Carbohydrate Polymers 5 (1985) 223–236

A Comparison of the Reactivity of Alginate and Pectate Esters with Gelatin

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(Received: 29 September 1984)

SUMMARY

Aqueous solutions of highly esterified propylene glycol alginate and gelatin interact rapidly in mildly alkaline conditions to form a gel with a very high melting point. The interaction involves the formation of amide bonds between the ester and uncharged amino groups on the protein.

Neither high-methoxyl pectin nor highly esterified propylene glycol pectate formed thermostable gels with gelatin, and the lack of reactivity was not due to differences between pectate and alginate in viscosity, rate of depolymerisation or rate of saponification. Pectate esters will react, however, with low molecular weight diamines in anhydrous conditions.

It is suggested that the different reactivity of the uronides in water reflects differences in the geometries of their glycosidic links between monomers, and that in alginate it is the mannuronic residues that are involved in these reactions.

INTRODUCTION

The introduction of new polysaccharides in the near future would appear to be limited primarily by the high cost involved in proving their safety as food additives (Sandford, 1979).

Thus work directed towards extending the application of the range of polysaccharides currently in use would appear to be particularly pertinent.

The study of the interactions of a polysaccharide with other polysaccharides, or with other macromolecules such as proteins, has already

Carbohydrate Polymers 0144-8617/85/$03.30

proved useful in this context, but is far from being fully explored. Widespread use has been made of synergistic interactions between different polysaccharides. For example, the interaction between locust bean gum, a non-gelling polysaccharide, and carrageenan results in gels forming at concentrations far below those required for gelation of the carrageenan alone.

Within the area of polysaccharide–protein interactions attention has been focused predominantly on non-specific ionic interactions (Stainsby, 1980; Imeson, 1984). Examples of the use of these interactions include:

(i) the extraction of proteins by precipitation with polysaccharides as seen in the precipitation of proteins from soybean whey with sodium alginate (Shank, 1968);
(ii) the synthesis of polysaccharide–protein gels as seen in the formation of an alginate–gelatin gel which was stable to temperatures as high as 80°C (Muchin *et al.*, 1976).

However, an interaction between propylene glycol alginate and gelatin, under slightly alkaline conditions, which results in the formation of a gel with a melting point in excess of 100°C, has been described (Agfa AG, 1964). The predominant interaction in this gel would appear to have the thermal stability associated more with covalent bonds than with ionic bonds. Although the patent describing this interaction was concerned with the development of a stable and insoluble photographic film, it seemed possible that the same reaction might be applied to the formation of texturised protein foods, providing the pH of the product was then reduced to an acceptable level. Patents describing the use of this interaction for

(i) binding fish protein concentrate into a product resembling fish muscle tissue (Unilever, 1976), and
(ii) forming sausage casings (or strings for sports racquets) by interacting propylene glycol alginate with pastes, slurries or dispersions of collagen (Collagen Products Ltd, 1976),

confirm the feasibility of this approach.

In the present work, the nature of the interaction with alginate ester is examined and attempts are made to obtain a similar interaction between pectate esters and gelatin, with the aim of diversifying the potential uses of these polyuronides.

MATERIALS AND METHODS

Materials

Propylene glycol alginate (Manucol Ester, E/RE; DE 80%; M/G ratio not known) was obtained from Alginate Industries Ltd, London, UK. High-methoxyl pectin (citrus, type 104; DE 68%) and sodium pectate (citrus) were obtained from H. P. Bulmer Ltd, Hereford, UK. The gelatin was a commercial sample of high viscosity and low isoelectric point, manufactured from ox-hide for photographic use. Other chemicals – 1,6-diaminohexane, 1,2-diaminoethane, propylene oxide, acetic anhydride and sodium acetate – were all Analar grade obtained from BDH Chemicals Ltd, Poole, UK. Alginates of high and low guluronate content (R3534, M/G 34/66; R3535, M/G 56/44) were obtained from Alginate Industries Ltd.

Gel formation

Quick-set method
Equal volumes of 2% aqueous propylene glycol alginate (PGA) and 10% aqueous gelatin were heated to 50°C, and then adjusted to pH 9·6 by the addition of 1 M NaOH, immediately prior to mixing. A strong gel formed within 4–5 s of mixing.

Dialysis method
Equal volumes of 2% aqueous PGA and 10% aqueous gelatin were heated to 50°C, adjusted to pH 6·5 with 1 M NaOH, and then mixed. No gel forms at this stage. The solution was poured into dialysis sacs (approx. 40 ml/sac) made from $\frac{18}{32}$ in Visking tubing, and the pH raised by immersing the sacs for 24 h in 3 litres of 0·032 M Na_2CO_3 and 0·104 M $NaHCO_3$ buffer (pH 9·6) which was constantly stirred. The temperature was maintained at 15°C and a trace of thymol was added to prevent microbial growth. As the pH rose gelation occurred.

Acetylation of gelatin

The method described by Kenchington (1958) was followed. This readily blocks all the side-chain amino groups of lysine and hydroxylysine.

Films for infra-red analysis

Films of sufficient thinness and strength could not be obtained simply by pouring the mixture on to a glass plate, inducing reaction and drying, but the following procedure proved satisfactory. Solutions of PGA, with and without 1,6-diaminohexane, were pipetted onto a clean mercury surface and dried under vacuum. The dry film was then clamped between two perspex O-rings and immersed in sodium hydroxide solution briefly to cause reaction. (The clamping was necessary, or the films wrinkled in the alkali.) The alkali contained 60% propan-2-ol to prevent the PGA from dissolving. After reaction the film was washed with 60% alcohol and then pure alcohol, before drying and analysing with a Perkin Elmer model 237 spectrophotometer.

Synthesis of propylene glycol pectate (PGP)

The method used was a modification of that described by Steiner *et al.* (1950). Sodium pectate (4 g) was dissolved in 210 ml of distilled water and 300 ml of isopropanol was then added, together with 10·5 ml of conc. HCl. The resultant pectic acid precipitate was successively centrifuged and washed in 60%, 80%, 90% aqueous isopropanol and finally in pure isopropanol before being dried under vacuum at 35°C.

The dried pectic acid (3 g) was mixed with 3 ml of water and then refluxed with 20 ml of propylene oxide for 2 h at 50°C with continuous stirring. After standing overnight at room temperature, the resultant gum was soaked in isopropanol to remove water, and then dried.

Degree of esterification

A titrimetric method similar to that described by Doesburg (1965) was used. Before titration, interfering substances were removed by prolonged washing with 65 : 33 : 2 propan-2-ol : water : HCl, the residual acid was carefully washed out and the purified sample dried. Solutions (0·2%) were then titrated to pH 7·5, with 0·1 N NaOH, and then saponified for 30 min at room temperature with 0·1 N NaOH before exact neutralisation and titration of the released uronic acid. Carbon dioxide was excluded by bubbling nitrogen. It is reasonable to assume that there was no interference from acetyl groups on the citrus pectates.

Viscosity measurements

Inherent viscosities, expressed as $(t_s - t_0)/t_0c$, were determined using an Ostwald capillary viscometer at 30·0°C; t_s is the flow time of the solution, c the concentration in g/100 ml polymer, while t_0, the flow time of the solvent, was approximately 60 s. These dilute solutions are Newtonian.

Intrinsic viscosities were obtained from plots of inherent viscosity against concentration.

Rate of depolymerisation of polyuronide esters at pH 9·6

A beaker containing 300 ml of either 0·2% PGA, or 0·2% PGP, in 0·05 M phosphate buffer and 0·2 M NaCl was immersed in a waterbath maintained at 15·0°C. The mixture was adjusted to pH 9·6 by the addition of 0·2 M NaOH and then maintained at this pH by the addition of 0·02 M NaOH, dispensed by means of an autotitrator (Radiometer). At various time intervals, 25 ml aliquots were removed and the pH immediately adjusted to pH 7·0 by the addition of 0·2 M HCl. These aliquots were diluted with water to a polyuronide ester concentration of 0·1%, and their viscosities determined.

Extent of interaction with 1,6-diaminohexane

PGA-1,6-diaminohexane films and PGP-1,6-diaminohexane films were prepared by drying mixed solutions of the components on glass plates. The ratio of ester to diamine was 20 : 1 (w/w). One film of each type was immersed in 1·5% NaOH for 30 s, to induce reaction.

Samples of the reacted and unreacted films were then washed in isopropanol : 0·05 M phthalate buffer, to remove unreacted 1,6-diaminohexane. The 1,6-diaminohexane remaining in the films (i.e. by reaction with ester) was then determined using the Kjeldahl nitrogen method. The analysis of the film which had not been in alkali served to show the effectiveness of the procedure for removing unreacted amine.

Measurement of mannuronate/guluronate ratios

The ratios were determined by circular dichroism spectroscopy in the region 200–250 nm according to the method of Morris *et al.* (1975).

Alginate solutions (3 mg/ml) were prepared in distilled water, adjusted to pH 7·0 with 0·1 N NaOH and then filtered with a micropore (5 μm) syringe. The spectra were recorded at 25°C on a Cary 61 CD spectrophotometer. Precautions were taken to ensure that the concentration of calcium ions present was insignificant.

RESULTS AND DISCUSSION

Dissolved gelatin interacts with alginate ester to form homogeneous gels only in mildly alkaline conditions (Ranganyaki & Stainsby, 1978). Below pH 8 no reaction occurs, whilst at pH 11 or higher the reaction is so fast that proper mixing of the reagents is almost impossible. In this work gels formed at pH 9·6, by the quick-set or by the dialysis method, were stable in boiling water (i.e. there was neither melting nor dissolution) provided the system was brought to neutrality by washing with phosphate buffer. This suggests that the gel network contains covalent bonds. However, a proportion of the strength of the gel at room temperature arises from the mainly hydrogen bonding interactions observed for gelatin alone (Stainsby, 1977), since the water-holding capacity of the gel is almost twice as great at 40°C (when H-bonding is absent) as at 15°C. Ionic interactions play an insignificant role: both polymers are negatively charged in neutral and alkaline solutions and the gel which forms is not disrupted by concentrated salt solutions.

McDowell (1970) has shown that a greatly enhanced thickening occurs when PGA reacts with polyvinyl alcohol, thin boiling starch or carboxymethyl cellulose in alkaline solution, and that the thickening does not persist for long. He has suggested that the increased molecular size, giving an increase in viscosity, is due to transesterification. Presumably the effect is made possible by the high density of reacting groups when segments of the polymer molecules inevitably come into close proximity, and becomes readily noticeable because polymers are involved. At longer reaction times, however, both de-esterification and chain fission must be dominant reactions and lead to thinning. The use of higher temperatures, too, reveals the transient nature of the aggregates.

When PGA and protein meet in alkaline solution the analogous reaction would involve the side-chain hydroxyl groups of serine and threonine (and tyrosine, if present, but it is almost entirely absent in

gelatin). These amino acids are reasonably evenly distributed with an average of one residue in twenty. At such a low density it is unlikely that significant association of protein and polysaccharide occurs.

Similarly, although alkaline depolymerisation of the ester leads to the formation of an unsaturated carboxyl group at the non-reducing end of the polysaccharide, particularly when the temperature is raised (BeMiller & Kumari, 1972), and such groups can attach to gelatin via nucleophilic attack by —OH or $-NH_2$ side chains, the very low concentration of the groups makes it unlikely that measurable thickening can be observed. Moreover, none of these possible reactions is likely to lead to gelation through network formation.

Gelation involves multifunctional reagents, i.e. the carboxyl or ester groups on PGA. The former can react only ionically with gelatin, and alginate itself is quite unreactive in mildly alkaline condition: both polymers then carry a nett negative charge. Highly esterified alginates are essential (McDowell, 1970), and the most probable reaction therefore is amide formation between these ester groups and uncharged amino groups on the protein. In view of the pH that is needed, the main sites of reaction are lysine (and, on gelatin, hydroxylysine) residues (ϵ-amino groups). Arginine side chains are still fully ionised and histidine side chains, though unionised, are infrequent. At pH 9·6 the initial ratio of ester groups to uncharged ϵ-NH_2 groups in the system (2% PGA/10% gelatin) is close to 5 : 1. At higher pH values the ratio diminishes and the rate of reaction increases as more uncharged groups become available. Since both polymers are multifunctional, thickening can be followed by gelation, which requires only a few crosslinks. The majority of the amide bonds that form, therefore, need not take part in the network. Again, de-esterification and chain fission are competing reactions, as is deamidation by alkali. Unless the pH is lowered, therefore, once a gel of sufficient strength has formed, the system will inevitably weaken and eventually liquefy.

The need for ϵ-NH_2 groups on gelatin is shown by the fact that if these are completely blocked by acetylation then the gel which forms at room temperature, by mixing the ester and derived protein at pH 9·6, melts at 30°C. This gel is devoid of covalent crossbonds.

Mohamed & Stainsby (1984), who converted the amino groups of gelatin to hydroxyl, using nitrous acid, have given more convincing support to the view that ϵ-NH_2 groups are of major importance. Again

only a gel of low melting point could be formed in the presence of alginate ester, but in this modification only amino groups are changed: in acetylation both hydroxyl (serine, threonine) and amino groups are blocked.

Further confirmation of the involvement of uncharged amino groups comes from the formation of insoluble films when the ester is reacted with 1,6-diaminohexane. The pK values of these amino groups are higher than those of the ϵ-amino groups of lysine and hydroxylysine and more alkaline conditions (pH 12) are needed to provide sufficient uncharged (reactive) groups.

Evidence for amide groups

The detection of additional amide groups is bound to be extremely difficult in a system containing protein, so evidence was sought using films formed with 1,6-diaminohexane. Infra-red spectroscopy was selected as the method of analysis. The region of particular interest is from 1500 to 1900 cm^{-1} (667 to 526 μm). As Fig. 1 shows, the strong absorption (1) of $>C=O$ stretching at about 1750 cm^{-1}, associated with the ester grouping, has been lost after immersion in alkali, and that of the carboxylate anion (2), at about 1600 cm^{-1}, strengthened. This absorption band now has two shoulders. That at about 1500 cm^{-1} (3) is probably due to a mixture of N–H bending and C–N bending and C–N stretching (an amide-II band; Rao, 1963), and may include N–H deformation of the ϵ-NH_2 group. The second shoulder, at about 1670 cm^{-1} (4), is also the result of reaction, and is thought to represent the C=O stretching of an amide group attached to a pyranose ring, i.e. an amide-I band.

Bociek & Welti (1975) have shown how the amide content of soluble polyuronides, in D_2O-phosphate, may be determined with precision using the amide-I peak, but this approach cannot be utilised for cross-linked films and gels. As the amide-I peak (KBr disc method) for pectin with a level of amidation of 11·7% is small but distinctly separate, at about 1670 cm^{-1} (Lockwood, 1976), it is evident that the degree of amidation in Fig. 1 is considerably lower.

Comparison of pectate and alginate ester reactivities

The reaction between alginate ester and gelatin at pH 9·6 might be expected to occur also between high-methoxy pectin (HMP) and gelatin

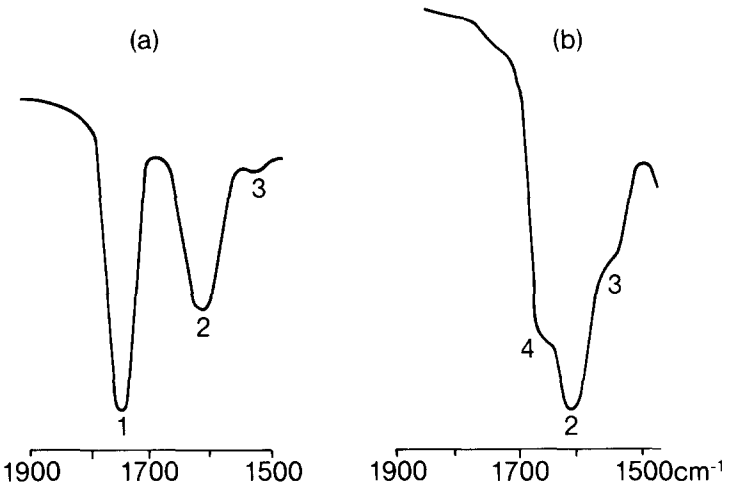

Fig. 1. Infra-red absorption patterns for PGA–1,6-diaminohexane films: (a) before reaction; (b) after reaction in alkali.

as the two polyuronides are rather similar, both chemically and physically. However, if HMP is substituted for PGA, using the dialysis method, then only a low melting point (~30°C) gel results. A factor which may be relevant is the nature of the ester grouping – the inductive effect of the hydroxy grouping in PGA may help stabilise the alkoxy group needed for amidation. A propylene glycol ester of pectate, with a degree of esterification of 92%, was therefore synthesised and used in place of PGA. Again only a low melting point gel formed.

It was then considered that the pectate ester might have a much lower molecular weight than the alginate ester, and that this was hindering gelation. However, the intrinsic viscosities in phosphate buffer and 0·1 M NaCl were very similar (2·85 dl/g for PGP, 2·90 dl/g for PGA) and, when the effects of rhamnose inserts into the pectate backbone are taken into account (Hallman & Whittington, 1973) it is probable that the pectate ester had the higher molecular weight.

Rates of depolymerisation were then compared, and are shown in Fig. 2. Clearly the rate is much greater for the alginate ester in the crucial early period at pH 9·6, so this cannot account for the inability of pectate ester to crosslink gelatin.

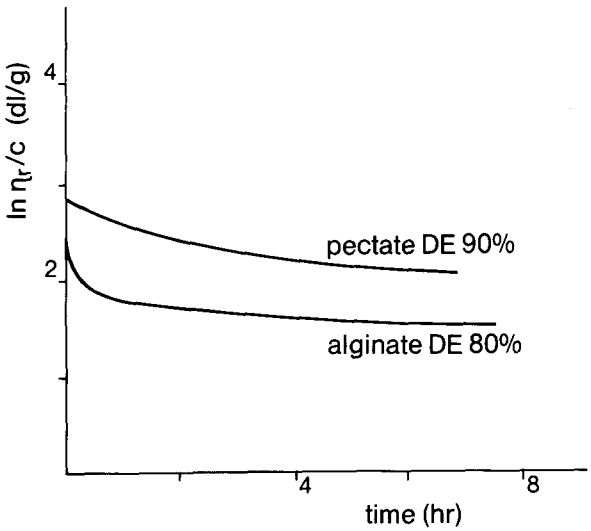

Fig. 2. Rates of depolymerisation of PGA and PGP. The uronate concentration, *c*, was 1 mg/ml.

When rates of saponification are considered (Fig. 3), the pectate ester was considerably more stable than the alginate ester. (It is noteworthy that the extent of de-esterification is quite trivial over the time-scale of the reaction by the quick-set method.)

Finally, as there seemed to be more than sufficient ester groups present, the concentration of amino groups was raised by increasing the gelatin concentration to 30%, giving the most concentrated solution that was readily practicable. Only a low melting point gel formed, even when the pH was raised to 11 to ensure that almost all the amino groups were instantly available in an uncharged form.

The PGP–diamine system was then examined, using film formation, as this enabled the reactants to be brought into closer proximity than in solution. At a ratio of ester to diamine of 20 : 1 (w/w), as used for alginate ester, the pectin–diamine film was water-soluble. Raising the diamine content by a factor of eight, to the highest level that could be obtained before it crystallised out, still gave a soluble film.

Kjeldahl nitrogen content was used to monitor the content of diamine bound to the polysaccharide but the level of residual nitrogen (0·06%)

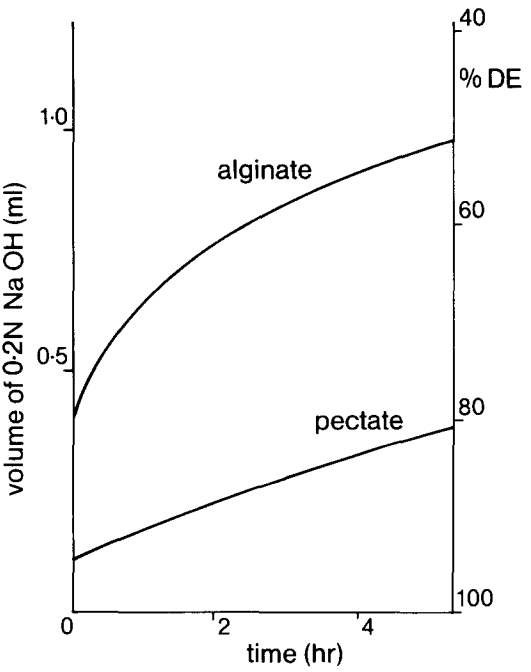

Fig. 3. Saponification of 1% solutions of PGA and PGP, by autotitration at pH 9·60.

was equal to that remaining with the polysaccharide when the diamine had been leached out before the pH was raised to promote reaction.

Finally a liquid diamine – 1,2-diaminoethane – was tried, since Carson (1946) has shown that it is possible to amidate dry high-methyl pectin with liquid *n*-butylamine. A solution of pectin was dried on a glass plate and then immersed in the diamine. After a very extended time (minimum 90 min) a film which was stable to boiling water was produced. If dry ester and liquid amine (1,2-diamine or *n*-butylamine) are shaken together for 3 days at room temperature and the unused amine removed with ethanolic hydrochloric acid, the nitrogen content (Kjeldahl) shows that all the ester groups have reacted and become amidated. Quite clearly water inhibits the amidation of pectate ester much more strongly than that of alginate ester.

Structural reasons for differences in reactivity

The inability to obtain high melting point gels from gelatin and propylene glycol pectate may well reflect the different molecular conformations of alginate and pectate in aqueous solutions. Almost all the sugar residues in pectate are glycosidically linked by diaxial bonds, whereas in alginate only the guluronate-rich regions are linked in this way. The mannuronate-rich regions are linked di-equatorially.

Theoretical calculations (Whittington, 1971) have shown that poly β-D-mannuronate would be rather more flexible than poly α-L-guluronate, and experimental evidence in support of this conclusion has been obtained from light-scattering and viscometric studies (Smidsrod, 1974). Thus there may be less steric restriction to reaction in the flatter and more flexible ribbon which results from di-equatorially linked residues. If this is so then pectate, and the guluronate-rich regions of alginate, would be less reactive than mannuronate-rich regions.

Some evidence in support of this view has been obtained using propylene glycol esters prepared from alginates having different compositions, in terms of the overall ratio of mannuronate to guluronate. Two of the alginates were commercial samples whilst the third (M/G = 70/30) was an extract from the receptacles of freshly harvested *Ascophyllum nodosum*. High melting point gels were made by the dialysis method and the stiffness of each gel, at room temperature, assessed by determining the load needed to compress the gel by 20% in an Instron.

The results (Table 1) show a correlation between stiffness (and hence the extent of covalent interaction with gelatin) and the proportion of mannuronate residues. It is probable that an even sharper dependence of strength on mannuronate content would have been observed if it had

TABLE 1

M/G ratio of alginate	*Load (g)*	*Overall DE (%)*
34/66 (commercial)	43	77
56/44 (commercial)	53	86
70/30 (*A. nodosum*)	74	60

proved possible to esterify the *A. nodosum* alginate to a level more comparable with that for the commercial alginates. Full confirmation of the view that it is the mannuronate-rich regions of alginate which react with ϵ-NH_2 groups of gelatin will require further work on the ease of esterification (which should parallel the ease of protein reactivity), and this in turn will require consideration of the difference in pK (approx. 0·3: Stainsby, 1980) for mannuronate and guluronate groups, so that esterification reactions may be carried out under comparable conditions. Alternatively, methods will need to be devised to determine the distribution of ester groups among the mannuronate and guluronate residues of a propylene glycol alginate.

ACKNOWLEDGEMENTS

E.L.W. wishes to thank H. P. Bulmer Ltd for supporting these studies through a research studentship, and Unilever Ltd (Colworth House) for providing a c.d. spectrometer.

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