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Characterisation of a novel gel system containing pectin, heat inactivated pectin methylesterase and NaCl

T. Schmelter[a,1], R. Vreeker[a], W. Klaffke[a,b,*]

[a]*Unilever Research Vlaardingen, P.O. Box 114, NL-3130 AC Vlaardingen, Netherlands*
[b]*Organisch-Chemisches Institut, Westfälische Wilhelms-Universität Münster, Corrensstrasse 40, D-48149 Munster, Germany*

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Abstract

An interesting and novel gelation system is described consisting of pectin, heat inactivated pectin methylesterase and NaCl. Gelation occurs at pH values ranging from 3 to 7, with gels formed at lower pH values being more stable. The rheological properties of the gel depend on the concentration of its components and the temperature at which gel formation takes place. Under optimum conditions, storage moduli can reach values of >1000 Pa. Disruption of the gel network by increasing the temperature to 65°C is reversible upon cooling, yielding gels whose strength is very similar to that of the original gel. Experimental data suggest that mainly hydrophobic interactions and hydrogen-bonding contribute to the formation and stabilisation of junction zones whereas electrostatic interactions play only a minor role.

Keywords: Pectin methylesterase; Gel system; Protein-carbohydrate interaction

1. Introduction

Pectins are primarily polymers of α-1,4-linked D-galacturonic acid residues interrupted by 1,2-linked rhamnose residues (Lau, McNeil, Darvill & Albersheim, 1985). Depending on the degree of methoxylation, pectins are classified into high-methoxyl pectin (methoxyl content >50%) and low-methoxyl pectin (methoxyl content <50%). The mechanism for gel formation is different in both pectin types. In the former case, gel formation is governed by both hydrogen bonds and hydrophobic interactions (Oakenfull, 1991), whereas in the latter case gels are formed in the presence of Ca^{2+}, which acts as a bridge between pairs of carboxyl groups of different pectin chains. These interactions between Ca^{2+} ions carboxyl groups are described by the 'egg box' model (Grant, Morris, Rees, Smith & Thom, 1973). Due to their unique gel forming properties, pectins are widely utilised as ingredients in many food products. In some food products containing pectin, protein is also present. Proteins and polysaccharides play an important role in the food industry because of their contribution to the textural and structural characteristics of food products (Mitchell & Ledward, 1986). The tremendous potential of protein–polysaccharide interactions for food processing is reflected by their wide range of applications such as: gelation, stabilisation of processed milk products, protein recovery, inhibition of protein precipitation, concentration and purification of proteins and thermoplastic extrusion (Samant, Singhal, Kulkarni & Rege, 1993). The texture of the food is influenced by the properties of the individual polymers, as well as by the nature and strength of the interactions between them (Dickinson & Euston, 1991). Depending on the characteristics of polysaccharide and protein solutions, the following systems can be achieved upon mixing (Tolstoguzov, 1986):

1. A liquid two-phase system can be obtained in which the two polymers are primarily in different phases.
2. A two-phase system may be obtained with both macromolecular components in the same phase (complex coacervation).
3. Homogeneous stable solutions can be achieved in which the two components do not interact or exist as soluble complexes.

The nature of polysaccharide–protein interactions is often of electrostatic origin and based on opposite charges carried by the polysaccharide (negative) and the protein (positive). The strength of the resulting complexes depends on the

* Corresponding author. Tel.: +31-10-460-5259; fax: +31-10-460-5236.
E-mail address: werner.klaffke@unilever.com (W. Klaffke).
[1] Present address: DMV International, P.O. Box 13, NL-5460 BA Veghel, Netherlands.

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distribution and number of these charges. Their stability is influenced by the concentration of the components, pH, ionic strength and temperature. These parameters also govern the anionic pectin–protein interactions and can result in the formation of nonequilibrium complexes. Whether these complexes are soluble or insoluble depends on the mixing conditions and solvent conditions. At the same pH and polysaccharide protein ratio, complexes of widely different solubilities can be formed (Tolstoguzov, 1986).

We discovered that gelation occurred in a system containing pectin, heat inactivated pectin methylesterase (PME) and sodium chloride. Varying the concentration of each of its components can readily modify the interesting rheological properties of the gel. This prompted us to embark upon investigating the nature of the interactions that contribute to gel formation.

2. Experimental

2.1. Materials

The PME expressed in *Aspergillus oryzae* was obtained from Novo Nordisk and the PMEs from orange peel or tomato were purchased from Sigma. Pectins with a degree of methylation (DE) between 31.1 and 76.8% were provided by Tim Foster (Unilever Research, Colworth House, UK). Citrus pectin (DE 28, 70, 93%) was purchased from Sigma and apple pectin from Fluka (DE 70–75%) and Sigma (DE 70%). Diethyl pyrocarbonate and phenylglyoxal monohydrate was purchased from Aldrich. Sulfosuccinimidyl acetate was obtained from Pierce.

2.2. Heat inactivation of pectin methylesterase

The PME (Novo) was inactivated by heating an enzyme solution in buffer for 15 min at 90°C. The inactivation was performed at the enzyme concentrations indicated in the text. PME activity was determined according to the method of Wood and Siddiqui (1971).

2.3. Test system for pectin–pectin methylesterase interactions

The following standard test system was used to study the interactions between pectin and PME (Novo). To 3 ml of a 1.5% solution of citrus pectin (DE 70%) in 50 mM ammonium acetate (pH 4.5), 200 μl of a 4% solution of heatinactivated PME in 50 mM ammonium acetate (pH 4.5) and 150 μl of a 2 M NaCl solution in water were added. The mixture was kept at room temperature. This test system was varied as described below, altering only one component at a time.

- Pectin: between 0–2.69%
- DE (pectin): between 28–93%.
- Methylesterase: between 0–0.23%.
- NaCl: between 0–358 mM.
- Temperature: between 5–65°C.
- pH: between 3–7.

Test systems lacking one of these components served as the corresponding control experiments.

2.4. Chemical modifications

The basic amino acids of PME (Novo) were chemically modified.

Arginine: the reaction was performed using a modification of the procedure described by Adachi and Marklund (1989). To 10 ml of a 50 mM phenylglyoxal monohydrate solution, 2.0 ml of water containing 50 mg of PME was added. The mixture was stirred gently for 30 min at room temperature, dialysed against water and subsequently lyophilised.

Histidine: the reaction was conducted using a modified version of the procedure described by Daron and Aull (1982). Ethanolic diethyl pyrocarbonate solutions were freshly prepared by diluting diethyl pyrocarbonate 1:19 (v:v) with cold absolute ethanol. Buffered diethyl pyrocarbonate solutions were prepared by adding 200 μl of ethanolic diethyl pyrocarbonate solution to 800 μl of 50 mM Tris/HCl (pH 7.5). To 1 ml of buffered diethyl pyrocarbonate solution, 50 mg of PME dissolved in 2 ml of 50 mM Tris/HCl (pH 7.5) was added. The incubation mixture was allowed to react for 20 min at 0°C and then warmed up to room temperature over a time period of 10 min. It was dialysed against water and subsequently lyophilised.

Lysine: the reaction was performed using a modification of the procedure described by Adachi and Marklund (1989). 50 mg of PME was dissolved in 2 ml of 0.2 M sodium hydrogen carbonate to which 50 mg of sulfosuccinimidyl acetate was added. The mixture was stirred gently for 45 min at room temperature, dialysed against water and then lyophilised.

2.5. Stability of pectin gels in the presence of different salts

Preformed gels obtained under the standard conditions (see 'Test system for pectin–PME interactions') were overlaid with either 1 ml of 2 M NaCl or 1 ml of 8 M urea.

2.6. Gel formation in the presence of ethanol

The gelation system contained the components listed in Section 2.3. However, instead of NaCl, ethanol was added to a final concentration of 20%.

2.7. Rheological measurements

Rheological measurements were conducted by employing a CarriMed CSL 500 controlled stress rheometer in the oscillation mode. The instrument was equipped with a

cone-plate device having a diameter of 4 cm and an angle of 2°. Gel formation was followed by recording the storage modulus G' and the loss modulus G'' as a function of time at an oscillation frequency of 1 Hz. Measurements were started 1 min after the sample was poured onto the rheometer plate. The stress applied was 0.25–1.0 Pa which was in the linear regime. The experiments were performed at 20°C, unless otherwise indicated.

2.8. Circular dichroism

Circular dichroism (CD) experiments were performed using a Jobin Yvon CD-6 spectrophotometer with a temperature-controlled cell holder. A scan program was employed with sequenced scanning (scan range 200–450 nm) and an interval between scans of 3000 s. Initially a baseline was established. After transferring the sample into a 0.05 cm Suprasil cuvette, the cuvette was placed in the holder (20°C) and the scan program was started immediately. Spectra were also recorded after the gelation was allowed to proceed for 11 and 22 h, respectively.

2.9. Electrophoretic measurements

A 0.15% solution of citrus pectin (DE 70%, Sigma) in 50 mM ammonium acetate and a 0.25% solution of heat inactivated PME (Novo) in the same buffer were applied separately to a Zetasizer 3 (Malvern). The experiments were conducted at 25°C with an electrode potential of 200 V.

3. Results

3.1. Interactions between pectin and heat inactivated pectin methylesterase

The interactions between pectin and PME were studied using the test system described above. Under the conditions referred to as the standard test system, a gel was formed at room temperature within 10 min. It is important to note that this gel formation is not due to methylesterase *activity* since the enzyme employed was heat inactivated. In order to investigate the contribution of the individual components of the gel system to gelation, their concentration was varied or modified or similar compounds replaced them, respectively. Gelation was abolished in the absence of either pectin, PME or NaCl.

The gelation properties of different pectins. The rheological evaluation of pectin-containing systems over a time period of 10 h is shown in Fig. 1. Citrus pectin (DE 70%) resulted in the formation of a gel having the highest G' value (circa 250 Pa). Apple pectin (Fluka) yielded a gel whose G' value was about 60% lower. None of the other pectins tested led to gel formation under these conditions. To ensure that gelation is not due to impurities in the pectins or PME (Novo), the polysaccharides and esterase were dialysed extensively against water, lyophilised and then used for the rheological studies. This treatment had no effect on the corresponding storage moduli (data not shown).

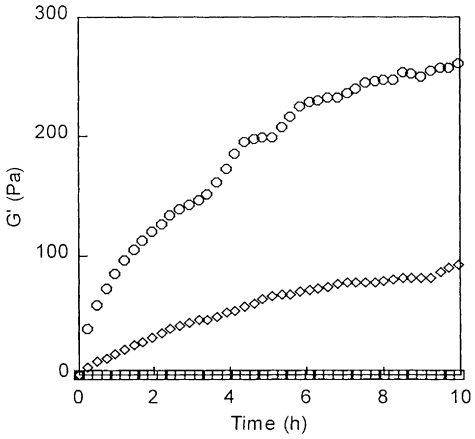

Fig. 1. The gelation properties of different pectins under standard conditions: (○) citrus pectin ex Sigma; (◇) apple pectin ex Fluka; and (□) apple pectin ex Sigma.

The impact of the degree of methylation on the gel strength. Citrus pectins having a DE of 28, 70 and 93%, respectively, were tested under standard conditions. The recording of the storage modulus is depicted in Fig. 2. Only the pectin with a DE of 70% formed a gel; in the case of a DE of 28 or 93% gelation was abolished.

Gelation in the presence of different pectin concentrations. The citrus pectin concentration was varied as indicated in Fig. 3. No gelation was observed with a 0.45% pectin solution. However, performing the reaction in the presence of 1.34 or 2.7% pectin solutions furnished gels with G' values of about 200 and 4000 Pa, respectively. Thus doubling the pectin concentration resulted in a 20-fold increase of gel modulus.

Gelation in the presence of different PME (Novo) concentrations and other PMEs. Lowering the amount of esterase (Novo) delayed the onset of gelation (Fig. 4). Gels with a storage modulus of about 100 and 250 Pa were obtained when the gelation was conducted for 10 h in the presence

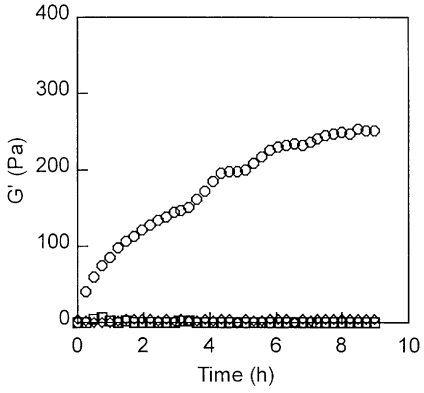

Fig. 2. The influence of the DE of citrus pectins on the gel modulus: (◇) DE 28%; (○) DE 70%; and (□) DE 93%.

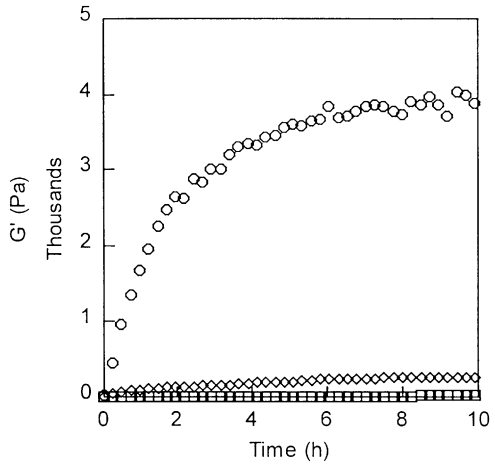

Fig. 3. The influence of the citrus pectin (DE 70%) concentration on the storage modulus of the corresponding gels: (○) 2.69%; (◇) 1.34%; and (□) 0.45% pectin.

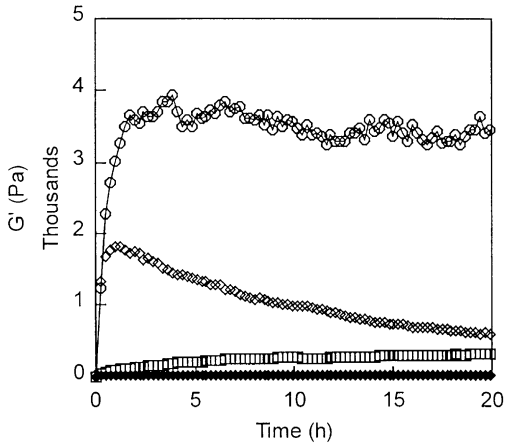

Fig. 5. The effect of the ionic strength on the gelation of citrus pectin (DE 70%)/PME (Novo): (◆) 44.8 mM; (□) 89.6 mM; (○) 179.2 mM; and (◇) 358.4 mM NaCl.

of 0.012 or 0.23% solutions of this enzyme. Reducing the PME concentration to 0.0058% yielded gels with a G' of approximately 70 Pa. No gelation occurred when the PME from Novo was replaced by PME from tomato or orange peel (data not shown).

The impact of the modification of PME on its gel forming properties. PMEs whose basic amino acids had been modified were tested for their gelation properties. Lysine-modified PME gelled like the native PME. However upon alteration of the arginine and histidine residues, gel formation was abolished completely.

The effect of ionic strength on the storage modulus. The ionic strength of the gelation system was altered by varying the concentration of NaCl between 44.8 and 358.4 mM (Fig. 5). Gelation was abolished in the presence of 44.8 mM NaCl. However, the storage modulus was significantly enhanced by increasing the NaCl concentration from 89.6 to 179.2 mM (from about 200–3900 Pa). Unexpectedly, at a NaCl concentration of 358.4 mM, the G' value reached a maximum at 1900 Pa after about 30 min and decreased continuously over 20 h (Fig. 5).

Stability of pectin–PME gels towards different salts. Pectin–PME gels were overlaid with NaCl or urea as described (vide supra). Addition of NaCl had no effect. However upon addition of urea, which disrupts noncovalent bonds, the gel dissolved within a few minutes.

The effect of temperature on gelation. Gelation was allowed to take place under the standard conditions at 10, 20 and 30. The results are depicted in Fig. 6 which reveals that gelation at 10 and 20°C yielded gels having a G' value of about 200 Pa. However, when the gelation was conducted at 30°C, the storage modulus increased rapidly between a gelation time of 5–8 h reaching a G' of 900 Pa.

Thermal stability of a pectin–PME gel formed in the presence of 179.2 mM NaCl. In order to study the thermal stability of pectin–PME gels, a gel was formed under standard conditions in the presence of 179.2 mM NaCl and allowed to gel for 30 min. This gel was subjected to the

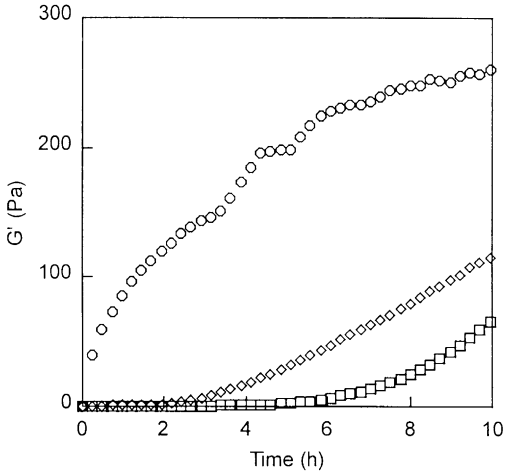

Fig. 4. The impact of the PME (Novo) concentration on the storage modulus of the corresponding gels: (○) 0.23%; (◇) 0.012%; and (□) 0.0058%.

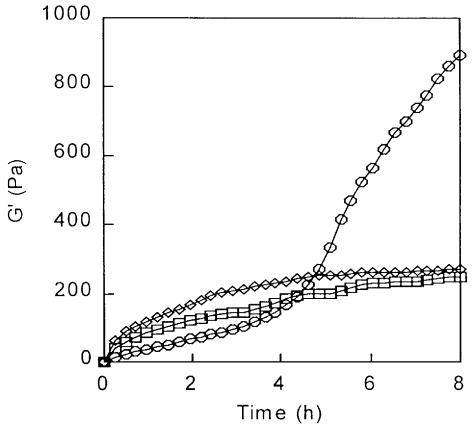

Fig. 6. Gelation under standard conditions at different temperatures: (○) 30°C; (□) 20°C; and (◇) 10°C.

following temperature sweep experiments: First, the gel was cooled to 5°C and then heated to 65°C at a rate of 1°C/min (Fig. 7). The figure shows that the G' value decreased from 5000 to 200 Pa. Second, the same gel was subsequently cooled from 65 to 5°C at a rate of 1°C/min. As illustrated in Fig. 7, the storage modulus increased continuously until it reaches its original value at 5°C. These results demonstrate that the gel melted in the temperature range used in these experiments and that the melting is thermoreversible.

Effect of the pH value on pectin–PME gelation. The gelation properties of pectin were studied by adjusting the pH value of the ammonium acetate buffer to 3, 4, 5, 6 and 7 using acetic acid or sodium hydroxide, respectively. Gelation occurred at all pH values, however the gels formed at a pH exceeding 5 were noticeably weaker than those formed at lower pH values. These gels began to dissolve slowly within 24 h after formation.

Gelation in the presence of ethanol. This experiment showed that gelation also occurred when NaCl was replaced by ethanol. Gelation did not take place in the absence of PME (Novo).

3.2. Chemically modified pectin methylesterase

Modification of the arginine, histidine and lysine residues of PME (50 mg) from Novo gave the following yields of altered protein.

- Arginine-modified enzyme: 36 mg.
- Histidine-modified enzyme: 45 mg.
- Lysine-modified enzyme: 39 mg.

In the case of arginine, a heterocyclic condensation product was formed by reaction with phenylglyoxal that blocks the amino groups in the side chain. The nitrogen atoms in the imidazole side chain of histidine were modified with diethyl pyrocarbonate and the amino function of lysine was blocked by acetylation with sulfosuccinimidyl acetate.

The gelling properties of these esterases are described above.

3.3. Characterisation of pectin–PME–NaCl-gels

In order to gain a better understanding of the underlying gelation mechanism, the pectin gels were further characterised by applying the following techniques.

The formation of a gel according to the standard procedure was monitored by CD at 0, 11 and 22 h after onset of gelation.

As shown in Fig. 8, only slight spectral changes were observed between 0 and 22 h. This suggests that no detectable conformational changes occurred during the formation of the gel network.

Determination of the electrophoretic mobility of citrus pectin (DE 70%) and PME (Novo) at pH 4.5 revealed that both macromolecules have very similar mobilities of -0.51×10^{-8} and -0.62×10^{-8} m^2/V, respectively. Thus, both macromolecules are negatively charged at this pH. Evaluation of pectin gels, formed in the presence of either 89.6 or 179.2 mM NaCl, by confocal scanning laser and optical microscopy demonstrated that the pectin/PME mixture was homogeneous and that no phase separation occurred (data not shown).

4. Discussion

Investigation of the gelation system containing citrus pectin, heat inactivated PME (Novo) and NaCl revealed that each component plays a pivotal role in gelation since it is abolished in systems lacking one of them. Since the contribution of methylesterase *activity* to the gelation process can be strictly ruled out, it seems likely that this particular enzyme has a unique primary structure

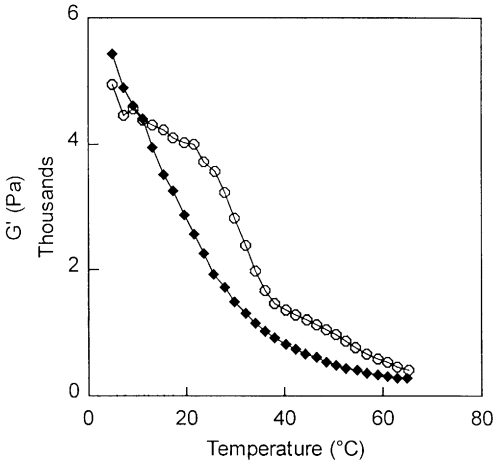

Fig. 7. Effect of temperature sweep on the storage modulus of a citrus pectin (DE 70%)/PME (Novo) gel formed in the presence of 179.2 mM NaCl: (○) increasing temperature from 5 to 65°C; and (◆) decreasing temperature from 5 to 65°C at 1°C/min.

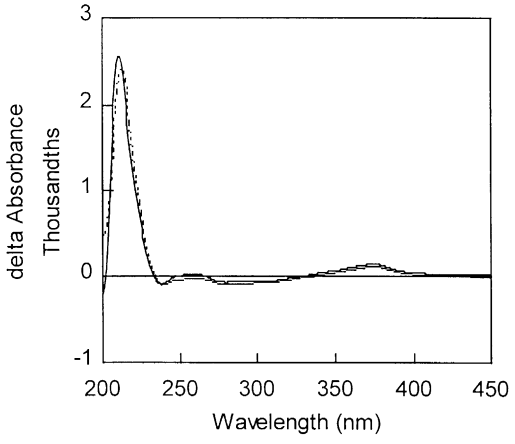

Fig. 8. Monitoring of the gel formation under standard conditions by means of CD recorded directly (—) and 22 h (– –) after start of gelation. The delta absorbance values were calculated per gram pectin in solution.

which facilitates gel formation. This notion is further supported by the observation that other methylesterases or pectin-modifying enzymes (arabinanase, galactanase, endoglucanase, pectin lyase, rhamnogalacturonase A,B and xylanase, all from Novo) were not able to form a gel under these conditions. Comparison of the amino acid sequence of the Novo enzyme (Novo Patent, 1994) with those of other PMEs (Duwe & Khanh, 1996) showed that the sequence of the former enzyme is very homologous to that of, for example, the esterase from *Aspergillus niger*. The C-terminus of the Novo esterase however contains more basic amino acid residues than that of the *Aspergillus* enzyme. It is conceivable that this difference in charge distribution has an impact on the gelation properties of the former enzyme. Further supporting evidence is provided by the observation that modification of arginine and histidine residues of the enzyme from Novo resulted in the loss of gel formation.

Fig. 4 reveals that stronger gels were obtained at higher PME concentrations. Furthermore, the onset of gel formation is delayed with decreasing enzyme concentration. From these findings it can be deduced that the PME has an impact on the formation of the three-dimensional network. Varying its concentration is a means to control a gel's rheological properties and the speed at with gelation proceeds.

Evaluation of the gels by means of confocal scanning laser and optical microscopy showed that the gels have a homogeneous appearance, with no indication of phase separation (data not shown). The absence of phase separation renders limited thermodynamic compatibility of the polymers or the occurrence of complex coacervation unlikely.

As illustrated in Fig. 3, the citrus pectin concentration had a major impact on the storage modulus. This is expected since an increase in pectin concentration is paralleled by an increase in potential sites for interchain interactions. Furthermore, gelation seemed to be strongly influenced by the degree of methylation of the pectin polymer (Fig. 2). The fact that gelation was abolished at a DE of 28% suggests that hydrophobic rather than electrostatic interactions contribute to gel formation. In addition, intermolecular electrostatic repulsion is increased with a decreasing ester content, which reduces the tendency for gelation. Of the citrus pectin samples tested here, that having a DE of 28% is the least flexible (Cros, Garnier, Axelos, Imberty & Pérez, 1996) which might also hinder gel formation. The highest storage modulus was obtained at esterification levels of about 70%, which resembles the gelation of pectins under conditions of low water activity (Morris, Gidley, Murray, Powell & Rees, 1980). If gel formation is only governed by hydrophobic interactions, the highest gel strength should be achieved with the citrus pectin having a DE of 93%. However, this was not found but it can be speculated that if hydrogen interactions also contribute to the gelling mechanism, a higher DE (e.g. 93%) will prevent more the possibility of establishing this type of linkages than a pectin with a lower DE (e.g. 70%).

The differences in the gelation behaviour of pectins from various sources (Fig. 1) reflect the importance of other factors for gel formation. No explanation for the loss of gelation with other pectins, except citrus pectin (Sigma) and apple pectin (Fluka), can be given. However, it is possible that the length and distribution of side chains, rhamnose content of the backbone, molecular weight and charge distribution have an impact on the formation of junction zones and pectin–PME associations.

The gelation mechanism and rheological properties of the gel also depended strongly on the ionic strength of the reaction mixture (Fig. 5). The effect of salt addition can be accounted for by screening charges which leads to a decreased electrostatic repulsion between polymer chains. Doubling the NaCl concentration from 89.6 to 179.2 mM resulted in a drastic increase of G'. However, further increase of the ionic strength (358.4 mM) furnished a gel whose maximal storage modulus was reduced by 50%. Furthermore, the decrease of gel strength with time indicates nonequilibrium behaviour resulting from continuous reorganisation of the network. Since this phenomenon was only observed under high salt conditions, it is possible that the underlying gelation mechanism differs from that at lower ionic strength or that the tendency to reorganise increases with increasing salt concentration. Several experiments, including CD, were carried out in order to gain a better understanding of the nature of the interactions that contribute to gel formation. CD is known to be a sensitive probe of the local environment of uronic acid carboxy chromophores. Gidley, Morris, Murray, Powell and Rees (1979) showed that calcium-induced gelation of sodium pectate solutions is accompanied by a large change in CD behaviour. Unfortunately, no further information could be gained from CD (Fig. 8) measurements in this study. In contrast to changes of the G' values during gelation, which indicate an ongoing molecular rearrangement, no CD-detectable conformational changes occurred under similar conditions.

Based on the following observations, a major contribution of electrostatic interactions in the formation of a three-dimensional network seems unlikely: (I). Citrus pectin (DE 70%) and PME (Novo) have an almost identical electrophoretic mobility at pH 4.5. Both polymers are negatively charged, which prevents very strong electrostatic attractive forces between them. However, it can not be ruled out that attractive interactions between both polymers occur due to patches of positively charged residues on the protein surface. (II). Gelation takes place between pH values of 3 and 7. Since the pK_a value of the carboxyl group in pectins is ~3.6 (Plaschina, Braudo & Tolstoguzov, 1978), its protonation should vary considerably over this pH range. This renders pectins less amenable to charge-dependent

associations at low pH values, but does not rule out the ± interactions completely. However, there seems to be a direct correlation between the acidity of the reaction medium and the strength of the corresponding gels which cannot be reconciled with strong electrostatic interactions. (III). Electrostatic interactions should show a sensitivity to ionic strength, but overlaying a preformed gel with 2 M NaCl had no effect. Moreover, stronger gels are formed in the presence of NaCl (Fig. 5). The opposite effect is anticipated when electrostatic (±) interactions are of importance.

In contrast, the gel dissolved quickly after being mixed with urea. Urea is a strong hydrogen-bonding compound, which might provide alternative, more energetically favourable hydrogen bonds than those stabilising the polymer interactions. The instability of the gel in the presence of urea suggests therefore, that hydrogen bonding contributes to gel formation.

It has been described in literature that junction zones in gels of high methoxyl pectins are additionally stabilised by hydrophobic interactions between the methyl ester groups (Walkinshaw & Arnott, 1981; Oakenfull & Scott, 1984). These interactions are necessary to overcome the entropic barrier to gelation (Oakenfull & Scott, 1984). The involvement of hydrophobic interactions in polymer network formation can be studied by the effect of variation of temperature on the rheological properties of the corresponding gels. Since hydrophobic interactions become stronger at higher temperature, the resulting storage modulus is increased as well (Lopes da Silva & Gonçalves, 1994). The available data (Fig. 6) provide evidence that these interactions contribute to the gelation observed here. As illustrated in Fig. 6, G' is significantly increased when gelation is performed at 30°C instead of 10 or 20°C. Further support for the role of hydrophobic interactions in this gel system can be derived from the fact that gelation also occurred upon addition of ethanol, instead of NaCl. According to Oakenfull and Scott (1984), small concentrations of ethanol strengthen hydrophobic interactions between high methoxyl pectin chains.

The reversible nature of the interactions contributing to gel formation is demonstrated by temperature sweep experiments (Fig. 7). Upon cooling and heating of gels, storage moduli were obtained which are very similar to their initial values.

Surprisingly, increasing the temperature causes a monotonous decrease in G' (Fig. 7), whereas, on the basis of the results shown in Fig. 6 an increased G' might have been expected. The origin of theis evident contradiction remains still unclear.

5. Conclusions

It has been demonstrated that gelation occurred in a system comprising pectin, heat inactivated PME and NaCl at pH values ranging from 3 to 7. The rheological properties of the corresponding gels are governed by the concentration of their components as well as the temperature at which gelation takes place. Investigation of the underlying gelation mechanism suggests that junction zones are primarily formed and stabilised by hydrophobic interactions and hydrogen-bonding whereas electrostatic interactions play only a minor role.

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