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Gelation of Sugarbeet and Citrus Pectins using Enzymes Extracted from Orange Peel

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ABSTRACT

Sugarbeet pectin is shown to form gels in the presence of calcium using an enzyme preparation extracted from orange peel. The gels were transparent and exhibited no syneresis. The mechanism of gelation is chain association arising from both lowered pectin solubility and from formation of a limited network of calcium-linked junction zones. The gelation reaction involves limited pectin demethoxylation, the release of acetate presumably from C-2 or C-3 of galacturonyl residues, and a decrease in pH. The enzymes responsible are pectinesterase (EC 3.1.1.11) and pectin acetylesterase. We suggest that the latter is a novel activity associated with triacetin acetylesterase (EC 3.1.1.6). The gels are compared to citrus pectin gels made in the same way.

INTRODUCTION

Pectins consist of an α-1,4-glycosidically linked D-galacturonic acid backbone interrupted by rhamnose residues. Neutral sugar side chains are covalently attached to rhamnose residues. The galacturonyl residues may be methoxylated at C-6 or acetylated at C-2 and/or C-3 (Fig. 1). The extent and distribution of substitution depends upon the source of the pectin and on the method of isolation (Nelson *et al.*, 1977).

The gelation properties are dependent upon the degree and distribution of methoxylated residues. Citrus pectins readily form gels — high methoxy derivatives gel in the presence of concentrated sugar solutions, whereas low methoxy pectins gel in the presence of Ca^{2+} (Nelson *et al.*,

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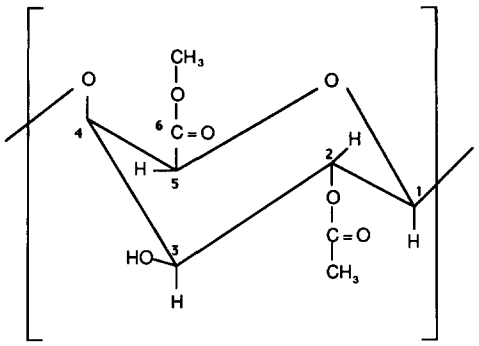

Fig. 1. Structure of an α-1,4-linked galacturonyl residue methoxylated at C-6 and acetylated at C-2.

1977). In contrast, pectin from sugarbeet (*Beta vulgaris*) has poor gelling properties (Michel *et al.*, 1985). This has been attributed to a high degree of acetylation (up to 4·5% w/w) (Pippen *et al.*, 1950), a low molecular weight (Thibault, 1986) and a large proportion of 'hairy regions' containing arabinose side chains (Keenan *et al.*, 1985). A small improvement of gelation in concentrated sugar solution was achieved by a mild acid hydrolysis for 11 days (Pippen *et al.*, 1950). Treatment of sugarbeet pectin with an enzyme preparation from *Aspergillus niger* modified the pectin to give improved gelling with calcium (Matthew *et al.*, 1990). Gels have also been made by cross-linking ferulic acid residues (contained within 'hairy regions') using ammonium persulphate (Thibault & Rombouts, 1986).

We report here a method suitable for producing calcium-dependent sugarbeet pectin gels at relatively low pectin concentration (down to 1% w/v) using enzymes readily extracted from orange peel. Good gels are obtained at ambient temperature. The rheological properties of these gels are compared to gels made with citrus pectin.

EXPERIMENTAL

Pectin

The orange peel pectin was type 105 'rapid set' citrus pectin (HP Bulmer Ltd, UK) with a degree of methyl esterification of 69–71%. Sugarbeet pectin was extracted from the pulp by heating with acid as previously described (Matthew *et al.*, 1990). It contained 5·6% (w/w) of methoxyl groups, equivalent to a degree of methyl esterification of 37%, and 1·8%

(w/w) of acetyl groups (about 7% acetylation) (Matthew *et al.*, 1990). Sugarbeet pectin thus extracted has been fully characterised by a ^{13}C-NMR study (Keenan *et al.*, 1985).

Solutions were prepared by dispersing the appropriate polymer in warm distilled water. In experiments where gels contained a high final concentration of sucrose or ethanol, the pectins were dispersed in sucrose (up to 3 M) or 30% ethanol respectively.

Enzymes

Pectinesterase and 'acetylesterase' from orange peel were obtained from Sigma Chemical Co., Dorset, UK. Both were supplied in ammonium sulphate. Samples were centrifuged, and the pellet resuspended in buffer to the required concentration.

Enzyme assays

Pectinesterase activity was measured by the method of Kertesz (1955), using a 0·5% citrus pectin solution at pH 8 and 30°C. 'Pectinase' activity was estimated by measuring the release of reducing groups using dinitrosalicylic acid (Miller, 1959). The reaction mixture contained 2 ml polygalacturonic acid (0·1% w/v) in 0·1 M sodium acetate pH 5·0 and 0·2 ml enzyme. Increase in reducing groups was measured after 15 min at 40°C using galacturonic acid as a standard. Deacetylation of triacetin (Jansen *et al.*, 1947) was detected by measuring the liberation of acetate from triacetin (100 mM) in 50 mM sodium succinate/50 mM sodium oxalate pH 6·2 using an acetate determination kit from Boehringer–Mannheim, East Sussex, UK. Deacetylation of pectin was measured in 0·28% sugarbeet pectin/50 mM sodium oxalate/50 mM sodium succinate at pH 6·2. Controls were run using enzyme solutions which had been boiled for 2 min. The release of acetate was quantified using the acetate kit described above. For both acetate-releasing assays, the reaction was stopped by treatment at 100°C for 1 min. For all enzyme assays, 1 unit of activity is the amount of enzyme that releases 1 μmol of product per minute under the conditions given above.

Protein assay

Protein was estimated using the Coomassie Protein Assay Reagent from Pierce, Luton, UK.

Pectin gelation

Pectin solutions, water and other additives were mixed before adding $CaCl_2$ and pectinesterase, respectively, to a final volume of 5 ml. The mixture was vortexed and immediately poured onto a 50 mm diameter petri dish. Unless otherwise indicated, it was left for 3 h prior to measurement of rheological properties. The pH of the gel was measured using a surface electrode (LKB Pharmacia, Milton Keynes, UK) placed either in the reaction mixture immediately upon pouring the gel, or on the surface of a set gel.

Measurement of rheological properties

A mechanical spectrometer (Instron 3250) was used to monitor the shear modulus (gel stiffness, G') of the contained pectin mixtures. This involved measuring the response of the gel to a mechanical disturbance (shear) to its free surface. All measurements were at room temperature using a 40 mm diameter flat platen in contact with the upper surface of the gel. To avoid irreversible damage to the gel phase only low amplitude oscillatory motion of the platen was employed. Increased gel stiffness was evidenced by a greater reaction to this applied motion and a consequently larger measure of gel shear modulus. Confirmatory evidence for the existence of a gel phase rather than a viscous solution was achieved by performing both frequency and strain oscillatory sweeps. The presence of a gel was taken as there being a low phase-angle and a markedly reduced dependence of G' on oscillatory frequency. The recorded gel stiffness was taken as the limiting value at low applied deformation which, by showing a plateau value, is in itself evidence of a permanent network formed within the system.

RESULTS

Gelation of citrus and sugarbeet pectins

In the presence of calcium, a solution of citrus pectin formed a gel upon addition of a commercial preparation of pectinesterase from orange peel. The gel stiffness after 3 h was dependent on the concentration of enzyme, pectin and $CaCl_2$ (Fig. 2).

Under the same conditions, solutions of sugarbeet pectin also formed good gels. These gels were clear and did not show syneresis. As above, gel stiffness was dependent upon the concentration of enzyme, pectin

and $CaCl_2$ (Fig. 2). Sugarbeet pectin gels were somewhat less stiff at a given pectin concentration, but the stiffness was less sensitive to variations in calcium concentration.

Effect of sucrose, NaCl and ethanol on gelation

In order to establish whether the mechanism of gelation of sugarbeet pectin was the same as citrus pectin, we examined the effect of sucrose and ethanol on gelation. These compounds affect pectin solubility. Figure 3 shows that sucrose only slightly affected gel stiffness. In contrast, ethanol markedly decreased the stiffness of citrus pectin gels, but had no effect on sugarbeet pectin gels. Na^+, which displaces Ca^{2+} from polygalacturonic acid (Rees, 1982), had no effect on gel stiffness of sugarbeet pectin, but considerably weakened citrus pectin gels (Fig. 3).

Enzyme action during gelation

Although both citrus and sugarbeet pectins gel on the addition of pectinesterase, the contrasting effects of sucrose, NaCl, ethanol and $CaCl_2$ could suggest that the molecular mechanisms of gelation might be

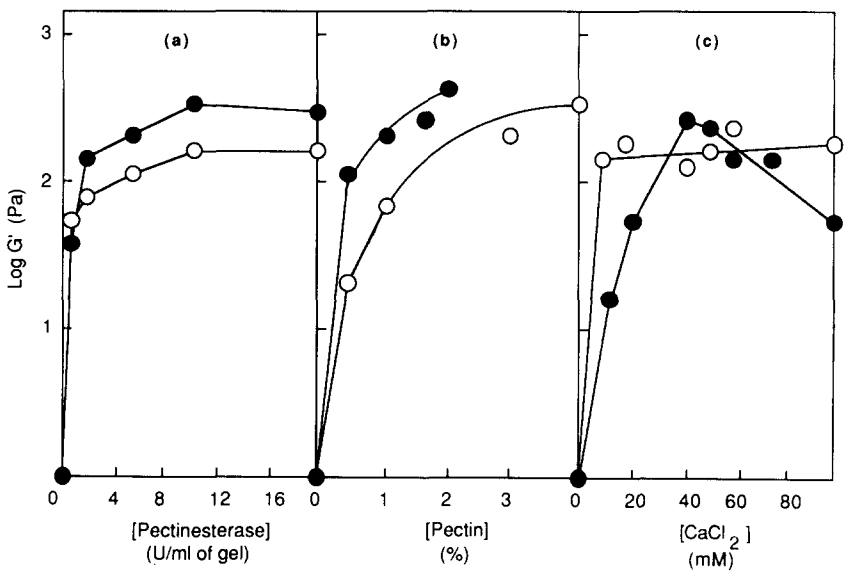

Fig. 2. The effect of various compositions of reactants on the gel stiffness of sugarbeet pectin (○) or of citrus pectin (●). (a) Citrus pectin, 1·2% (w/v); sugarbeet pectin, 3% (w/v); $CaCl_2$, 40 mM. (b) Pectinesterase, 9 U/ml; $CaCl_2$, 40 mM. (c) Citrus pectin, 1·2% (w/v); sugarbeet pectin, 2% (w/v); pectinesterase, 6 U/ml.

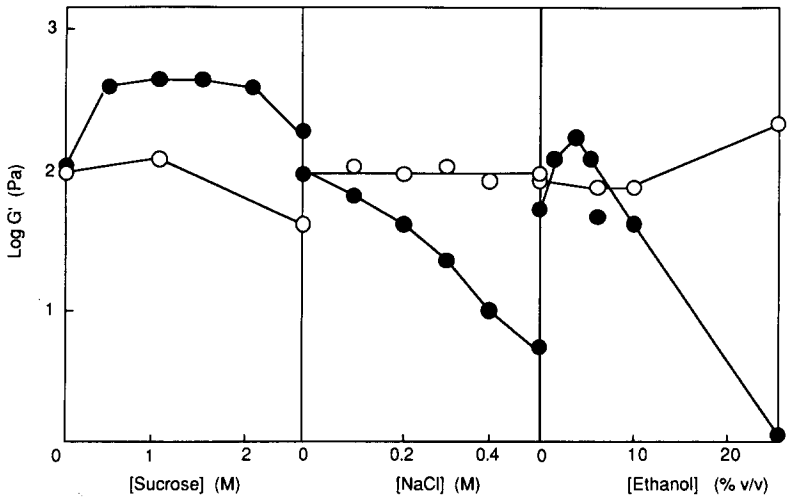

Fig. 3. The effect of sucrose, NaCl and ethanol on the stiffness of citrus pectin gels (1·2%, w/v) (●) and of sugarbeet pectin gels (2·5%, w/v) (○) in the presence of pectinesterase (2 U/ml) and $CaCl_2$ (40 mM).

subtly different for each pectin. Apart from the demethoxylation reaction catalysed by pectinesterase, other reactions that might occur are hydrolysis of the main chain or of side chain substituents, or deacetylation. We found no evidence of any hydrolysis, since incubation of 0·8 mg pectinesterase with 2·5 mg of citrus or sugarbeet pectin at 40°C gave no increase in reducing groups. Deacetylation of pectin, however, was detected. Pectinesterase (50 U/ml) was incubated with 1 ml sugarbeet pectin (0·5%) at 30°C, pH 6, for 30 min: 0·3 mg acetate was released. Since a boiled enzyme sample gave no release of acetate, the pectinesterase preparation must contain pectin acetylesterase activity, either as an integral activity of pectinesterase or as a separate enzyme. A similar experiment using citrus pectin did not yield a significant amount of acetate. This demonstrated that, whereas deacetylation may play a role in sugarbeet pectin gelation, it does not occur during citrus pectin gelation.

The deacetylation reaction

Orange peel extracts have been shown to contain an acetylesterase. It is active on triacetin (Jansen *et al.*, 1947), Cephalosporin C (Abraham & Fawcett, 1975), acetyl esters of 1-hydroxypyren-3,6,8-trisulphonic acid trisodium salt (Wolfbeis & Koller, 1983) and 3,5-diacetoxycyclopent-1-

ene (Miura *et al.*, 1976). Pippen *et al.* (1950) found no activity of this acetylesterase on sugarbeet pectin nor on acetylated citrus pectin. Their only indicator of activity, however, relied upon acetylesterase action inducing gelation in 65% sucrose, which involves a different mechanism to calcium-dependent gelation of low-methoxy pectins (Rees, 1982). We therefore re-tested the acetylesterase for activity on sugarbeet pectin.

Acetylesterase from orange peel is available commercially, but contains some pectinesterase. Figure 4 shows that this crude acetylesterase deacetylated sugarbeet pectin; after 60 min, 4 mg of acetate was released from 1 g of pectin, equivalent to ~ 20% of the total acetyl content. The extent of deacetylation was proportional to the triacetin acetylesterase activity and not to the amount of pectinesterase activity.

pH changes during gelation

Pectinesterase action releases protons which decrease the pH of the surrounding medium:

$$RCOOCH_3 + H_2O \rightarrow RCOO^- + H^+ + CH_3OH$$

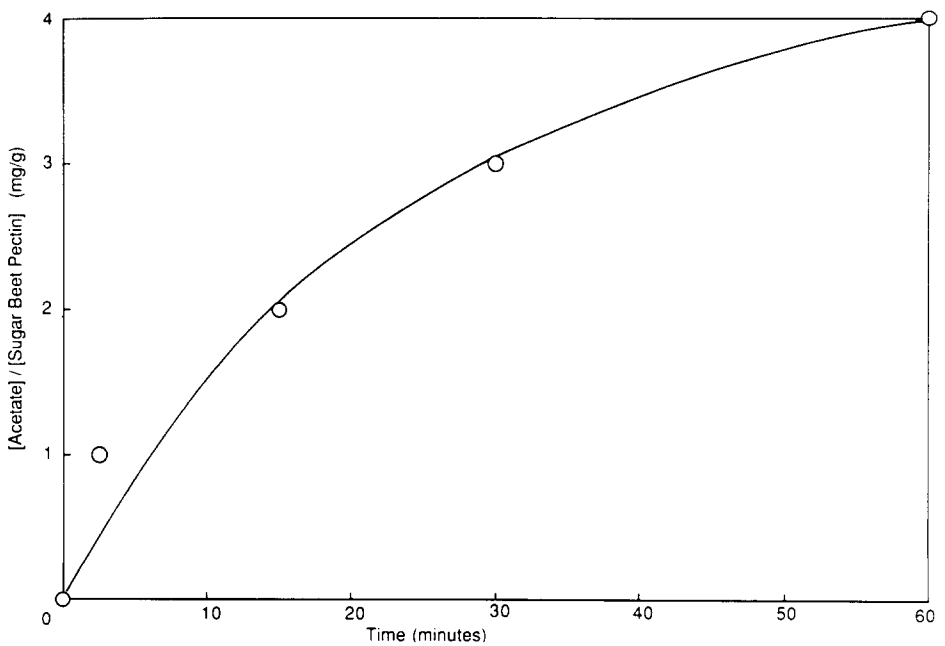

Fig. 4. Release of acetic acid from sugarbeet pectin (0·5%, w/v) by crude acetylesterase (0·4 μg/ml, 4·3 U triacetin hydrolysis/ml) in 25 mM sodium succinate, pH 6·0, at 30°C.

The pH thus decreased during the setting of pectin gels (Fig. 5). The pH of citrus pectin dropped from 3·5 to 2·8 and of sugarbeet pectin from 5·2 to 3·8. Although the optimum pH of orange peel pectinesterase is 7·6–8·0 (Veersteeg *et al.*, 1978), well away from the acidic pH values above, it clearly has sufficient activity to demethoxylate enough galacturonyl residues to induce gelation.

We attempted to measure the pH dependence of gelation, but because of the pH drop, it was not possible to buffer the solution sufficiently without introducing unacceptably large buffer concentrations. Even in 0·2 M tris/citrate buffer, the pH fell from 9 to 6 over the course of citrus pectin gelation. A higher concentration of buffer interfered with gelation possibly owing to a high ionic strength. In 0·2 M tris/citrate, citrus pectin formed a gel at all pH values (initial pH 4–9); however, sugarbeet pectin only formed a gel below pH 5. In the same buffer, but with 140 mM $CaCl_2$ instead of 40 mM, sugarbeet pectin gelled below pH 6. In both cases, the gel stiffness was less than in unbuffered solution.

DISCUSSION

At near neutral pH, orange peel pectinesterase demethoxylates in a blockwise fashion (De Vries *et al.*, 1986). At lower pH values, the demethoxylation becomes more random, and smaller blocks of C-6 unsubstituted galacturonyl residues are produced (De Vries, 1988). Acid deesterification is random (De Vries *et al.*, 1986), so that the type of pectin resulting from the reaction reported here is intermediate between

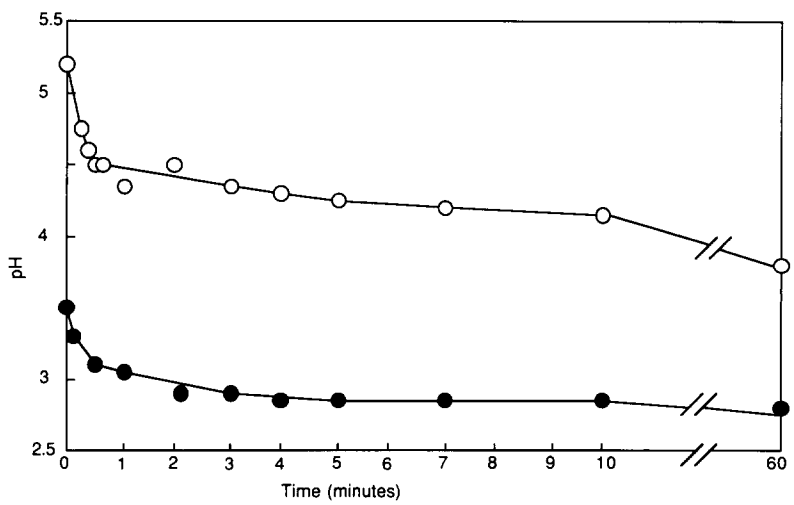

Fig. 5. pH change during gelation of citrus pectin (1·2%, w/v) (●) or of sugarbeet pectin (3%, w/v) (○) in the presence of pectinesterase (4 U/ml) and $CaCl_2$ (40 mM).

pectin produced by acid treatment and by enzyme deesterification at neutral pH. The lack of syneresis may be partly due to this distribution of C-6 unsubstituted galacturonic acid. It also suggests that the enzyme is switched off after a certain time which prevents extensive demethoxylation. At pH 3, the activity of all pectinesterase isoenzymes is almost zero (Veersteeg, 1979), so that the pH drop is responsible for halting enzyme action. Pectinesterase is unlikely to be inhibited by immobilisation within the set gel, since enzyme motion within polysaccharide gels is rapid, as shown by comparable studies on the motion of bovine serum albumin in amylose gels (Leloup *et al.*, 1987).

Pectin with a very low degree of esterification gels in the presence of Ca^{2+}/K^{+} using EDTA/glucano-δ-lactose in the system to release Ca^{2+} (S. Buhl, personal communication). This is achieved by a slow pH decrease. The drop in pH may thus play a further role in gelation by making the pectin less soluble. The pK_a of the pectin carboxylate group is ~3·1 (Thibault, 1986). As the pH approaches the pK_a, the pectin becomes less charged and, hence, less soluble. This is comparable to the gelation of high methoxypectin in concentrated sugar solution where the pectin gels by partial dehydration (Morris *et al.*, 1980) only below pH 3·5. Lowering of solubility may be critical for the gelation of sugarbeet pectin which does not gel above pH ~5–6. In this system, it is relatively unimportant for citrus pectin which gels at all pH values tested.

The effect of ethanol on gelation is also linked to solubility. Citrus pectin, dissolved in 30% ethanol, did not gel but changed into aggregates on addition of pectinesterase and calcium. Sugarbeet pectin, however, formed a good gel in 25% ethanol. The gel stiffness was slightly increased, owing to limited dehydration induced by the solvent. This phenomenon helps to explain the effect of NaCl. For both sugarbeet pectin and citrus pectin, NaCl replaces some Ca^{2+} from the galacturonic acid carboxylate group. But with sugarbeet pectin, the additional effect of partial pectin dehydration (which is increased in the presence of ethanol) is sufficient to maintain gel structure. This arises from differences in hydration of citrus and sugarbeet pectin. The former contains fewer acetyl groups and fewer neutral sugar side chains and is consequently more hydrated and more soluble. Therefore, the gelation of sugarbeet pectin reported here is due to a chain association by a combination of both short calcium-linked junction zones and limited dehydration. The relative importance of each effect is determined by gelation conditions.

Acetyl groups inhibit gel formation of both high- and low-methoxypectins by steric hindrance of chain association (Rombouts & Thibault, 1986). The method of extraction used here yields sugarbeet pectin with only 1·8% (w/w) acetylation — some methods have given up to 4·5%

(w/v) (Pippen *et al.*, 1950). Thus less pectin acetylesterase is needed to produce a given level of acetylation compared to a sugarbeet pectin isolated with a higher degree of acetylation. We have not determined whether C-2 or C-3 acetylation blocks C-6 demethoxylation by pectinesterase. If this is so, the pectinesterase might require the prior action of pectin acetylesterase, giving rise to synergy between the two enzymes. Furthermore, sugarbeet pectin isolated by different methods has different levels of acetylation (B. J. H. Stevens & R. R. Selvendran, personal communication), and so high acetyl forms may require a pre-treatment with pectin acetylesterase for successful gelation.

Deacetylation also affects pectin solubility, making it more soluble in water by decreasing the pectin backbone hydrophobicity (Dea & Madden, 1986). It does not affect the pH-dependence of gelation *per se*, since acetylation does not significantly alter the C-6 carboxylate pK_a (Thibault, 1986).

Results obtained at this laboratory using an enzyme preparation derived from *A. niger* (Matthew *et al.*, 1990) showed that the ability of the modified sugarbeet pectin to form calcium gels was due to loss of some acetyl groups, a degree of demethoxylation and the removal of arabinose residues. The effect was attributed to steric considerations, increase in the acidic nature of the modified pectin and the decrease in the non-uronide content of the sugarbeet pectin. The gelation process described here requires less concentrated pectin solutions and offers a way of easily and cheaply improving the quality of sugarbeet pectin. Pectinesterase is food approved, and it might be possible to simply use a 'natural' extract from orange peel as a suitable enzyme source.

Whether the pectin acetylesterase is significant *in vivo* remains to be seen. It would be interesting to determine if its presence was inversely related to the degree of pectin acetylation. Moreover, elucidation of its mode of action requires purification of pectin acetylesterase to homogeneity.

In summary, sugarbeet pectin forms gels in the presence of crude orange peel pectinesterase and $CaCl_2$ by a combination of several concomitant reactions — demethoxylation to give small calcium-linked junction zones, deacetylation to reduce steric hindrance of chain association, and a decrease in pH to lower pectin solubility. These reactions are catalysed by pectinesterase and by pectin acetylesterase.

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