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Synergistic interactions between the genetically modified bacterial polysaccharide P2 and carob or konjac mannan

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Abstract—Rheological studies have confirmed that the bacterial polysaccharide P2, a genetically modified variant of the *Acetobacter xylinum* polysaccharide acetan, undergoes synergistic gelation with either of the plant polysaccharides carob or konjac mannan. X-ray fibre diffraction data shows that P2 can form a 5-fold helical structure of pitch 4.7 nm and an axial rise per disaccharide repeat of 0.92 nm. Optical rotation data demonstrate that P2 undergoes a coil–helix transition in solution and that deacylation enhances the stability of the helical structure in solution. Studies made on mixtures prepared at different temperatures and ionic strengths suggest that denaturation of the P2 helix favours interaction and gelation. Deacetylation of P2 enhances gelation. X-ray diffraction data for oriented fibres prepared from deacetylated P2–konjac mannan mixed films reveal a 6-fold helical structure of pitch 5.54 nm with an axial rise per disaccharide repeat also of 0.92 nm. This mixed helix provides direct evidence for binding between the two polysaccharides. P2 contains two sites of acetylation: one on the backbone and one on the sidechain. The former site of acetylation inhibits helix formation for P2. It is suggested that this site of acetylation also inhibits formation of the mixed helix, explaining the enhanced gelation of mixtures on deacetylation.

Keywords: Rheology; Bacterial polysaccharide; Konjac mannan; Mixtures; Carob

1. Introduction

The bacterial polysaccharide P2 is a genetically modified variant[1,2] of the *Acetobacter xylinum* polysaccharide acetan. Acetan (Fig. 1a) is similar in structure to that of the commercially important polysaccharide xanthan gum[3] (Fig. 1b), produced by *Xanthomonas campestris*. The biosynthetic pathways for the production of these two polysaccharides are very similar.[4] Indeed, the first four steps in the assembly of sugars in the repeat unit are identical in both bacteria and the polysaccharides thus possess a conserved structural region, excluding noncarbohydrate substituents. The genes involved in the biosynthesis of xanthan have been identified in collections of natural and induced mutant strains[5–7] and this provided a rational basis for the isolation of the cluster of genes involved in the biosynthesis of acetan.[8–12] It was established through chemical mutagenesis that *A. xylinum* can assemble and export variants of the acetan structure with truncated sidechains.[13] The polysaccharide P2 was obtained by inactivation of the *aceP* gene, which encodes for a glycosyl transferase involved in acetan biosynthesis.[2] This enzyme attaches the β-(1→6) linked D-Glc residue to the sidechain. Structural analysis confirmed that P2 is a xanthan mimic, in which the tri-saccharide sidechain is terminated by a glucose residue, and there is additionally partial acetylation at C6 on the (1→3,4) linked backbone glucose residue (Fig. 1c). For the structural analysis of the P2 polysaccharide by NMR it was necessary to partially degrade the polysaccharide. In this degraded product small quantities of the acetan trimer (Fig. 1e) and the acetan tetramer, also called CR1/4 (Fig. 1d) were detected.[1] These fractions may have been present in the original sample, but it is possible that they were induced by the degradative conditions (sonication, high

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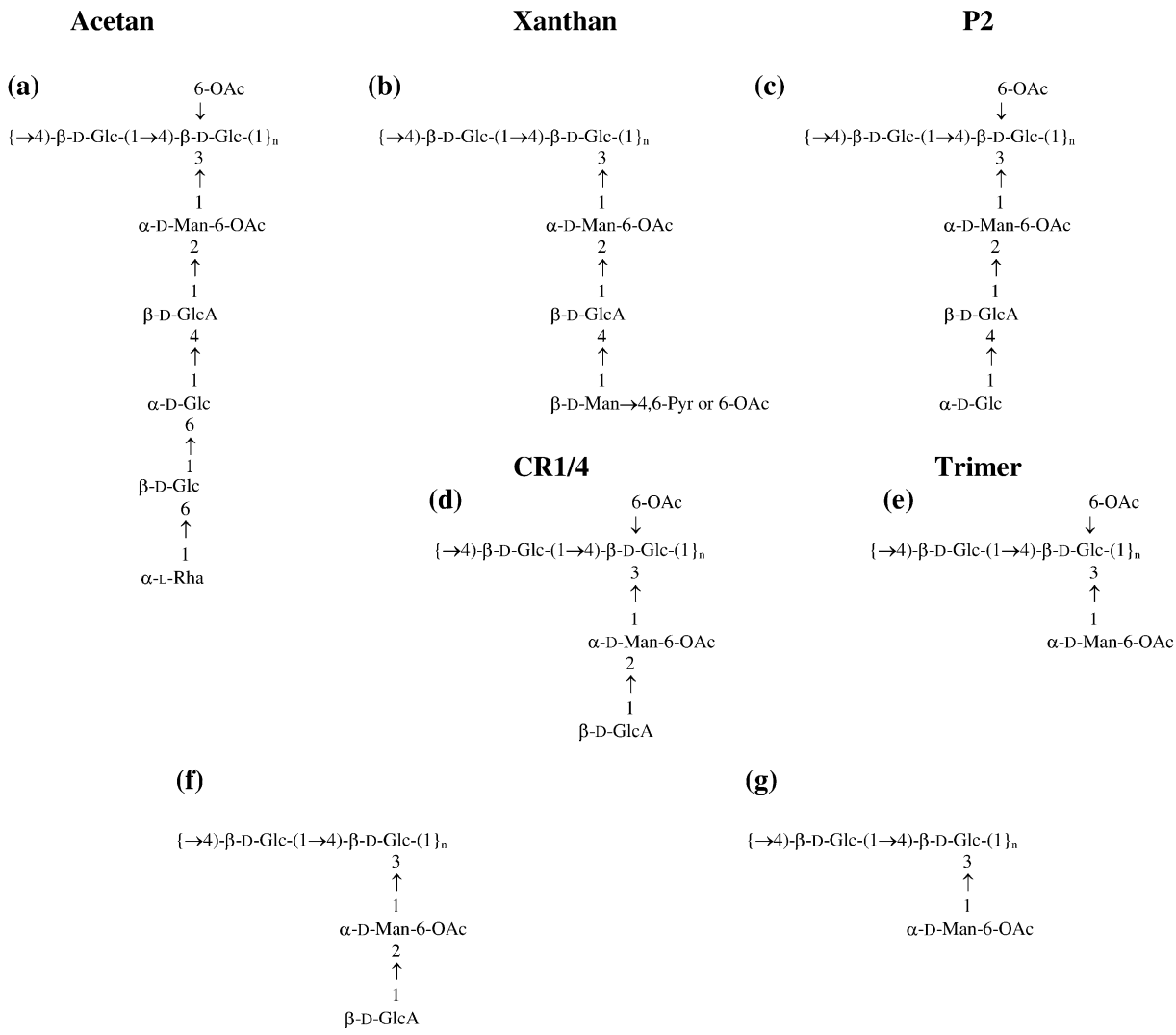

Figure 1. The chemical repeat units of the related bacterial polysaccharides (a) acetan, (b) xanthan, (c) P2, (d) CR1/4 or the acetan tetramer, (e) the acetan trimer, (f) the xanthan tetramer and (g) the xanthan trimer. Ac—acetyl and Pyr—pyruvate substituents. Note the noncarbohydrate substitution is usually nonstoichiometric.

temperature) needed to obtain high quality NMR spectra.

Xanthan gum is unusual in exhibiting synergistic gelation with certain plant galactomannans and glucomannans. Under the experimental conditions for which these mixtures gel, the individual components do not gel. Several recent review articles[14–17] summarise the experimental data on these systems and discuss current models for their gelation. Similar synergistic behaviour has been reported[16] for the family of xanthan polysaccharides with truncated sidechains, for acetan and for the acetan variant CR1/4.

The xanthan and acetan families consist of a cellulosic backbone (β(1→4)-linked D-glucose) solubilised by attachment of a charged sidechain to every second glucose residue (Fig. 1). X-ray diffraction studies[18–20] of oriented fibres prepared from xanthan mixed gels have provided direct evidence for intermolecular binding between xanthan chains and certain galactomannans and glucomannans. Experimental studies of gels suggest that factors destabilising the xanthan helix favour gelation and the formation of new heterotypic junction zones.[15,16] Similar behaviour is observed for mixed gels formed with the polysaccharides acetan[21–23] and CR1/4[24]. Analysis of the X-ray diffraction data for oriented fibres prepared from mixed gels of xanthan–konjac mannan[16,20] or deacetylated acetan–konjac mannan[16,20] suggest that the new junction zones are novel 6-fold mixed helices. Recent studies have confirmed the stereochemical feasibility of these mixed helical structures[25] by modelling of the mixed helix structure formed between deacetylated acetan and konjac mannan. For mixed gels formed between acetan or xanthan and certain galactomannans it has been suggested that the junction zones arise from a co-crystallisation of denatured xanthan or acetan chains with segments of the galactomannan

molecules.[16,18,19] It has been shown that the denatured xanthan chain can approximate to the cellulosic backbone structure,[26] which is stereochemically equivalent to the backbone structures of the galactomannans and glucomannans.

The present study provides further information on the conformation of P2 and new information on the nature of the interactions between P2 and the plant polysaccharides carob or konjac mannan.

2. Materials and methods

The isolation of the *A. xylinum* strain CKE5, inactivation of the *aceP* gene, growth, isolation, purification and characterisation of the P2 polysaccharide is described in detail elsewhere.[2] The purified product is in the sodium salt form and was stored freeze dried. Deacetylated P2 was prepared using the method described by Colquhoun et al.[1] Samples of konjac mannan and carob were, respectively, purchased from Senn Chemicals (Switzerland) and Sigma Chemicals (UK). Samples of acetan were prepared in the sodium ion form and deacetylation achieved as described by Colquhoun et al.[1] Xanthan was purchased from Kelco and prepared and stored as the sodium salt form. Both polysaccharides were stored as a freeze-dried material.

Stock samples of konjac mannan or carob were prepared by adding the powdered material to water at room temperature. Samples in sealed glass tubes were heated to 85 °C and held at this temperature under agitation to disperse the polysaccharides. The hot samples were centrifuged at 5000*g* for 1.5 h to remove insoluble material and then cooled to room temperature. Sample concentrations were determined by evaporation to dry weight. Xanthan, acetan, deacetylated acetan, P2 and deacetylated P2 were prepared in a similar fashion but the centrifugation step was not needed for these samples. Final sample concentrations were determined by evaporation to dry weight. Mixtures were prepared by mixing the sols at room temperature, adding the required diluent, mixing and then heating to the required temperature T_M. These hot mixed sols were then poured into 5 cm Petri dishes used as gel moulds, allowed to cool to room temperature and stored covered overnight.

Gels were characterised using an Instron 3250 mechanical spectrometer in the plate–plate (20 mm diameter) mode, under conditions of oscillatory shear. Optical rotation measurements on polysaccharide sols were made using a JASCO DIP-1000 polarimeter (Hg line at 436 nm).

X-ray diffraction patterns were recorded photographically using a pinhole camera on a microfocus X-ray generator using nickel filtered Cu K_α radiation of wavelength 0.15418 nm. Calibration of the camera used powdered calcite as a reference standard. The camera was flushed with dry helium. Hot sols (1% w/w) of P2, carob or 1:1 mixtures of P2 or xanthan with either carob or konjac mannan were poured onto flat PTFE sheets, allowed to cool to room temperature and partially dried to produce thin films. Thin strips were cut from the films and stretched (100–200%) to produce oriented fibres. The carob fibres were stored for up to 1 year to enhance crystallinity.

3. Results and discussion

Figure 2a shows an X-ray diffraction pattern for P2 indicating that P2 can form a 5-fold helix of pitch 0.92 nm. This is equivalent to the helical structures formed by xanthan, acetan, CR1/4 and the xanthan variants.[23,27,28] The X-ray diffraction patterns obtained for 1:1 mixed deacetylated P2–konjac mannan gels provide direct evidence for intermolecular binding between deacetylated P2 and konjac mannan chains. Note the mixed gel pattern is unique and is not the sum of the patterns for P2 (Fig. 2a) and konjac mannan (Fig. 2d). Analysis of the diffraction pattern shown in Figure 2b identifies the formation of a new 6-fold helical structure in which the axial rise per backbone disaccharide repeat unit is 0.92 nm. This is the same as that seen for mixed gels formed between xanthan[20] or acetan and konjac mannan.[22] Figure 2c shows the equivalent 6-fold helical structure formed for xanthan–konjac mannan mixtures. Given the similarity of the present X-ray patterns and those obtained for deacetylated acetan–konjac mannan mixtures, it is likely that the double helix structure proposed for the deacetylated acetan–konjac mannan system is also valid for the present deacetylated P2–konjac mannan system. However, the patterns obtained for P2–carob 1:1 mixtures were poor when compared with pure carob alone or with those obtained previously with xanthan–carob or xanthan–tara mixtures.[16,19] The suggestion is that the P2–carob interaction is the same as that seen for xanthan–galactomannan mixed gels. Here, a co-crystallisation of the two polysaccharides into a galactomannan-like structure, with the xanthan sidechains on the denatured xanthan chains disrupting the lattice, caused systematic diffraction peak absences in the mixed gel patterns.

Figure 3 shows optical rotation data for P2 and deacetylated P2 sols. The significant transition in optical rotation on heating, with a midpoint transition temperature T_C of ~55 °C supports a previous suggestion,[1] based on changes in the broadening of NMR lines on changing the temperature, that P2 shows an order (helix)–disorder (coil) transition in solution. Deacetylation of P2 is seen to increase slightly the midpoint transition temperature ($T_C \sim 60$ °C), indicating that removal of the acetate substituents stabilises the helical structure. Additionally, the ordered conformation of a polymer, if

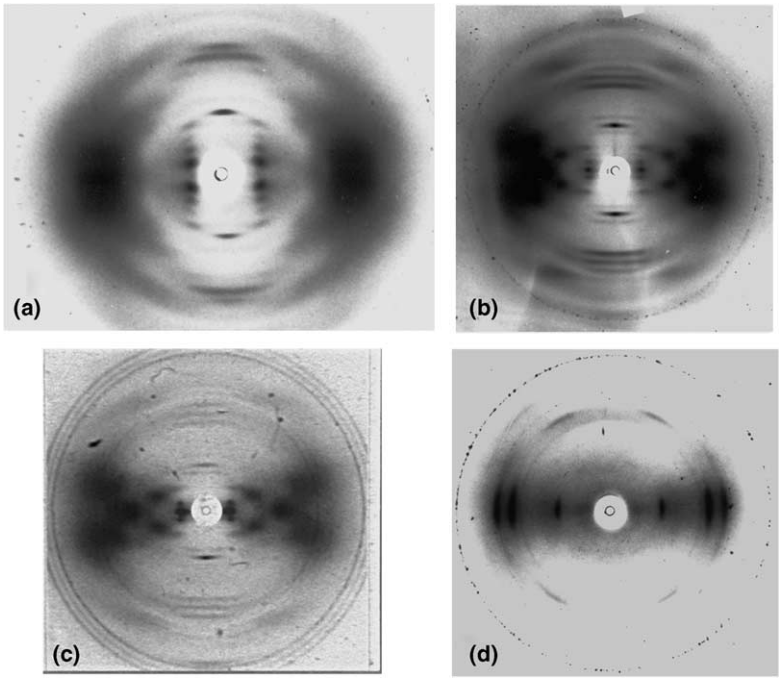

Figure 2. X-ray fibre diffraction patterns for (a) P2 polysaccharide, (b) deacetylated P2–konjac mannan (1:1) mixture, (c) xanthan–konjac mannan (1:1) mixture and (d) konjac mannan. The films were cut into strips and were stretched under controlled relative humidity. The samples stretched (a) 100%, (b) 200% and (c and d) 100%.

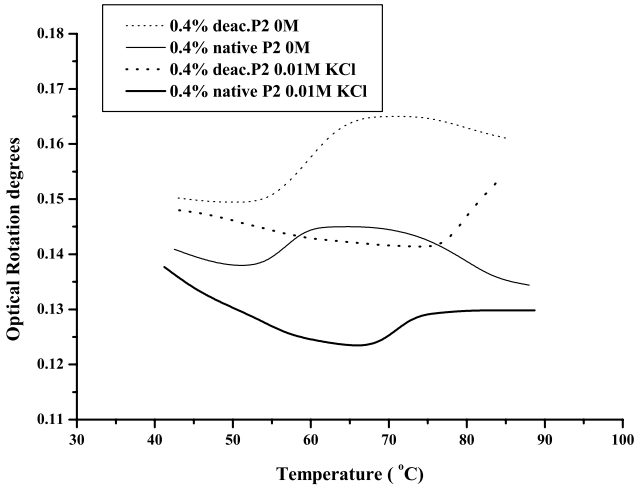

Figure 3. Optical rotation data as function of temperature for native 0.4% P2 in water (—), 0.4% deacetylated P2 in water (···), 0.4% P2 in 0.01 M KCl (—), and 0.4% deacetylated P2 in 0.01 M KCl (---).

charged, will be stabilised by increasing the ionic strength of the solvent phase. The polysaccharide P2, in common with the other biopolymers such as acetan, CR1/4 and xanthan has a polyelectrolytic character due the glucuronic acid residue in the sidechain. As expected (Fig. 3) addition of salt (0.01 M KCl) to P2 stabilises the helix and raises the transition temperature for both the native and deacetylated forms.

Mixtures of P2 (0.5% w/w) with either carob or konjac mannan at 0.5% w/w at a fixed ratio of 1:1, prepared at room temperature, do not form detectable gels, even when left over periods of 24 h. However, there are detectable increases in the viscosity of the mixtures above the expected value for simple mixtures, which could indicate intermolecular interaction. Importantly, when the polysaccharide mixture is prepared at elevated temperature, significant gelation is observed. Figure 4 shows measurements of the dynamic moduli (G^* at 1 Hz) of P2–carob and P2–konjac mannan mixtures as a function of composition. These mixtures were

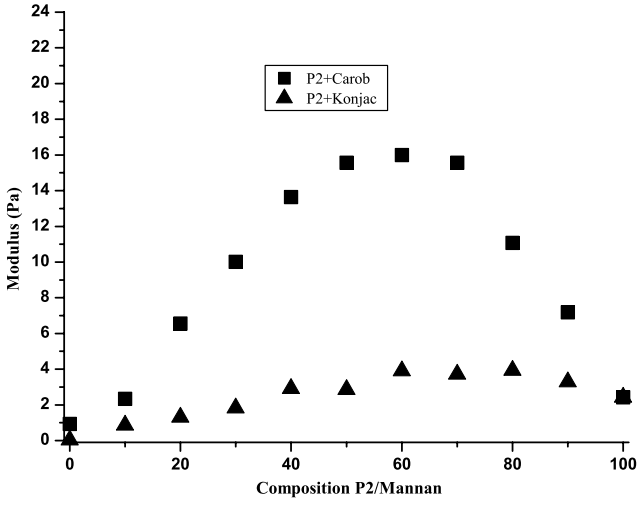

Figure 4. Rheological data for 0.5% P2–carob (■) and 0.5% P2–konjac mannan mixtures (▲).

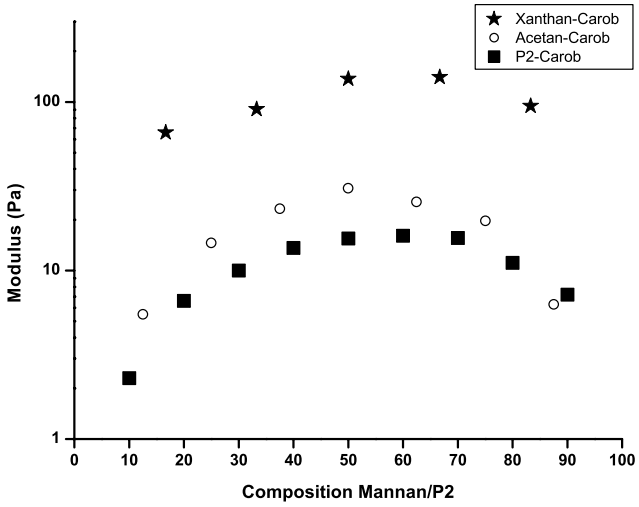

Figure 5. Comparison of the rheological behaviour of 0.5% xanthan–carob (■), acetan–carob (★), and P2–carob (○) mixed gels.

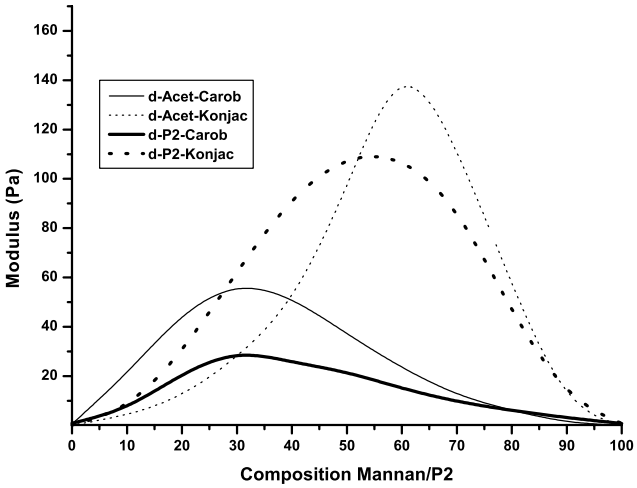

Figure 6. Comparison of the properties of gelled 0.5% mixtures formed between deactylated P2 and carob (—), deacetylated P2 and konjac mannan (---), deactylated acetan and carob (—), and deacetylated acetan and konjac mannan (···).

prepared by mixing aqueous solutions at room temperature, mixing and heating to 85 °C with subsequent retorting into plastic Petri dishes and allowing to stabilise overnight at room temperature. The mixtures with carob form stiffer gels than the P2–konjac mannan mixtures. Both mixtures show maxima in the moduli at about 50% P2. In the case of P2–carob mixtures the behaviour seen is very similar to that reported previously for acetan–carob mixtures[23] and, in both cases, the resultant 'gels' are significantly weaker than those formed with xanthan gum (Fig. 5). The important difference in the chemical architecture between xanthan and P2 is that the former polysaccharide lacks acetyl substitution on the backbone.

With acetan, deacetylation was found to enhance gelation, particularly for acetan–konjac mannan mixtures.[23] Indeed, this effect could be followed for a cold mixed system over a period of days. Heating the mixture enhanced the gelation. A synergistic interaction between deacetylated P2 mixed with carob or konjac mannan, which leads to gelation was also observed (Fig. 6). The increases in dynamic modulus are accompanied by shifts in mixed composition at which the maximum moduli are attained: for deacetylated P2–carob this moves to about 30% and for deacetylated P2–konjac mannan the maximum is at about 60%. Once again this behaviour mirrors that seen with acetan mixed gels.[23] The maximum interaction of the deacetylated ionic polysaccharide (acetan or P2) is much greater with konjac than with carob after heat treatment. The relatively poor interaction between P2 and konjac mannan, when compared to that of deacetylated P2 and the glucomannan, was why X-ray patterns of the latter mixtures were used to investigate the structural origins of the synergistic interaction.

These studies suggest that the heterotypic junction zones arise because of the stereochemical similarity between the cellulosic backbone of xanthan, acetan, CR1/4 or P2 and the backbones of the galactomannans and glucomannans. This is consistent with the observation that for mixtures containing xanthan or acetan, destabilisation of the 5-fold helix by raising the temperature, promotes gelation[18,19,22] whilst increasing the ionic strength has the opposite effect.

Similar temperature controlled studies have been made for P2 mixtures with carob and konjac mannan. Figure 7 shows the effect of mixing temperature (T_M) on the gelation of 0.5% (1:1) P2–carob, deacetylated P2–carob and deacetylated P2–konjac mannan mixtures. The samples were mixed at T_M poured into moulds, cooled to room temperature and allowed to set overnight. For preparation temperatures

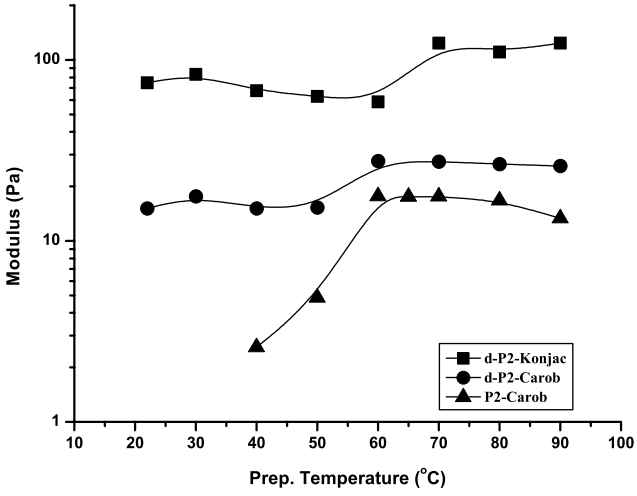

Figure 7. Effect of mixing temperature on final modulus of 1:1 mixtures of P2 with carob and konjac mannan.

$T_M < 40\,°C$ no detectable gelation was observed for P2–carob mixtures. For $T_M > 40\,°C$ the measured modulus increased with increasing T_M, reaching a plateau value for $T_M > 60\,°C$. The transition region mirrors the order–disorder transition seen for P2 by optical rotation. The result demonstrates that denaturation enhances gelation. The data for P2–konjac is not shown on Figure 7 since the maximum modulus of this hot set, no salt system was ~4 Pa. Accordingly, lowering T_m or increasing the ionic strength would result in an even lower modulus. For deacetylated P2–carob mixtures a similar transition is observed, coincident with the order–disorder transition seen in the sol state, but in this case gelation is also observed for mixtures prepared below the midpoint of the order–disorder transition. Similar results are seen for deacetylated P2–konjac mannan mixtures although the transition is seen at slightly higher temperatures. This 'cold-setting' effect, in which interaction, binding and gelation occurs below the helix–coil transition temperature, has also been seen for synergistic interactions of acetan with galactomannans or glucomannans.[23] Clearly the formation of heterotypic junction zones can drive denaturation of the P2 helix. For acetan mixed gels it was shown that gelation of mixtures for which $T_M > T_C$ occurs very rapidly in the order of hours, whereas gelation of mixtures prepared for $T_M < T_C$ can take several days for the modulus to achieve plateau values.[23] Thus the transitions in the moduli values seen in Figure 6 could reflect such time-dependent changes in binding and gelation or ageing effects. The ageing effects for $T_M < T_C$ will be determined by the kinetics of unfolding of the P2 helix driven by the formation of new heterotypic junction zones. Thus the extent of ordering of the P2 helix on mixing affects gelation primarily through determining the rate of association of the chains rather than the level of association attained.

Increasing the ionic strength should stabilise the P2 helix and decrease the level of denaturation at a given mixing temperature. Figure 8 shows the effects of increasing the ionic strength on the gelation of P2–carob, deacetylated P2–carob and deacetylated P2–konjac mannan mixtures. The moduli are, in all cases, found to decrease with increasing ionic strength. However, even at the highest ionic strengths, where the level of denaturation would be expected to be very small, gelation can be observed. These results are consistent with the interpretation of the 'mixing temperature' experiments. The sols are prepared at 85 °C. At low ionic strengths the extent of denaturation should be high and gelation can occur rapidly. Association on cooling will rapidly lock-up the gel network preventing substantial rearrangement with time. Hence the modulus will reflect the degree of denaturation on mixing. However, at higher ionic strengths the level of denaturation on mixing will be smaller but gelation will occur more slowly.

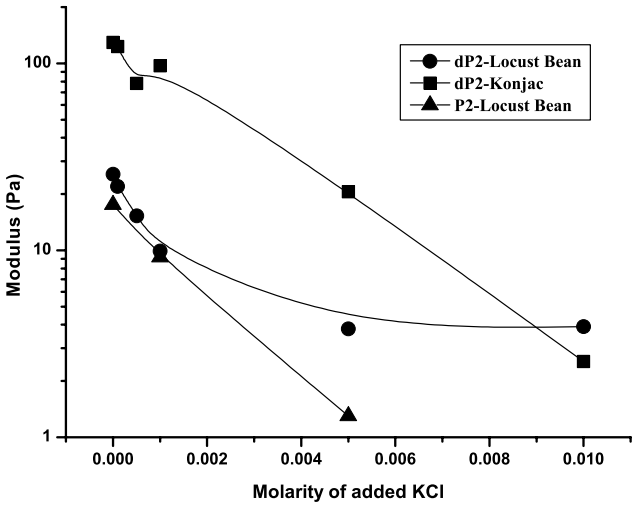

Figure 8. Effect of ionic strength on the gelation of mixtures of P2 with carob and konjac mannan.

Hence the final moduli values will be higher than might be expected for the level of denaturation on mixing, since the slowly forming network can accommodate further association due to additional unfolding of the P2 helix with time during cooling.

What is at first sight surprising is that deacetylation, which stabilises the P2 helix, should substantially enhance gelation, particularly for P2–konjac mannan mixtures. This effect of deacetylation is similar to that seen for acetan and CR1/4, but unlike that seen for xanthan.[15,16] Although some strains of xanthan can contain acetyl groups on the terminal mannose residue of the sidechain,[29] most samples of xanthan are partially acetylated on the mannose residue adjacent to the backbone. Studies on native and deacetylated xanthan clearly show that deacetylation destabilises the helix and enhances gelation. P2 in common with acetan and CR1/4 contains two sites of acetylation: acetyl groups on the sidechain mannose residue as for xanthan, plus an additional substituent at C-6 on the (1→3,4) backbone glucose residue. Deacetylation of P2, as with acetan and CR1/4 leads to a slight increase in the stability of the helix. Clearly the stabilising effect of the sidechain acetate is more than offset by the destabilising effect of the acetate substituent on the backbone. If this substituent inhibits formation of the P2 helix then it is understandable that it would inhibit formation of the mixed P2–konjac mannan helix. The modelling of the X-ray patterns for deacetylated acetan–konjac mannan confirm that acetylation at C-6 interferes with hydrogen bonding between the acetan backbone and the glucomannan. The similarity of the P2 and acetan structures supports this explanation for the action of the backbone acetate in P2. This steric effect is distinct from the effects of the sidechain acetate, which will act through moderating the level of denaturation under given mixing conditions. If the

suggested explanation for the formation of P2–galactomannan gels is correct, any steric effects would be dominated by the length of the sidechains rather than the presence of acetate on the backbone. Hence the effects of deacetylation of P2 would be more significant for the glucomannan mixed gels than for the galactomannan mixed gels.

4. Conclusion

Synergistic gelation has been observed in mixtures of the bacterial polysaccharide P2 with carob and konjac mannan. Deacetylation has been found to enhance gelation, particularly for the P2–konjac mannan mixtures. X-ray diffraction data for oriented fibres of P2 demonstrate that it forms a 5-fold helical structure of pitch 4.7 nm. The X-ray fibre diffraction patterns obtained for the mixed gels show evidence for intermolecular binding between P2 and carob or konjac mannan. For the P2–carob gels it is suggested that the junction zones arise due to co-crystallisation of denatured P2 and carob into a partially disrupted carob lattice. For P2–konjac mannan gels the junction zones have been shown to be mixed chain 6-fold helices of pitch 5.54 nm. The use of genetically modified polysaccharides provides a basis for selectively modifying the structures of polysaccharides in order to determine structure–function relationships.

Acknowledgements

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