Synergistic Interactions of Acetan with Carob or Konjac Mannan

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ABSTRACT: New experimental evidence has been obtained which demonstrates synergistic interaction of mixtures of acetan with carob or konjac mannan. These binary mixtures have been shown to form thermoreversible gels under conditions for which the individual components do not gel. The rheological properties of acetan−carob and acetan−konjac mannan mixtures have been studied at various concentrations above and below the threshold concentration for gelation. In dilute solutions, the synergisms manifested themselves as an enhancement of the viscosity, whereas at higher concentrations gelation occurred. The synergistic effects were much weaker for acetan−konjac mannan mixtures than for acetan−carob mixtures. Deacetylation of acetan enhanced the synergistic interactions, particularly for acetan−konjac mannan mixtures, and also altered the polymer ratio at which the maximum interactions were observed. The influence of the stability of the acetan helix on the gelation of the binary mixtures was investigated by varying the mixing (preparation) temperature and the level of salt added to the mixtures. These studies suggest that factors destabilizing the acetan helix favor gelation.

Introduction

Only a few binary mixtures of polysaccharides exhibit synergistic interactions which lead to or enhance gelation.[1−4] Many such blends are important as industrial gelling agents. In addition, there is an academic interest in trying to identify the molecular mechanism(s) of gelation. Several recent review articles summarize the experimental data on these systems and discuss current models for their gelation.[1−4] Most of the known synergisms involve mixtures of an anionic "helix-forming" polysaccharide (e.g., xanthan, kappa carrageenan, agarose, and furcellaran) with certain "nongelling" plant galactomannans (e.g., carob and tara) or glucomannans (e.g., konjac mannan).

Galactomannans are neutral polysaccharides consisting of a mannan $\beta(1 \rightarrow 4)$ linked D-mannose ribbonlike backbone solubilized by partial substitution with $\alpha(1 \rightarrow 6)$ linked D-galactose side chains.[5] The galactomannans are characterized by different average levels of substitution and different distributions of side chains along the backbones. Partial substitution can result in blocks of completely unsubstituted backbone or unsubstituted faces of the backbone. These blocks are believed to be of importance in determining self-association of galactomannans and also in their interactions with other polysaccharides.[5−10] Glucomannans contain essentially a backbone of irregularly distributed $\beta(1 \rightarrow 4)$ linked D-glucose and D-mannose solubilized by partial O-acetyl substitution.[11] The backbone is an extended ribbon, stereochemically very similar to the mannan backbone of the galactomannans.

The earliest molecular models for the gelation of these polysaccharide−polysaccharide mixtures suggested an intermolecular binding between the helix of the helix-forming polysaccharide and the glucomannan backbone, or between the helix and unsubstituted blocks or faces of the galactomannan backbone.[5−10,12] The latter effect was used to explain why highly substituted galactomannans (e.g., guar) do not participate in gelation, whereas less substituted galactomannans (e.g., carob and tara) do form mixed gels, and why galactomannans with similar levels of substitution, but with different distributions of substituents, show different affinities for forming mixed gels.

Gel-forming mixtures containing galactomannans or glucomannans can be divided into two broad types. In the first type the helix-forming polysaccharides are themselves gelling polysaccharides (e.g., agarose, κ-carrageenan, and furcellaran). Addition of the galactomannan or glucomannan modifies and/or enhances gelation or leads to gelation at lower concentrations.[1−4] For these mixtures, it has proved impossible to find direct evidence for stereochemically compatible specific intermolecular binding between the two polysaccharides.[13−15] It has been suggested that either such binding does not occur or that any binding is transient, or that there exists a nonspecific binding of the galactomannan or glucomannan to aggregates of the anionic polysaccharide.[14] The bulk of the experimental evidence summarized in recent reviews[1−4] and supplemented by newer theoretical[16] and experimental[17−20] studies has been taken to suggest that the mixed gels contain junction zones in which segments of the galactomannan or glucomannan chains adsorb to the surfaces of aggregates of the anionic polysaccharide helices.

In the second type of mixture, the anionic polysaccharide (usually xanthan) is nongelling. Xanthan consists of a cellulosic backbone ($\beta(1 \rightarrow 4)$ linked D-glucose) solubilized by an attachment of a charged trisaccharide side chain to every second glucose residue (Figure 1).[21−23] X-ray diffraction studies of fibers prepared from xanthan-mixed gels have provided direct evidence for intermolecular binding between xanthan and certain galactomannans or glucomannans.[14,24,25] Experimental studies of these gels suggest that factors destabilizing the xanthan helix favor gelation and the formation of new heterotypic junction zones.[2,3] Qualitative analysis of the X-ray diffraction data suggests that these new junction zones may involve a novel "mixed" helix for xanthan−konjac mannan interactions[3] and possibly cocrystallization of denatured xanthan with segments

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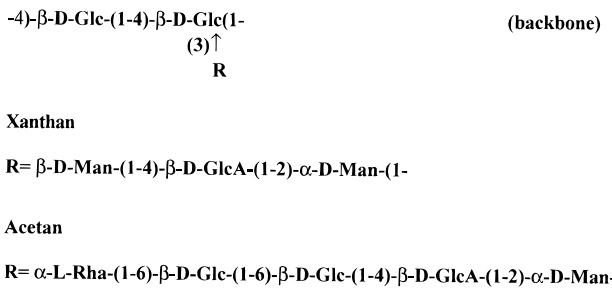

Figure 1. Chemical repeat units for xanthan and acetan. The noncarbohydrate decoration of the repeat units has been omitted.

of tara or carob molecules for these xanthan−galactomannan interactions.[3] The latter model provides a physical explanation for the sensitivity of gelation to the level of galactose substitution of the galactomannan, based on the ability to incorporate the extended xanthan side chain into the galactomannan crystal lattice.[3] The need to destabilize the xanthan helix is consistent with the stereochemically acceptable model for intermolecular binding. It has been shown that denatured xanthan can approximate to the cellulosic backbone conformation compatible with the backbone structures of the galactomannans and the glucomannans.[26]

Further information can be obtained by investigating possible synergisms between galactomannans or glucomannans with "xanthan-like" polysaccharides. Acetan is an anionic heteropolysaccharide secreted by the bacterium *Acetobacter xylinum*.[27] It is structurally similar to xanthan (Figure 1) but contains a pentasaccharide side chain.[27−30] Both xanthan and acetan contain noncarbohydrate substituents.[23,29,30] In both cases, the (1 → 2) D-mannose residue is partially O-acetylated. Acetan shows no equivalent to the acetyl or pyruvate substitution of xanthan on the terminal mannose residue. However, acetan is partially acetylated on the backbone.[29,30] It adopts a xanthan-like 5-fold helical structure, stabilized by the addition of salt and favored at reduced temperature, and exhibits similar rheological properties to xanthan.[27,31,32] Deacetylation of acetan slightly raises the helix-coil transition temperature (T_c) while deacetylation of xanthan lowers the value of T_c, thus destabilizing the helix.[33] This suggests that whereas acetyl substitution on the mannose residue of acetan stabilizes the helix this effect is offset by the destabilizing effect of acetyl substitution on the backbone. Preliminary studies[33] have suggested that acetan only exhibits synergistic interactions with galactomannans or glucomannans when the polymer has been deacetylated. Isolation and purification of larger quantities of acetan have permitted a more complete and detailed investigation of potential synergistic interactions of acetan−carob and acetan−konjac mannan mixtures and these studies are reported in this article.

Experimental Section

Native acetan was prepared by batch fermentation of *A. xylinum* according to the procedures described by MacCormick and co-workers.[34] The polymer was further purified by selective precipitation with hexadecyltrimethylammonium bromide (CTAB) using the methods described by Scott[35] and then freeze-dried. This preparative procedure yields the sodium salt form of the polysaccharide. Deacetylation of acetan was achieved by alkali treatment. A 0.25% (w/v) solution was prepared in water by heating an aqueous dispersion to 90 °C. The solution was then cooled to 1 °C and titrated to pH 12.5 using 0.1 M NaOH and left stirring at this temperature for 16 h. Previous studies[30] have shown that these conditions result in complete deesterification. The pH of this sample was then adjusted to pH 8.5 by the addition of 0.1 M HCl, and the solution dialyzed against distilled water before freeze-drying.

The molecular weight of native acetan was determined by light scattering (Fica 5000, 436 nm) and found to be 1.4×10^6 Da. This value compares favorably with, but is slightly lower than, those of Smidsrød et al.[36,37] However, different growth conditions and purification procedures are known to affect polysaccharide properties such as molecular weight. The native and deacetylated polysaccharides were compared by size exclusion chromatography using a TSK G5000PWXL column and Gilson refractive index detector. The peaks of the respective samples eluted coincidentally, inferring that negligible depolymerization of the polysaccharide had occurred during deesterification. Solutions of acetan or deacetylated acetan were prepared by dispersing the freeze-dried polymer in distilled water at 90 °C in a sealed tube with occasional shaking.

Carob gum was purchased from Sigma Chemicals (Dorset, U.K.). The mannose:galactose (M:G) ratio was determined by optical rotation measurements[1] and found to be 3.7:1. Samples of konjac mannan were bought from Senn Chemicals AG (Dielsdorf, Switzerland). Both gums were used without further purification. Solutions of the gums were prepared by heating aqueous dispersions at 90 °C in sealed tubes with occasional shaking. These samples were centrifuged (4500 g for 1.5 h) to remove insoluble matter. The concentration of the supernatant was determined by evaporation of a small fraction of the sample to dry weight, and then the remainder of the stock sample was used for preparation of mixtures. Salt was added to the acetan, galactomannan, or glucomannan solutions as appropriate before mixing the final polymer solutions. The acetan−carob or acetan−konjac mannan mixtures were prepared at room temperature, which is below the helix-coil transition temperature for acetan. Samples of the mixtures were either poured directly into cylindrical molds (5-cm diameter), or taken through an appropriate heating cycle, and then poured into molds and cooled to room temperature. Samples were covered and stored overnight at room temperature. Time-dependent studies of gelation were made after preparing the mixtures in situ in the rheometer. The exposed surfaces were covered with silicone fluid in order to prevent the samples from drying out.

Rheological measurements were made using an Instron 3250 mechanical spectrometer. For gelled samples the molds were glued to the lower platen. All measurements on gelled samples were made using a parallel plate configuration under oscillatory shear at a strain of 0.1 which is within the linear viscoelastic region. Solutions were monitored in cone and plate mode under conditions of steady shear.

Results and Discussion

Figure 2 shows that deacetylation of acetan alters the rheology of acetan solutions. The high viscosity and marked shear-thinning behavior suggests that, at the concentrations of 1.0% and 0.5% (w/v), both native and deacetylated acetan adopt the ordered helical structure. This is consistent with previous studies[33] which also showed that deacetylation stabilized the acetan helix. The high shear rate viscosities are similar for the native and the deacetylated acetans, providing further evidence that no significant depolymerization occurred on alkali treatment as demonstrated by size exclusion chromatography. The major differences in behavior are seen for the low shear rate, with deesterification reducing the low shear rate viscosity values. For native acetan, the viscosity continues to increase with decreasing shear rate. Similar rheological behavior for the related polysaccharide xanthan is explained on the assumption of

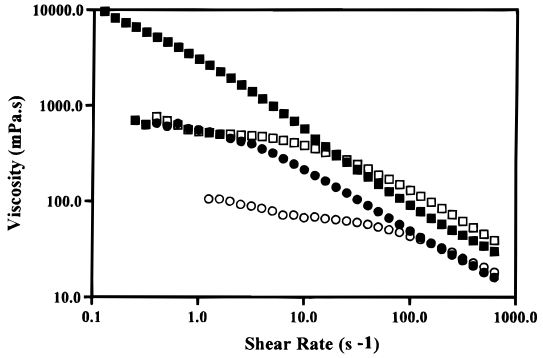

Figure 2. Comparison of viscosity-shear rate dependence for native and deacetylated acetan. Data are shown for native acetan at 1.0% (■) and 0.5% (●) concentrations and for deacetylated acetan at 1.0% (□) and 0.5% (○) concentrations.

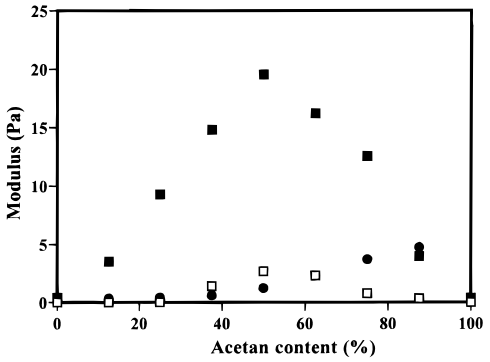

Figure 3. Shear modulus data for native acetan−carob and native acetan−konjac mannan mixed gels. Data for acetan−carob mixtures at 0.5% (■) and 0.1% (□) polymer concentrations. The data for acetan−konjac mannan gels (●) is for a total polymer concentration of 0.5%. The data were collected at a frequency of 1 Hz.

intermolecular association.[38] Thus, for acetan it seems reasonable to suggest that deesterification reduces intermolecular association.

Preliminary studies on dilute mixtures of native acetan with carob or konjac mannan failed to detect synergistic interactions on the basis of viscosity measurements.[33] For most of these studies, the samples were mixed, stored, and measured at room temperature. In previous studies[14,24,39] on xanthan mixed gels, it has been reported that heating and mixing the polysaccharides above the T_c enhances the intermolecular interaction and gelation. Thus, the effects observed for native acetan in these preliminary experiments may have been small and difficult to detect. In the preliminary studies on acetan mixtures,[33] it was reported that heating (to 60 °C) and cooling to room temperature did not lead to detectable synergistic effects. However, it has since been shown[30] that these early samples of acetan were "contaminated" with a low level of excess NaCl, which would have raised the value of T_c. Thus, the mixtures may not have been heated to a sufficiently high temperature to enhance the synergistic interaction.

Figure 3 shows that native acetan exhibits synergistic interactions with both carob and konjac mannan. For these studies, the mixtures were heated to 90 °C, well-above the reported T_c value for native acetan,[30] before cooling, storing, and measuring. At total polymer concentrations of 0.5% and 0.1% (w/v) native acetan−carob mixtures gel and show a maximum in the modulus at a polymer ratio of acetan:carob of 1:1. This is similar to the behavior observed for xanthan−carob mixtures,[39]

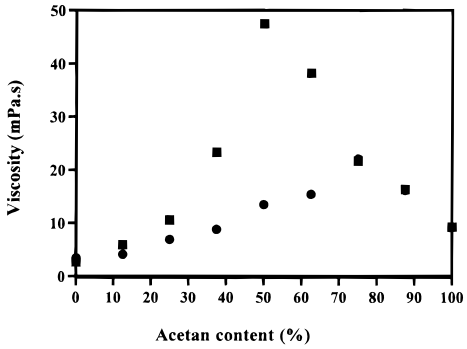

Figure 4. Viscosity data for dilute acetan−carob and acetan−konjac mannan mixtures. Data collected for native acetan−carob mixtures native acetan−konjac mannan mixtures at a total polymer concentration of 0.05% (■) and data for native acetan−konjac mannan mixtures at a total polymer concentration of 0.05% (●).

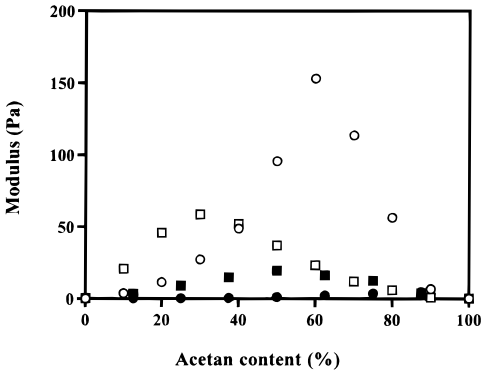

Figure 5. Effect of deacetylation on the gelation of acetan−carob and acetan−konjac mannan mixtures (0.5% polymer concentration). Data are presented for native acetan−carob (■), deacetylated acetan−carob (□), native acetan−konjac mannan (●), and deacetylated acetan−konjac mannan (○) gels. Experimental data was collected at a frequency of 1 Hz.

although the modulus values are considerably lower for the acetan mixed gels. At the lower polymer concentration of 0.05% (w/v), gelation does not occur, but an enhancement of viscosity, maximal at an acetan−carob ratio of 1:1, is still observed (Figure 4). The interaction between native acetan and konjac mannan is much weaker. At 0.5% (w/v), gelation is observed (Figure 3), but the gels are weak with a maximum modulus value at an acetan−konjac mannan ratio of 7:1. For total polymer concentrations of 0.05% (w/v), the mixtures do not gel but do show an equivalent enhancement in viscosity (Figure 4). Thus, the differences in gel stiffness are most likely due to the influence of different aspects of xanthan/acetan structure on the formation of new heterotypic junction zones. The principal differences between xanthan and acetan are the mass per unit length, the charge density, the length and composition of the side chain, and the acetylation of the acetan backbone. At present, it is not possible to distinguish the relative effects of these variables on the formation of mixed gels.

Although it is not possible to selectively remove acetyl groups from either the side chain or the backbone, it is possible to study the effect of complete deacetylation on gelation. Figure 5 compares mixed gels prepared with native or deacetylated acetan. It can be seen that deesterification enhances gelation for acetan−carob and acetan−konjac mannan mixtures. For deacetylated acetan−carob mixtures, the gels are stiffer at all

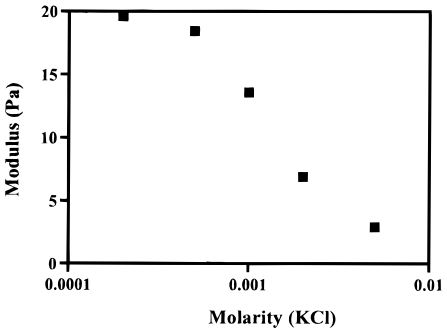

Figure 6. Effect of added salt on the gelation of 0.5% 1:1 native acetan−carob mixtures. Data collected at a frequency of 1 Hz.

polymer ratios and the maximum modulus value occurs at the acetan−carob ratio of 3:7 instead of 1:1 as seen for the native acetan mixed gels. The effect of deesterification on acetan−konjac mannan mixtures is much more pronounced. There is a substantial increase in gel stiffness and the maximum modulus value occurs at an acetan−konjac mannan ratio of 6:4 rather than 7:1 as seen for the native acetan mixtures (Figure 3). These effects parallel those observed for xanthan mixed gels.[2,3] For xanthan mixed gels, the effects of substituents on gelation can be explained in terms of their effects on helix stability. Deacetylation, which destabilizes the xanthan helix, enhances gelation whereas removal of pyruvate, which stabilizes the helix by reducing the charge density, inhibits gelation.[40−43] Similarly, the xanthan tetramer, which lacks the terminal mannose and hence pyruvate substituents, has a more stable helix and gelation with carob or konjac mannan is inhibited.[2] The effect of deacetylating acetan is more difficult to explain. The acetate substituent on the side chain should stabilize the helix as with xanthan and its removal should lower the value of T_c. However, deacetylation of acetan slightly increases the T_c, suggesting that the acetyl substituent on the backbone destabilizes the helix and that this effect is dominant. Thus, the removal of the acetyl substituent on the backbone must play a special role in enhancing the formation of heterotypic junction zones.

Effect of Order/Disorder of Acetan. The effect of helix stability on mixed gel formation can be probed by altering the preparation conditions.[39] For example, addition of salt (KCl) will screen the charged polysaccharide (acetan), raising T_c and sharpening the transition.[33] Hence, with increasing levels of added salt the level of denaturation at the mixing temperature of 90 °C will decrease sigmoidally. If the level of denaturation on mixing determines the amount of heterotypic junction zones formed, then the addition of salt should progressively inhibit gelation as observed for xanthan−carob mixtures.[39] The variation of modulus with log of ionic strength (added salt) should follow a sigmoidal curve. This is indeed observed for 0.5% 1:1 native acetan−carob gels (Figure 6) and for 0.5% 3:7 deacetylated acetan−carob gels (Figure 7). Similar results are observed for 0.5% 6:4 deacetylated acetan−konjac mannan gels (Figure 7) which support the proposal that denaturation of the acetan helix prior to mixing favors gelation. These results therefore favor a model for heterotypic junction zones based on the stereochemical compatibility between the cellulosic backbone of acetan and the backbones of the galactomannans and glucomannans.

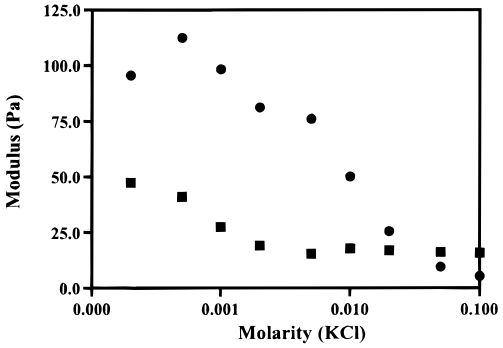

Figure 7. Effect of added salt on the gelation of 0.5% 3:7 deacetylated acetan−carob mixtures (■) and 0.5% 6:4 deacetylated acetan−konjac mannan mixtures (●). Data collected at a frequency of 1 Hz.

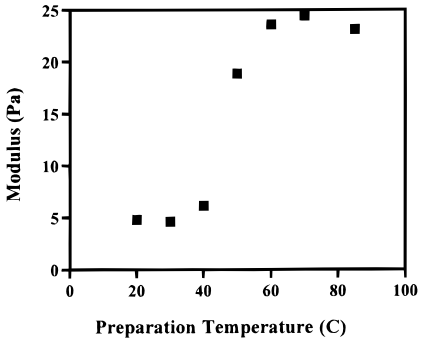

Figure 8. Effect of preparation temperature (mixing temperature) on the elastic modulus of 0.5% 1:1 native acetan−carob mixed gels. Data measured at a frequency of 1 Hz.

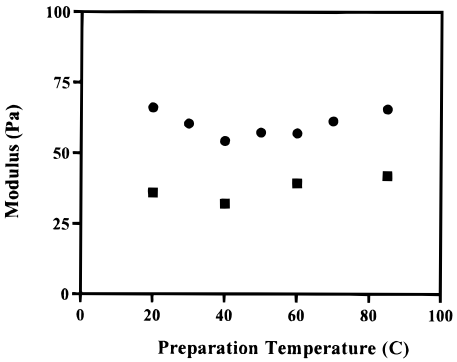

Figure 9. Effect of preparation temperature (mixing temperature) on the elastic modulus of 0.5% 4:6 deacetylated acetan−carob (■) and 0.5% 4:6 deacetylated acetan−konjac mannan (●) mixed gels. Data measured at a frequency of 1 Hz.

An alternative experimental approach to studying the effects of helix stability on gelation is to fix T_c but vary the temperature at which the components are mixed before cooling and testing for gelation. This approach has been used previously to study xanthan mixed gels.[39] In this case, the measured modulus should increase sigmoidally, following the degree of denaturation and thus mapping onto the helix−coil transition curve. This is observed for 0.5% 1:1 native acetan−carob gels (Figure 8). However, similar behavior was not observed for 0.5% 4:6 deacetylated acetan−carob or 0.5% 4:6 deacetylated acetan−konjac mannan gels (Figure 9). This result is surprising in view of the data presented in Figures 7 and 8 where it is demonstrated that varying the salt content or varying the preparation temperature are apparently equivalent methods for sampling different levels of helix denaturation. The data in Figure 9

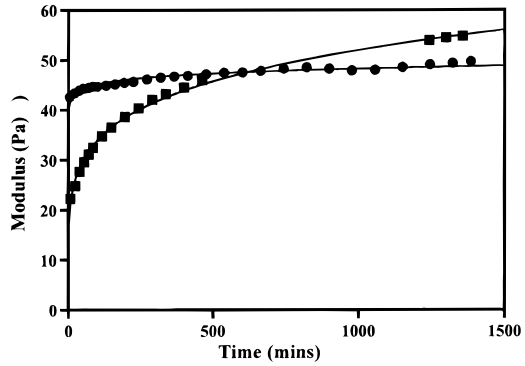

Figure 10. Development of the elastic modulus of deacetylated acetan−konjac mannan mixtures (0.5% total polymer concentration, 4:6 acetan−konjac mannan) with time after mixing. Data obtained for samples mixed at 70 °C (●) and 20 °C (■). Measurements were made at a frequency of 1 Hz.

imply that for these samples the modulus of the gels is independent of the level of denaturation of the acetan helix on mixing and appears to contradict the results shown in Figure 7.

The assumptions in the analysis of the above data are that the level of denaturation of the helix on mixing remains constant during preparation and storage and is the sole determinant of the level of formation of heterotypic junction zones. It has been suggested[39,44] that the formation of heterotypic junction zones can perturb the equilibrium state of the helix−coil transition. If the rate of formation of the heterotypic linkages is fast compared to the time scale for preparation and measurement of the gels, then the modulus will not be dependent on the initial level of helix denaturation and will not exhibit the expected sigmoidal behavior. If the rate of formation of heterotypic linkages is slow compared to the preparation time, then the expected sigmoidal behavior will be observed. The rate of formation of heterotypic linkages may itself vary with parameters such as salt content or preparation temperature. Thus, this could explain the apparent discrepancy between the results presented in Figures 7−9. This hypothesis can be tested by probing the time dependence of the formation of the mixed gels. Figure 10 shows data for 0.5% 4:6 deacetylated acetan−konjac mannan mixtures. The data compare the rates of gelation for mixtures prepared initially by mixing at 70 and 20 °C: above and below the value of T_c for deacetylated acetan. For samples mixed at 70 °C and then rapidly quenched, the modulus of the gels is essentially independent of preparation time up to at least 1500 min. For mixtures prepared at 20 °C, where the level of denaturation of the acetan helix is small, the earliest measurements of the modulus are low compared with the values for the 70 °C mixtures. This is what would be expected if the level of denaturation directly determines the modulus value. For the 20 °C mixtures, the modulus increases with time, approaching a plateau value slightly in excess of the values observed for the 70 °C mixtures. The modulus values for the 20 and 70 °C mixtures become comparable after about 420 min, which is short compared with the normal preparation time of 16 h (960 min). Thus, the absence of a sigmoidal transition in Figure 9 is due to the time-dependent denaturation of the acetan helix, driven by the formation of heterotypic linkages or junction zones. For the 70 °C mixtures, which were rapidly quenched, the high level of helix denaturation on mixing will allow binding sites between the two polymers to nucleate and grow simultaneously, thus freezing in the structure of the gel before it reaches its equilibrium value. For the 20 °C mixtures, the progressive denaturation will permit molecular rearrangement of the structure and a closer approach to an equilibrium structure, resulting in a higher modulus value.

Effects of Acetyl Groups. It is possible to use models for xanthan mixed gels to briefly attempt to analyze the behavior of acetan mixed gels. For xanthan−galactomannan mixed gels it has been suggested[3] that the two polymers may cocrystallize in a "galactomannan-type" structure. Whereas acetylation of the glucomannan backbone inhibits molecular packing in the mannan lattice,[45] the wider interplanar spacing in the galactomannan lattice, determined by the level of side chain substitution and water content,[46] should render the system insensitive to acetylation of the backbone as for acetan. Hence, it would be expected that acetylation of the acetan would play a minor role in cocrystallization compared to the effect of accommodating the much larger side chain into the interplanar space. For a given M:G ratio, the larger side chain of acetan would be harder to accommodate than that of xanthan. Thus, the gels formed with acetan should be, *and are*, weaker than those obtained with xanthan. Furthermore, deacetylation might, as is actually observed, have only a small effect on association and gelation. For acetan−konjac mannan mixtures, the effect of deacetylation is quite pronounced. The evidence here is that acetylation of the acetan backbone destabilizes the acetan helix. Hence, acetylation on the backbone might be expected to have a similar effect on the formation of a mixed helical structure. Such a mixed helix is a plausible model for intermolecular binding for xanthan−konjac mannan gelling mixtures.[3] In this case, deacetylation might then be expected to have a significant effect on mixed gel formation of acetan with konjac mannan.

Conclusions

Acetan polysaccharide shows synergistic interactions when mixed with carob or konjac mannan. In dilute solution, these synergisms manifest themselves as an enhancement of viscosity. At higher concentrations, the mixtures form thermoreversible gels. The gels formed are weaker than equivalent xanthan−carob or xanthan−konjac mannan gels. For native acetan, the interaction with konjac mannan is much weaker than that observed with carob. Deacetylation of acetan enhances the synergism, and this is most evident for the acetan−konjac mannan mixtures. Experimental studies support the general conclusion that destabilization of the acetan helix promotes gelation and that stabilization of the acetan helix inhibits gelation, provided that the special effects of backbone substitution with acetate and the rate of interaction (gelation) are taken in consideration. The data are consistent with intermolecular binding between the two polysaccharides (acetan−carob or acetan−konjac mannan), but direct evidence for such binding still needs to be obtained.

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