectable for 10 mM $Ca^{2+}$ at 30 °C. Under these conditions, progressive development of solid-like character (storage modulus, $G'$) was observed during 160 min of enzymic de-esterification, and the mechanical spectrum recorded at the end of the incubation period had the form typical of a biopolymer gel. On subsequent heating to 70 °C, dissociation of the gel network (sigmoidal reduction in $G'$ and $G''$) was observed. At or above the midpoint temperature of this melting process (~50 °C), there was no indication of gel formation on enzymic de-esterification (at 50 or 60 °C). At lower temperatures (20, 30 and 40 °C), the rate of gelation (assessed visually) showed no systematic increase as the incubation temperature was increased towards the temperature-optimum of the enzyme (~50 °C). This unexpected behaviour is attributed to competition between faster de-esterification and slower formation of $Ca^{2+}$-induced 'egg-box' junctions.



## 1. Introduction

The structure of pectin is complex,[1–4] and has been the subject of extensive research, notably by Professor W. Pilnik and his colleagues in Wageningen University. In production of commercial pectin[5–7] by the normal procedure of treating plant tissue (usually citrus peel or apple pomace) with mineral acid, however, much of the structural complexity is lost by hydrolysis of several of the component sequences in native pectin, including the neutral-sugar sidechains involved in attachment to the cellulose and hemicellulose constituents of the plant cell wall. The resulting extracts have comparatively low molecular weight (~50–150 kDa), and consist predominantly of α-D-galacturonate residues, most of which occur in (1→4)-linked, linear 'homogalacturonan' sequences.

Some (up to ~80%) of the galacturonate residues in commercial extracts occur as the methyl ester. The percentage of galacturonate in the ester form is known as the 'degree of esterification' (DE). Functional properties can be manipulated by controlled reduction in DE, either by treatment with pectin methyl esterase enzymes or by hydrolysis with acid or alkali. Commercial pectins[5–7] are classified as 'high-methoxy' (DE > 50%) or 'low-methoxy' (DE < 50%), and form gels in two entirely different ways.

Gelation of high-methoxy pectin occurs on cooling in the presence of high concentrations (typically ~60–65 wt %) of sucrose and/or other sugars under acidic conditions (as in production of jams and marmalade). Low pH (often induced by incorporation of citric acid to augment the inherent acidity of fruit) promotes association of pectin chains into gel junctions by converting unesterified galacturonate residues from the charged ($COO^-$) to the uncharged (COOH) form, therefore suppressing intermolecular electrostatic repulsion. High concentrations of sucrose (or other cosolutes) reduce the content of water available to maintain the pectin in the solution state. Differences in the effectiveness of different cosolutes,[7–10] however, suggest that they may also influence self-association of pectin chains by 'condensing' around them[9] or binding to them.[10]

Low-methoxy pectin forms gels in the presence of calcium ions. Although sucrose and/or other sugars may be added to confer sweetness, their presence is not required for gelation to occur, and low-methoxy pectin can therefore be used in production of low-calorie jams and jellies.[5–7] The poly-α-D-galacturonate (homogalacturonan) sequences of pectin[11,12] are almost the exact mirror image of the poly-α-L-guluronate sequences in alginate[13] (differing

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only in configuration at C−3). Both have the same (1→4)-diaxial linkage geometry, which promotes adoption of a 2-fold, buckled zig-zag conformation with cavities that can accommodate an array of site-bound $Ca^{2+}$ ions between pairs[12,13] of polyuronate chains to form the 'egg-box' junctions[14] of the gel network. However, while calcium alginate gels are usually stable to temperatures above 100 °C, the thermal stability of the $Ca^{2+}$-induced gels of low-methoxy pectin[15] is decreased by the presence of esterified galacturonate residues which, being uncharged, cannot contribute to electrostatic attraction between the polymer chains and the included array of divalent cations.

Although, formally, the division between high-methoxy and low-methoxy pectins is defined as coming at DE 50, in practice[5–7] commercial low-methoxy pectin normally has a DE of around 30–35, and high-methoxy pectins have DE values in the range 60–75, because preparations of intermediate DE (around 50) do not give useable gels either with $Ca^{2+}$ ions or with high concentrations of cosolute at low pH.

It has been shown previously[16] that strong 'synergistic' gels can be formed by using α-galactosidase to remove galactose sidechains from guar galactomannan in soluble mixtures with ordered xanthan (thus increasing the content of unsubstituted mannan sequences available to form heterotypic junctions with the xanthan chains). In the present work, we have evaluated the possibility of using an analogous procedure to induce gelation of high-methoxy pectin, by enzymic de-esterification in the presence of calcium ions (i.e., by conversion, in situ, from the high-methoxy form, where gelation with $Ca^{2+}$ does not occur, to the low-methoxy form, which gives stable egg-box junctions).

## 2. Materials and methods

The high-methoxy pectin used (Grinsted Pectin MRS 351 from citrus peel; batch number 0708 0590; production date 23 August, 2007) was a gift from Danisco, who also kindly provided the following analytical information: degree of esterification = 68.1%; US-SAG (IFT method) = 150; loss on drying = 8.5%; pH in a 1% solution = 2.99. The pectin methyl esterase was a commercial preparation, NovoShape, from Novozymes A/S, Bagsvaerd, Denmark. The gene encoding the esterase enzyme is derived from the fungus *Aspergillus aculeatus,* and is transferred into a strain of the food-grade organism *Aspergillus oryzae* for commercial production. The NovoShape preparation has a declared activity of 10 PEU/mL and a temperature optimum of ~50 °C. Calcium chloride was purchased as a 0.5 M solution from Reagecon, Shannon, Co. Clare, Ireland. Distilled deionised water was used throughout.

Pectin stock solution was prepared at a concentration of 1.25 wt % by overhead stirring at ambient temperature. Calcium chloride solutions were prepared at concentrations 10 times those required in the mixtures with pectin. NovoShape solutions were used either at the original concentration, as received, or after dilution by factors of 10, 50, 100 or 125. Mixtures were prepared in weighed beakers by addition, in sequence, of pectin stock solution (40 g), calcium chloride solution (5 g), water (4.5 g) and enzyme solution (0.5 g), to give a total mass of 50 g and a pectin concentration of 1.0 wt %. Timing was started on addition of the enzyme solution, and the constituents were mixed by brief manual stirring.

Gel time was estimated visually by periodically tipping each beaker a few degrees from vertical and noting the time at which the surface of the sample first remained perpendicular to the walls of the beaker, rather than flowing towards horizontal. Observations were made at temperatures of 20, 30, 40, 50 and 60 °C, by holding the beakers in thermostatted water baths. After gelation, the samples were stored overnight (~16 h) at 5 °C, and the extent of syneresis was determined by withdrawing any expressed fluid with a Pasteur pipette, measuring the reduction in mass, and expressing it as a percentage of the original mass of the sample.

Low-amplitude oscillatory measurements of storage modulus ($G'$), loss modulus ($G''$) and complex dynamic viscosity ($\eta^* = (G'^2 + G''^2)^{1/2}/\omega$, where $\omega$ is frequency of oscillation in rad/s) were made using cone-and-plate geometry (6 cm diameter; 2° cone angle) on a CarriMed CSL-100 rheometer, with temperature control by the Peltier system of the instrument. Mechanical spectra of pectin in the solution state, with no added enzyme, were recorded at 30 °C using highly truncated cone-and-plate geometry (5 cm diameter; 0.05 rad cone angle) on a sensitive prototype rheometer designed and constructed by Dr. R.K. Richardson, Cranfield University, UK. Measurements on both instruments were made at 1.0% strain, and the periphery of the sample was coated with light mineral oil to minimise loss of water by evaporation.

## 3. Results

### 3.1. Visual observations

It was immediately apparent that samples prepared using undiluted NovoShape formed gels very rapidly (typically within about 1½ min of adding the enzyme). The first target was then to identify an enzyme concentration that would induce gelation on a more experimentally tractable timescale. In these experiments only two incubation temperatures were used (20 and 30 °C), and the concentration of calcium chloride in the mixtures was held fixed at 10 mM (calculated as sufficient to give full dimerisation of poly-α-D-galacturonate sequences, except in the limit of almost complete de-esterification). The results obtained are shown in Figure 1.

As would, of course, be expected, there was a progressive increase in gel time (estimated visually) as the concentration of enzyme was decreased. At the highest levels of dilution (100-fold and 125-fold) gelation appeared to occur slightly sooner at 20 °C than at 30 °C, but no temperature-dependence was detected at the higher concentrations of enzyme (10-fold and 50-fold dilution). It must be emphasised that there was a considerable element of subjectivity in judging when a cohesive network had formed, but 50-fold dilution of the enzyme appeared to give a convenient rate

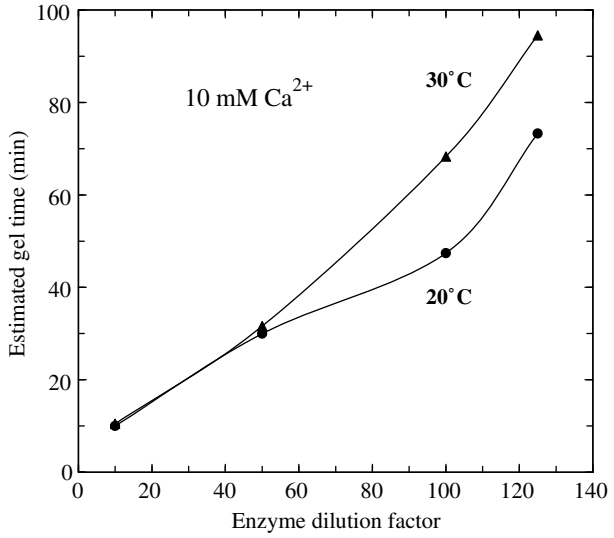


**Figure 1.** Effect of concentration of enzyme (fungal pectin methyl esterase) on the rate of gelation of 1.0 wt % high-methoxy pectin (DE ≈ 68%) in the presence of 10 mM $CaCl_2$, as judged by visual observation of gel times at 20 °C (●) and 30 °C (▲).

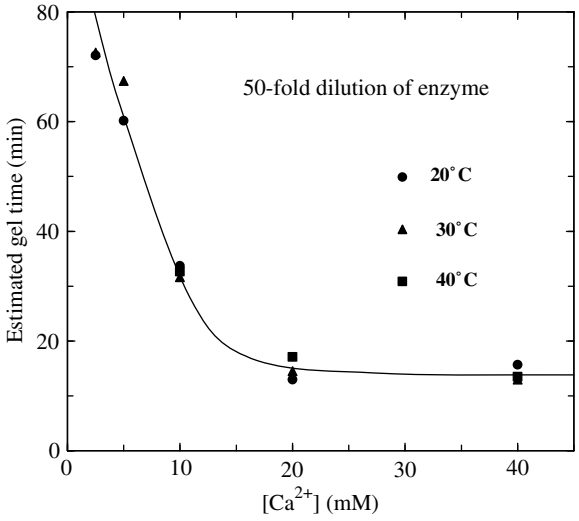


**Figure 2.** Effect of calcium chloride concentration on the rate of gelation of 1.0 wt % high-methoxy pectin (DE ≈ 68%) by de-esterification with 50-fold dilution of NovoShape (0.2 PEU/g pectin), as judged by visual observation of gel times at 20 °C (●), 30 °C (▲) and 40 °C (■).

of gelation, and was used in evaluation of the effect of varying $Ca^{2+}$ concentration.

The concentrations of calcium chloride studied were 2.5, 5, 10, 20 and 40 mM, and mixtures were held at 20, 30, 40, 50 and 60 °C. At the two highest temperatures (50 and 60 °C) there was no indication of network formation, even after incubation for ~8 h or longer. The gel times observed (by visual inspection) at 20, 30 and 40 °C are shown in Figure 2. There is no evidence of any large or systematic effect of temperature or gelation rate. The gel times decreased sharply as the concentration of $Ca^{2+}$ was increased from 2.5 to 20 mM, but no further increase in gelation rate was detected on raising the $Ca^{2+}$ concentration to 40 mM.

### 3.2. Syneresis

Although, as shown in Figure 2, temperature (within the range 20–40 °C) had little effect on gel time, it had a substantial effect (Fig. 3) on the amount of fluid expressed by the same samples (50-fold dilution of enzyme; $Ca^{2+}$ concentration varied up to 40 mM). The extent of syneresis decreased systematically as the temperature of enzymic de-esterification was increased from 20 °C to 30 °C and then to 40 °C. At each temperature of incubation, the extent of syneresis decreased (Fig. 3) with decreasing concentration of calcium chloride. For the samples incubated with enzyme at 30 °C, there was no detectable syneresis on reduction of $Ca^{2+}$ concentration to 10 mM. Since release of fluid would, of course, be expected to cause slippage, this combination of experimental variables (50-fold dilution of enzyme; incubation at 30 °C; 10 mM calcium chloride) was used for the low-amplitude oscillatory measurements described below.

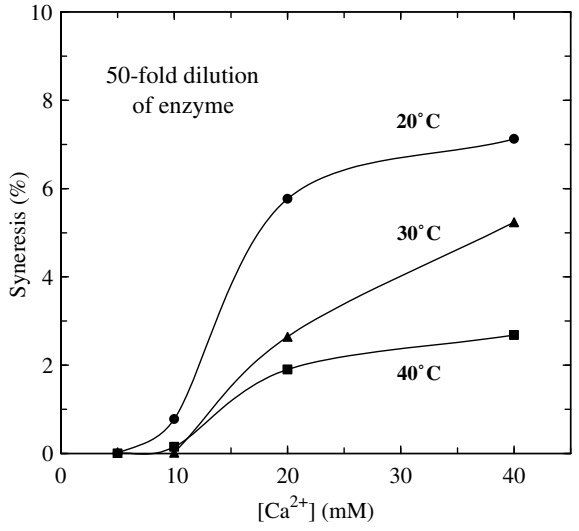


**Figure 3.** Effect of calcium chloride concentration on syneresis of gels formed by 1.0 wt % high-methoxy pectin (DE ≈ 68%) on de-esterification with fungal pectin methyl esterase (0.2 PEU/g pectin) at 20 °C (●), 30 °C (▲) and 40 °C (■). Syneresis was measured after samples had been held for ~16 h at 5 °C.

### 3.3. Oscillatory rheology

Figure 4 shows the values of $G'$ and $G''$ recorded at 1 rad/s and 1% strain during enzymic de-esterification of high-methoxy pectin (1.0 wt %) in the presence of 10 mM calcium chloride for 160 min at 30 °C. Throughout the incubation period there is a small, essentially linear, increase in $G''$. The increase in $G'$ (characterising development of solid-like structure) is initially slow, but becomes more rapid at longer times. The onset of the rapid increase in $G'$ is in reasonable agreement with visual observation (Fig. 2) of gel time under the same experimental conditions (50-fold dilution of enzyme; 10 mM $Ca^{2+}$; de-esterification at 30 °C).

The mechanical spectrum recorded (at 30 °C) after the 160 min incubation period (Fig. 4) is shown in Figure 5. The spectrum has the form typical[17] of a physically crosslinked biopolymer gel: $G'$ is about an order of magnitude higher than $G''$, with little frequency-dependence of either modulus, and the dependence of $\log \eta^*$ on $\log \omega$ is essentially linear, with a slope close to −1.

The changes in $G'$ and $G''$ (1 rad/s) observed on subsequent heating (1 °C/min) from 30 °C to 70 °C are shown in Figure 6. Loss of network structure is evident as a sigmoidal decrease in both moduli, centred at ~50 °C. Similar melting of calcium pectinate gels formed by cooling low-methoxy pectin in the presence of $Ca^{2+}$ cations has been reported previously.[15] The melting process shown in Figure 6 spans a temperature range of ~20 °C, which is consistent with the breadth of order–disorder and accompanying gel–sol

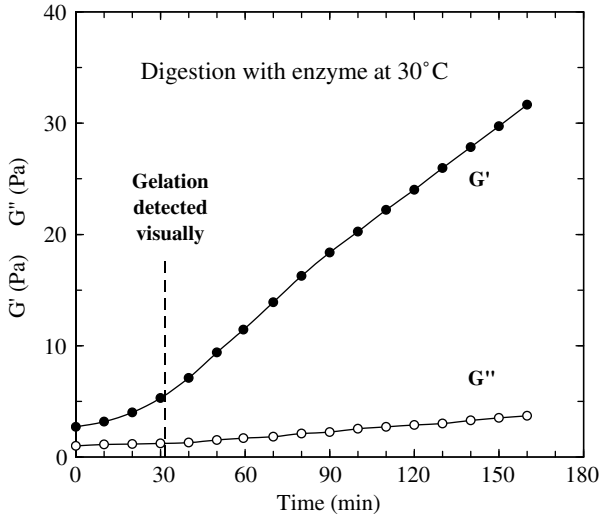


**Figure 4.** Changes in $G'$ (●) and $G''$ (○) measured at 1 rad/s and 1% strain during de-esterification of 1.0 wt % high-methoxy pectin (DE ≈ 68%) with fungal pectin methyl esterase (0.2 PEU/g pectin) in the presence of 10 mM $CaCl_2$ at 30 °C. The vertical dashed line shows the time at which formation of a cohesive gel network was observed visually (Figs. 1 and 2).

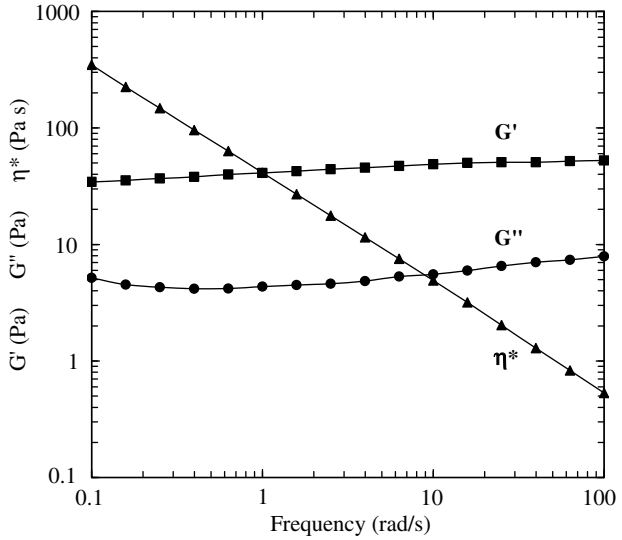


**Figure 5.** Mechanical spectrum (1% strain) recorded for 1.0 wt % high-methoxy pectin (DE ≈ 68%) after digestion (Fig. 4) for 160 min with fungal pectin methyl esterase (0.2 PEU/g pectin) in the presence of 10 mM $CaCl_2$ at 30 °C: $G'$ (■); $G''$ (●); $\eta^*$ (▲).

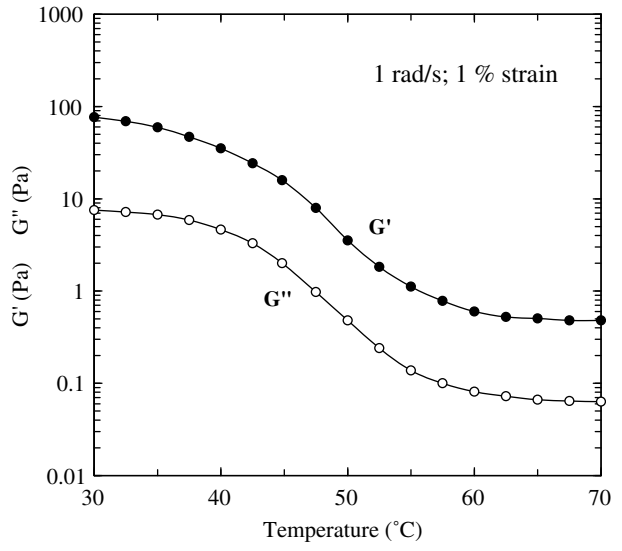


**Figure 6.** Changes in $G'$ (●) and $G''$ (○), measured at 1 rad/s and 1% strain during heating at 1 °C/min, for 1.0 wt % high-methoxy pectin (DE ≈ 68%) after digestion (Fig. 4) for 160 min with fungal pectin methyl esterase (0.2 PEU/g pectin) in the presence of 10 mM $CaCl_2$ at 30 °C. Heating commenced immediately after the mechanical spectrum shown in Figure 5 had been recorded.

transitions observed for other gelling polysaccharides, including carrageenans,[18,19] agarose,[20] gellan (acylated and deacylated)[21,22] and 'synergistic' mixtures of xanthan with konjac glucomannan.[23]

At the start of the enzymic incubation (1 wt % pectin at 30 °C) shown in Figure 4, solid-like character ($G'$) was already greater than liquid-like response ($G''$). To explore the origin of this effect, mechanical spectra (Fig. 7) were recorded (at 30 °C) for 1 wt % pectin, with no added enzyme, in water and in 10 mM $CaCl_2$ (as in the incubation experiment; Fig. 4). The commercial (CarriMed) instrument used to obtain the results shown in Figures 4–6 gave an unacceptable scatter of experimental points for these preparations, and measurements were therefore made using the prototype rheometer described in Section 2.

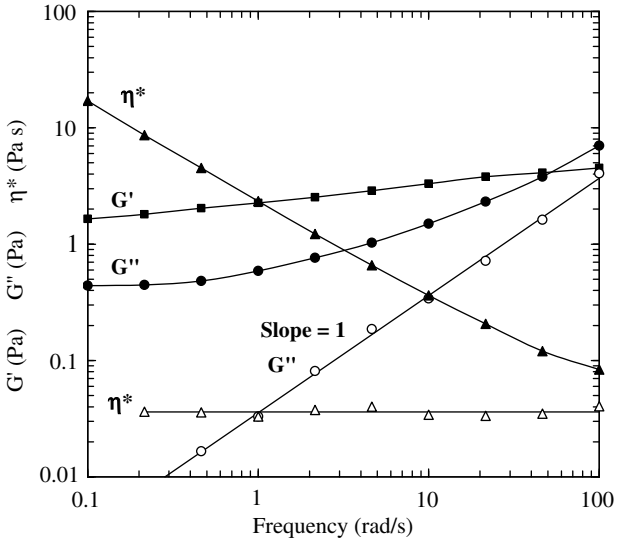


**Figure 7.** Mechanical spectra (1% strain) showing the frequency-dependence of $G'$ (squares), $G''$ (circles) and $\eta^*$ (triangles) at 30 °C for 1.0 wt % high-methoxy pectin (DE ≈ 68%) in water (open symbols) and in 10 mM $CaCl_2$ (filled symbols). Values of $G'$ for the solution prepared in water were too low to be measured.

Even on this highly sensitive instrument, $G'$ for 1.0 wt % pectin in water (at 30 °C) remained too low to be measured at any of the frequencies studied (0.1–100 rad/s). However, a linear relationship was observed (Fig. 7) between $\log\omega$ and $\log G''$, with a slope of almost exactly 1.0, and, in consequence, $\eta^*$ was independent of frequency (Newtonian). This behaviour is typical[17] of a dilute solution of non-entangled disordered coils, and is consistent with the low molecular weight (Section 1) of commercial pectins.

However, the sample incorporating 10 mM $CaCl_2$, although fluid, gave a gel-like mechanical spectrum (Fig. 7), with $G'$ greater than $G''$ throughout most of the accessible frequency-range. Polysaccharide preparations that can be poured but show predominantly solid-like response to low-amplitude oscillation are well known,[17] and are termed 'weak gels'. Their characteristic properties arise from a tenuous network structure formed by intermolecular junction zones that are similar to those in conventional gels, but less stable.

The mechanical spectrum shown in Figure 7 for 1 wt % high-methoxy pectin in 10 mM $CaCl_2$ is broadly similar to spectra recorded for other polysaccharide 'weak gels', such as those formed by ordered xanthan,[24] welan[21] and rhamsan,[21] with a smaller separation between $G'$ and $G''$ and greater frequency-dependence of both moduli than in 'true' (self-supporting) gels.[17] The up-turn in $G''$ at high frequency (Fig. 7) towards the slope observed for the same concentration of pectin in water, however, indicates a substantial 'sol fraction' of chains that do not form part of the network.

The values of $G'$ and $G''$ at 1 rad/s (Fig. 7) are in reasonable agreement with those measured at the same frequency at the start of the period of enzymic incubation (Fig. 4), and indicate that the content of unesterified galacturonate residues in the starting solution of high-methoxy pectin was sufficient to give a tenuous 'weak gel' with 10 mM $Ca^{2+}$, before conversion to a 'true' gel (Fig. 5) by creation of longer unesterified sequences capable of forming egg-box junctions of greater stability.

## 4. Discussion and conclusions

One immediate conclusion from this investigation is that cohesive gels can be formed by enzymic de-esterification of high-methoxy pectin in the presence of calcium ions. A convenient rate of

gelation for experimental studies and, perhaps, practical application was obtained by diluting the NovoShape preparation by a factor of 50 before incorporating it in mixtures with pectin and calcium chloride at a level of addition (Section 2) of 1 mL/g pectin. Since the declared activity of NovoShape (Section 2) is 10 PEU/mL, the enzyme/pectin ratio at 50-fold dilution (as in the rheological studies reported in Section 3.3) is 0.2 PEU/g pectin.

In a recent investigation of the mode of action of pectin methyl esterases,[25] the recombinant fungal esterase purified from commercial NovoShape was found to have a pH optimum of ~3.0 (with highly esterified citrus pectin as substrate). As reported in Section 2, the citrus pectin used in the present investigation has a natural pH of 2.99 in 1% solution, which was the concentration in all mixtures studied. Thus, although the precise value varies with substrate and ionic environment, it seems reasonable to conclude that our experiments were carried out close to the optimum pH of the enzyme.

De-esterification of pectin by the fungal esterase in NovoShape (cloned from *Aspergillus aculeatus*) is predominantly random ('multiple chain' mechanism), but with a limited contribution from a 'multiple attack' mechanism which produces short blocks of ~8–10 de-esterified galacturonate residues.[25] For non-esterified poly-α-D-galacturonate, the minimum sequence length for formation of stable egg-box junctions is ~14 residues,[26] corresponding to a minimum array of ~7 site-bound $Ca^{2+}$ cations between chains of 2-fold symmetry. From analysis of changes in circular dichroism accompanying formation of calcium pectinate gels, however, it has been concluded[27] that esterified galacturonate residues can be accommodated within the junction zones of the gel network, provided that each 'nest' in the egg-box structure has at least one non-esterified (charged) galacturonate residue. Formation of long blocks of fully de-esterified polygalacturonate does not, therefore, seem to be a necessary requirement for $Ca^{2+}$-induced gelation.

However, as discussed in Section 1, the presence of uncharged (esterified) galacturonate residues reduces the thermal stability of the egg-box junctions, with consequent dissociation of network structure on heating. Instability of $Ca^{2+}$-induced association at, or above, the midpoint-temperature of the melting process shown in Figure 6 would explain why no gelation was observed on de-esterification at 50 or 60 °C.

At the lower incubation temperatures studied (20, 30 and 40 °C), it might have been expected that gelation would become more rapid as the temperature was increased towards the optimum of ~50 °C for activity of the methyl esterase. However, as shown in Figure 2, no such effect was observed. We suggest the following, tentative, interpretation of this unexpected behaviour. Structural ordering of biopolymers by a co-operative array of non-covalent bonds does not follow normal Arrhenius kinetics. Instead, competition between propagation and thermal dissociation normally gives rise to an overall increase in the rate of ordering as temperature is decreased.[28,29] The insensitivity of gel time to increasing temperature (Fig. 2) therefore suggests fortuitous cancellation of two opposing factors: faster de-esterification and slower formation of intermolecular functions.

The primary mechanism of $Ca^{2+}$-induced association of homogalacturonan sequences in pectin (and polyguluronate sequences in alginate) is formation of dimeric junctions with a single array of site-bound $Ca^{2+}$ cations.[12,13] Since only the inner faces of the 2-fold polyuronate chains in the dimeric junctions participate in cation-binding, full dimerisation involves site-binding of only 50% of the total stoichiometric requirement of the two strands (i.e., one calcium cation for every four uronate residues). Taking account of moisture content and residual neutral sugars, the molecular weight per galacturonate residue in commercial pectins is around 200 Da, giving a galacturonate concentration of ~50 mM for the 1% solutions used in the present work. The concentration of $Ca^{2+}$ required for complete dimerisation is therefore ~12.5 mM, which is in reasonable agreement with the end of the steep reduction in gel time observed (Fig. 2) as $Ca^{2+}$ concentration was increased.

At higher concentrations of $Ca^{2+}$ (above 50% stoichiometric) further association can occur by incorporation of arrays of $Ca^{2+}$ cations between dimers. This secondary mechanism of association does not appear to affect the rate at which a cohesive gel is formed initially (Fig. 2), but it does seem to promote subsequent contraction of the gel network, as shown (Fig. 3) by development of substantial syneresis at $Ca^{2+}$ concentrations above those required for dimerisation. As discussed above, ordered association of biopolymers is normally promoted by reduction in temperature, which would explain the systematic increase in syneresis observed (Fig. 3) as incubation temperature was decreased from 40 to 20 °C. However, it seems possible to avoid syneresis by using a $Ca^{2+}$ concentration slightly below 50% stoichiometric (10 mM rather than 12.5 mM for the pectin concentration of 1% used in the present work) and an incubation temperature no lower than ~30 °C.

As indicated in the title of the paper, this investigation was carried out as a feasibility study, and there is clear scope for further research. In particular, now that we have identified the experimental conditions required to eliminate syneresis, and consequent slippage, it should be possible to supersede the visual observations reported in Figures 1 and 2 by objective rheological measurements. Another obvious continuation would be to use a fully, or almost fully, esterified pectin as starting material, to eliminate the effect (Fig. 7) of unesterified polygalacturonate sequences present in the commercial pectin used in the present work (DE ≈ 68%). A third line of continuation would be to make comparisons between fungal methyl esterase which, as discussed above, removes ester groups in a predominantly random way, and one of the plant methyl esterase enzymes, which give long runs of unesterified polygalacturonate.[25] The results of the present investigation, however, are sufficiently encouraging to justify such further studies.

In a previous investigation,[30] it was shown that treatment of sugarbeet pectin with an enzyme preparation extracted from orange peel allowed $Ca^{2+}$-induced gelation to occur, by removal of acetyl substituents (which block egg-box formation) under the action of pectin acetyl esterase present in the extract. The extract also contained a pectin methyl esterase enzyme, and comparative studies using pectin from orange peel demonstrated that it too formed $Ca^{2+}$-induced gels, showing that the gelation process reported here is not unique to pectin methyl esterase of fungal origin.

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