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# Calcium binding and swelling behaviour of a high methoxyl pectin gel

C. William Tibbits, Alistair J. MacDougall, 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**

Progress in understanding the structural role of pectic polysaccharides in plant cell walls is currently restricted by a lack of information on the molecular properties of undegraded cell wall pectins. We have examined, in solution, the calcium ion binding behaviour of a high methoxyl cell wall pectin from unripe tomato pericarp, and found it to be comparable to other pectins. After gelation through calcium addition, the affinity of the pectin for calcium ions was increased by at least an order of magnitude, with an estimated stability constant, $K$, of ~8000. At pH 6 calcium binding is directly related to crosslink formation and gel stiffness. The swelling of the gel in aqueous salt solution was also examined; the kinetics of swelling were comparable to other polyelectrolyte gel systems. A fraction of the galacturonate residues in the gel does not participate in crosslinking but can contribute to gel swelling through a general polyelectrolyte effect at low ionic strengths. With decreasing concentration of free calcium ions, further marked swelling, and eventually dissolution, occurs as a result of dissociation of calcium crosslinks. 

*Keywords:* Pectin; Gel, swelling; Cell wall; Tomato

## 1. Introduction

In plant cell wall research the molecular interactions that determine the mechanical properties of the wall, and the biochemical mechanisms by which the physical properties of the wall are controlled, are subjects of particular current interest. As major components of the primary cell wall and middle lamella of dicotyledonous plants, pectic polysaccharides are an important focus of attention in this work [1].

Pectic polysaccharides are structurally complex consisting of a backbone of (1→4)-α-D-galacturonosyl residues interrupted with typically a 10% substitution of (1→2)-α-L-rhamnopyranosyl residues. A portion of the rhamnosyl residues are branch points for neutral sugar side chains which contain L-arabinose and D-galactose. The rhamnosyl substitution is thought to cluster in "hairy" regions [2,3] leaving "smooth" sequences of the galacturonan backbone. The backbone may be partially acetylated and may be further substituted

* Corresponding author. Fax: +44-1603-507723; e-mail: steve.ring@bbsrc.ac.uk

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with terminal xylose. A portion of the galacturonosyl residues are methyl esterified.

Pectic polysaccharides appear to be crosslinked in the cell wall by several different interactions/linkages, since a range of chemical extractants (including cyclohexanediaminetetraacetic acid [CDTA], $Na_2CO_3$, and alkali) is required to solubilise them from purified plant cell walls. The observation that the addition of a chelating agent, CDTA, to parenchymatous tissues causes cell separation, and that the extracted pectic polysaccharide can form a gel, on removal of the CDTA and addition of calcium ions, underlines the importance of ionic interactions [4].

A classical picture of pectin gelation in concentrated solutions is that high methoxyl pectins gel at low pH (~3) in the presence of large concentrations of a solute such as sucrose, while low methoxyl pectins can form gels on addition of calcium ions at higher pHs (5–7). The general features of the ion binding behaviour of the pectic polysaccharides are known [5–7]. Their affinity for divalent ions such as $Ca^{2+}$ increases with decreasing average degree of methyl esterification of the pectic polysaccharide and increasing length of unsubstituted galacturonan backbone. In concentrated solutions of low methoxyl pectic polysaccharides this ionic interaction leads to the formation of chain association [8–12]. If the extent of association is limited as a consequence of molecular structure or kinetic factors, gels are formed. The affinity of high methoxyl pectins for $Ca^{2+}$ is generally not high enough to lead to sufficient chain association for gelation. The observation [4] that a high methoxyl cell wall pectin can gel on addition of $Ca^{2+}$ is therefore somewhat unusual. A model [12] of the crosslink in pectic gels is the "eggbox", in which charged polysaccharide segments enclose $Ca^{2+}$ ions, although other types of chain association can also occur.

Crosslinking is not the only way that pectic polysaccharides can affect the mechanical properties of the plant cell wall. One important additional factor is the extent of hydration of the pectic polysaccharide matrix. This will affect both the mechanical properties and the porosity of the cell wall. For gels formed from pectic polysaccharides, hydration and swelling are influenced by ionic interactions. The swelling of neutral polymer networks in a solvent is dependent on the balance between the affinity of the polymer for the solvent, (usually expressed through an interaction parameter, $\chi$, characterizing an energy of interaction between the solvent and a polymer segment) and an elastic, restorative force which is dependent on the extent of crosslinking. For polyelectrolyte gels, immersed in a solvent, an important additional contribution to gel swelling arises as a result of the Donnan effect producing an excess of ions within the gel compared to the external solution. The physical origins of these effects is well described and a topic of continuing theoretical and experimental study [13–15]. In addition to equilibrium swelling behaviour, also of relevance to the behaviour of pectins in the plant cell wall is the rapidity of response to a change in the ionic environment of the apoplast. Again the essential physics of the kinetics of polyelectrolyte gel swelling has been established [16,17].

In this article we present the results of a study on the ion binding and swelling characteristics of gels formed by the chelator extracted pectic polysaccharide from the pericarp of unripe tomato.

## 2. Experimental

*Source of materials*.—The CDTA extracted pectin was prepared from the cell wall material of unripe tomato pericarp tissue, purified and characterised as described [4]. Polygalacturonic acid (Fluka) had a neutral sugar content of $< 3\%$ (as assessed after acid hydrolysis and GLC of the alditol acetates). The analytical methodology for pectin characterisation is described in [4] and references therein.

*Preparation of gels and measurement of gel stiffness*.—Gels were prepared by the addition of $CaCl_2$ to concentrated solutions (1–3%) of the $K^+$ salt of the pectin, (pH 6.8) at 25 °C. The shear modulus of the gel was determined from the velocity of a small amplitude shear wave through the gel using a Rank Brothers' Pulse Shearometer as described [18].

*Swelling measurements*.—Gels were prepared in cylindrical moulds (0.8 cm diameter) with 25 mM $CaCl_2$ and then immersed in dilute salt solutions (3 mM and 25 mM NaCl), at 25 °C, to obtain a range of dilutions of the $CaCl_2$ present in the gel. As the affinity of the gel network for $Ca^{2+}$ ions is relatively high, this involves substantial dilution (up to $10^6$), and the dominant counterions are $Ca^{2+}$ and $Na^+$. After 24 h the swollen gels were weighed, their stiffness measured, and the calcium

and uronic acid content of the swollen gel and external solution determined. For the pH dependence of gel swelling, gels were prepared as above and then diluted in 100 times their own volume of dilute HCl solutions of the required pH. Swelling kinetics were determined by video photomicrography through the observation of the change in diameter of ~1 mm gel cylinders as a function of time at room temperature.

*Calcium determinations*.—The calcium binding of pectic polysaccharides in solution was determined using a calcium electrode (93-20 Orion) with a single junction reference electrode (90-01 Orion). For the swelling and gel dissolution measurements, calcium concentrations were determined using atomic absorption spectrophotometry at 422.7 nm with an air/$C_2H_2$ or air/$N_2O$ flame, in solutions containing respectively 3.5% HCl and 0.1% $LaCl_3$, or 3.5% HCl and 0.1% KCl. Gels were ashed at 480 °C prior to analysis.

*Stability constant calculation*.—For the interaction of pectin with $Ca^{2+}$ on gel dissolution, EQUIL, speciation software for complex ionic equilibria, (Micromath, UT, USA) was used to fit the experimental data to obtain an estimate of the stability constant. Allowance was made for the solubilisation of pectin from the gel network during the dissolution, and determined through the measurement of uronic acid contents. The amount solubilised ranged from 10% in the initial stages, to 45% uronic acid at the final stages of gel dissolution.

## 3. Results and discussion

*Calcium binding behaviour*.—Addition of the chelating agent, CDTA, to unripe tomato pericarp tissue results in cell separation and the extraction of a cell wall pectin. Details of the structure and composition of this fraction were reported previously [4]. The anhydrouronic acid content was 71% and, of this, 68% was methyl esterified. D-Galactose and L-arabinose were the main neutral sugars present. The intrinsic viscosity, measured in an aqueous salt solution (0.1 M $NH_4Cl$), of 810 mL/g, indicated that this pectin fraction was minimally degraded. Particular care was taken to purify the polysaccharide free from CDTA and it was estimated that this contamination was <1% [4,19].

The ion binding behaviour of the pectic polysaccharides is relevant to their gelation behaviour and behaviour in the plant cell wall [4,5,20,21]. For polyelectrolytes, ionic interactions are influenced by ionic strength. In salt-free solution, the macroion will have a relatively high affinity for counterions. As ionic strength increases and the charge becomes screened the affinity will be reduced. This reduction or anticooperative behaviour is characteristic of polyelectrolytes. Additional to this general behaviour is the possibility of more localised interactions. For this reason it is customary to investigate the specific binding of counterions in the presence of a supporting electrolyte which reduces the general polyelectrolyte effect. The calcium binding behaviour of the pectin in solution (0.2%), in the presence of supporting electrolyte (0.02 M KCl), was determined using a calcium electrode. Fig. 1 compares the calcium binding behaviour of the CDTA pectin and of a commercial pectate sample which was >95% polygalacturonic acid. The binding isotherm is a plot of $[Ca^{2+}]_b/[COO^-]$ versus $[Ca^{2+}]_t/[COO^-]$, where concentrations are expressed on a molar basis, subscripts b and t refer to bound and total, respectively, and $[COO^-]$ is the molar galacturonate concentration. The behaviour is qualitatively comparable to previous data on the calcium binding behaviour of pectic polysaccharides with the pectate having a higher affinity for $Ca^{2+}$ [6]. There is some evidence from the shape of the pectin binding isotherm of different binding behaviour at higher calcium levels, as observed previously. It is useful to attempt a more quantitative analysis through the determination of a stability constant, $K$, which describes the ionic equilibrium. This is usually carried out using Hill and Scatchard plots [6]. A Scatchard analysis of the present pectin data gives a value of log $K$ of ~2.7, for the interaction

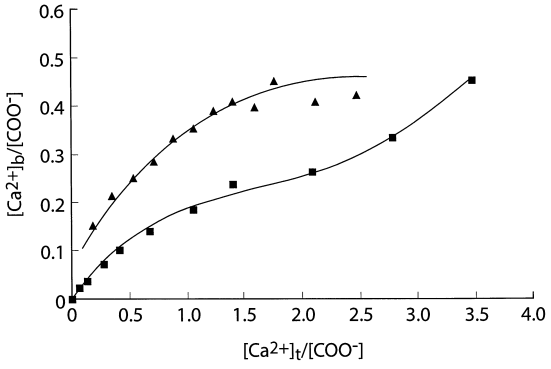


Fig. 1. Calcium binding of CDTA extracted pectin (■) and pectate (▲) expressed as a binding isotherm, $[Ca^{2+}]_b/[COO^-]$ versus $[Ca^{2+}]_t/[COO^-]$.

between $Ca^{2+}$ and a digalacturonic acid segment. This value is broadly comparable with earlier estimates [5] of the affinity of calcium ion for pectins with this degree of esterification.

A characteristic of this CDTA pectin fraction is that it forms elastic gels on addition of calcium to entangled polymer solutions ( > 0.5 to 0.6% for this molecular size). In the concentration range 0.6 to 3.2% the shear modulus, $G'$, increases with concentration, $c$, as $c^{1.9}$ (with the amount of $CaCl_2$ in the gel adjusted to give a calcium ion concentration equivalent, on the basis of charge, to 250% of the unesterified carboxyl groups). The dependence of normalised shear modulus (normalised with respect to the maximum observed modulus at high calcium concentration) on calcium concentration, expressed as $[Ca^{2+}]_t/[COO^-]$, is shown in Fig. 2 for pectin concentrations of 1.1 and 2.3% (maximum shear modulus, 1320 and 2700 $Nm^{-2}$, respectively). Observed behaviour does not show a strong dependence on pectin concentration in this range. If the gel is diluted, by addition of water or aqueous salt solution, the calcium concentration in the gel is reduced, and a new equilibrium is formed between bound and free calcium ions, resulting in dissociation of crosslinks at low $[Ca]_b$ (Fig. 2). The curves for gel formation and dissolution do not superimpose. Once formed, dissociation of the crosslink is observed only at much lower calcium concentrations. The calcium binding behaviour of this dissolution is shown in Fig. 3 as a binding isotherm of $[Ca^{2+}]_b/[COO^-]$ versus $[Ca^{2+}]_t/[COO^-]$. The affinity of the pectin in the gel for calcium is stronger than that of the polysaccharide in solution. The observed calcium binding behaviour on dissolution gives a log $K$ of ~3.9. The data shown in Figs. 2 and 3 are consistent with the proposal that initial binding of calcium to the pectin is followed by network formation through chain/chain association. Analysis of the calcium binding data on gel dissolution therefore gives information on the strength of calcium binding to the pectin gel network. While pectins with a high degree of methyl esterification show comparable affinities for calcium in aqueous solution, not all such fractions have the ability to form gels. One explanation for this behaviour is that structural variation (e.g., backbone substitution), inhibits network formation but not the initial binding of calcium to the pectin chain in solution.

*Swelling behaviour*.—Swelling is observed on dissolution of the pectin gel. The time dependence of swelling is shown in Fig. 4(a). In this case swelling is expressed as a relative increase in linear dimension, which approaches a plateau value after ~2 h. The rate of swelling is influenced by the dimensions of the gel and the rate at which water can diffuse into the polymer network. To permit comparison with other systems, a cooperative diffusion coefficient, $D$ of the gel at swelling equilibrium can be obtained from [16,17]

$$D = (3/8)\left(d_f^2/\tau_i X^2\right) \quad (1)$$

where $X$ is a function of the ratio of the shear to osmotic longitudinal modulus and, $\tau_i$, is a relaxation time of the gel obtained from

$$-\ln\left(d_f - d_{(t)}\right)/\left(d_f - d_i\right) = (t/\tau i) - B \quad (2)$$

where $d_f$ and $d_i$ are the initial and final diameters of a gel cylinder and $d_{(t)}$ the diameter at time, $t$.

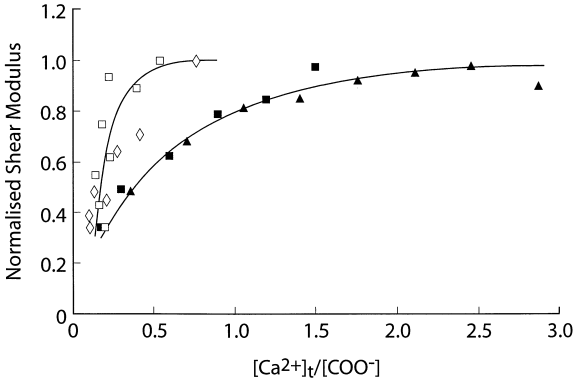


Fig. 2. Plot of normalised shear modulus versus $[Ca^{2+}]_t/[COO^-]$ for the formation (solid symbols) and dissolution (open symbols) of gels prepared from pectin solutions of different concentrations; ■, □, 1.1%; ◇, 2%; ◆, 2.3%.

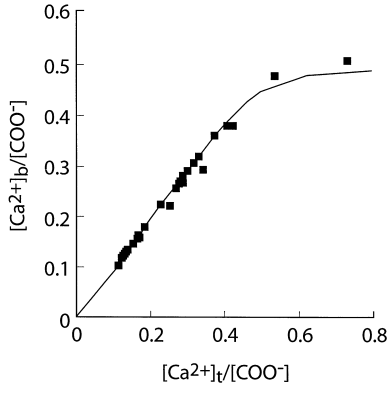


Fig. 3. Plot of $[Ca^{2+}]_b/[COO^-]$ versus $[Ca^{2+}]_t/[COO^-]$ observed on the dissolution of 2% pectin gels. The plotted line shows the predicted calcium binding behaviour for a log $K$ of 3.87.

A plot of $-\ln\left(d_f - d_{(t)}\right)/\left(d_f - d_i\right)$ versus $t$ is shown in Fig. 4(b) and has the form of the predicted behaviour. The value of $D$ obtained of $4.6\times10^{-11}\,m^2/s$ is comparable to that of covalently crosslinked synthetic polyelectrolyte gels and illustrates the generally fast swelling kinetics of these systems. This is relevant both to their technological usefulness, and to the behaviour in the plant cell wall where the potential rapidity of response to changes in apoplast environment may be important.

For a covalently crosslinked polyelectrolyte gel, the counterions can give rise to appreciable osmotic pressures, particularly at low ionic strengths [13–16]. If this pressure is greater than the stiffness of the gel, swelling will be observed. For pectin gels, ionic effects can affect swelling both through an effect on the magnitude of the swelling pressure and also the extent of crosslinking of the gel and hence its shear modulus. Significant swelling was not observed in high salt solutions (1–2 M) when the calcium level was maintained at 25 mM. This observation indicates that the neutral carbohydrate components of the network do not have a high affinity for water. With reducing salt and calcium content swelling is observed. Fig. 5 shows the swelling behaviour of pectin gels as a function of free calcium concentration in the gel, expressed as $[Ca^{2+}]_f/[COO^-]$. In 25 mM salt solution, when $[Ca^{2+}]_f/[COO^-]$ is $<0.05$ increased swelling is observed with decreasing calcium concentration. This is accompanied by a marked decrease in the stiffness of the gel [Fig. 5(b)] and indicates that at the lower concentrations of calcium the origin of the observed swelling is the dissociation of the calcium crosslink. At lower concentrations of supporting electrolyte (3 mM salt solution) an additional swelling effect is observed at values of $[Ca^{2+}]_f/[COO^-]$ $>0.05$. This indicates that there are a small number of carboxylate groups not

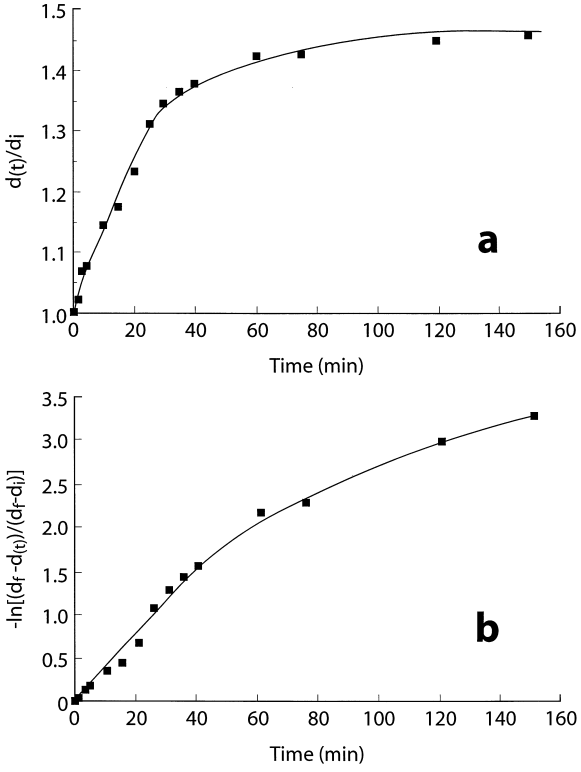


Fig. 4. Plot of (a) the time dependence of swelling, measured as the relative change in diameter $\left(d_{(t)}/d_i\right)$, of a 1.1% pectin gel and (b) analysis of this behaviour in a plot of $-\ln\left[\left(d_f - d_{(t)}\right)/\left(d_f - d_i\right)\right]$ versus time.

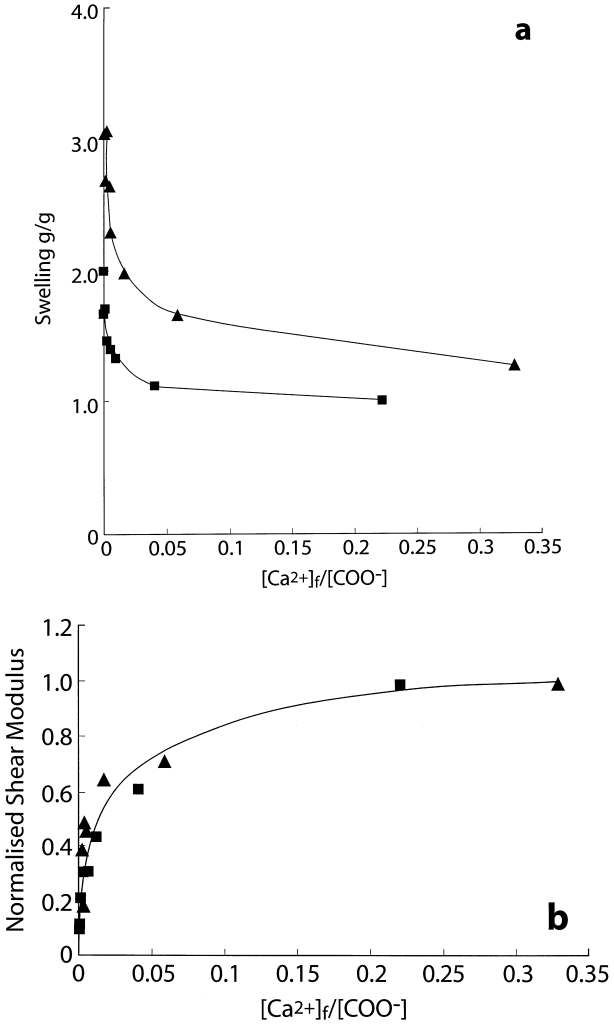


Fig. 5. The influence of external salt concentration on (a) swelling behaviour (expressed as the relative change in mass), and (b) normalised shear modulus of 1.1% pectin gels as a function of $[Ca^{2+}]_f/[COO^-]$. The bathing solutions contained 3 mM (▲) or 25 mM (■) NaCl.

involved in calcium crosslinking, their counterions exert an osmotic pressure which leads to gel swelling. As before, there is a marked increase in gel swelling at concentrations of $[Ca^{2+}]_f/[COO^-] < 0.05$.

It is useful to estimate the number of free carboxylate groups not involved in the calcium crosslink which would be required to generate an osmotic pressure, $\pi$, sufficient to swell the gel. This estimate can be obtained from [13]

$$\pi \approx RT(ic_2)^2/2\omega v c_s^* \quad (3)$$

where $i$ is the degree of ionization multiplied by the valency charge on the polymer, $c_2$ is the concentration of polymer charge, expressed as moles of repeating unit (the anhydrogalacturonic acid residue), $\omega$ the valency factor of the electrolyte, $v$ the number of cations and anions into which the electrolyte dissociates and $c_s^*$ the concentration of strong electrolyte external to the gel, with $R$ and $T$ having their usual meanings. To generate an osmotic pressure of 600 $Nm^{-2}$ (the shear modulus of the gel on initial swelling in 3 mM salt solution) requires that ~20% of the carboxylate groups in the gel are not involved in crosslinking. The purpose of making this estimate is to illustrate the dual role of the anhydrogalacturonate residues in affecting the functional properties of the gel in both participating in crosslinking and contributing to gel swelling at low ionic strengths. The structural origins of these differences in behaviour await determination.

The dependence of gel swelling and shear modulus on the pH of the supporting electrolyte was also examined (Fig. 6). The shear modulus of the gel falls with decreasing pH reaching a minimum at pH 3, but then increases again. From the known $pK_a$ of the carboxyl group of D-galacturonic acid of 3.23 [22] it would be expected that the amount of calcium anhydrogalacturonate would be rather small at pH 2. The minimum in gel shear modulus and maximum in swelling of the pectin gel occur in the vicinity of the expected $pK_a$ of the pectin. The observation that shear modulus increases with decreasing pH at pH < 3 indicates the occurrence in these systems of other types of crosslink. At these pHs the gels are slightly turbid indicating the formation of polymer aggregates of the order of the wavelength of light in size. This aggregation may be a further source of crosslinking in these low pH systems. The general trend for the swelling of the gel is to decline with decreasing pH, however a maximum swelling is observed at pH 3, where crosslinking is at a minimum. This further illustrates the importance of ionic crosslinking in influencing swelling behaviour in these systems, in addition to the Donnan effect which is so important in covalently crosslinked polyelectrolyte gels.

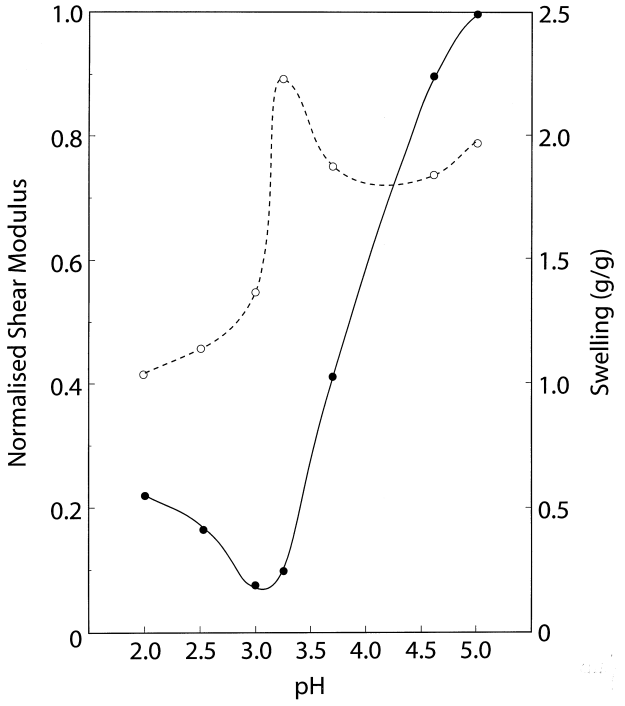


Fig. 6. Plot of normalised shear modulus (●) and swelling (○), as a function of pH, for a 1.1% pectin gel (25 mM $CaCl_2$) immersed in 100 times its own volume of dilute HCl of the required pH.

## 4. General discussion

The physicochemical behaviour of pectic polysaccharides is relevant to their industrial usage as isolated polysaccharides and also to their behaviour in the plant cell wall. In a previous article we demonstrated that a high methoxyl, CDTA-extractable pectic polysaccharide from tomato cell wall formed elastic gels on addition of calcium [4]. In this study we have shown that although the affinity of the polysaccharide for calcium ions is comparable to that of other pectic polysaccharides of similar degrees of methyl esterification when in solution, on gelation a stronger interaction is formed, characterized by a stability constant which

is at least an order of magnitude greater than for the solution case. The regulation of crosslinking and ionic interactions in the plant cell wall is a topic of continuing interest, with increasing information becoming available on the ionic composition of the apoplast. The availability of estimates of the strength of the interaction between pectins and calcium ions is necessary to model the complex ionic equilibria in the plant cell wall and, in turn, their effect on cell wall properties.

Another relevant property is the affinity of the cell wall polysaccharides for water. This will affect the polysaccharide concentration in the cell wall and consequently will affect both the mechanical properties of the cell wall, and its porosity to enzymes. If all free carboxylate groups of the pectin participate in calcium crosslinks the swelling of the network will be determined by the affinity of water for the neutral carbohydrate. Generally water is an indifferent solvent for polysaccharides and therefore this effect is expected to be small. Indeed minimal swelling of a pectin gel is observed when there is sufficient calcium present to ensure maximum crosslinking (as assessed by the shear modulus reaching its plateau value) and there is supporting electrolyte. Polyelectrolyte swelling, primarily arising as a result of a Donnan effect, is a potentially stronger driving force for gel swelling. The observation that at low ionic strengths this effect contributes to the swelling of pectin gels indicates that not all the carboxylate groups are involved in crosslinking and control of the fraction of these groups present would be a way of controlling the swelling properties and behaviour of the pectin gel.

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