ELSEVIER

Carbohydrate Polymers **23** (1994) 115–120

0144-8617/94/$07.00

Some observations on the nature of heated mixtures of bovine serum albumin with an alginate and a pectin

R. Kelly, E.S. Gudo, J.R. Mitchell & S.E. Harding

University of Nottingham, School of Agriculture, Sutton Bonington, UK, LE12 5RD

(Received 29 January 1993; revised version received 13 September 1993; accepted 19 October 1993)

The techniques of sedimentation analysis in the analytical ultracentrifuge, relative viscosity and dynamic light scattering were used to study the effect of heat treatment on dilute solution mixtures of (i) bovine serum albumin (BSA) and a sodium alginate ($M_w \sim 210\,000$) and (ii) BSA and a highly esterified (70%) pectin ($M_w \sim 130\,000$).

The two polysaccharides showed very different responses to thermal treatment, both in the presence of BSA and alone. The equivalent hydrodynamic diameter from dynamic light scattering of the pectin is reduced in the presence of the protein and both the mixture and pectin alone showed a reduction in apparent diameter with increasing heating temperature. In contrast the alginate mixture showed a larger apparent diameter than the polysaccharide alone at high heating temperatures, a value which appeared to be highly sensitive to the temperature of heat treatment. The maximum complex size was found at the highest heating temperatures (95°C), and higher than can be accounted for simply in terms of high temperature BSA aggregation phenomena. In contrast, at 85°C the size could be accounted for by simply a weighted average of free alginate and aggregated BSA, after comparison with the appropriate controls.

INTRODUCTION

There are many reasons for wanting to know how proteins and polysaccharides behave together in mixtures. In the food industry, for example, the physicochemical properties of foods can be dictated by potential interaction phenomena (see, for example, Tolstoguzov, 1986). This can be especially so when foods containing mixtures of these substances are heat processed.

Polysaccharides are employed as thickeners in food products containing proteins. Often these mixtures are subjected to heat. It has been shown that even in dilute solution complexes are formed between alginate and bovine serum albumin (BSA). The strenth of these complexes is greater when the protein is heat denatured (Imeson *et al.*, 1977).

It was suggested, in that paper, that electrostatic forces are responsible for the interaction. In contrast the attraction between pectin and serum albumin has been ascribed to the increase in the entropy of mixing as a result of dehydration of the macromolecules on complex formation (Semenova *et al.*, 1991).

The size of the complexes is an important parameter not only because size may affect thickening and gelation, but also because such complexes are surface active and might be expected to have useful foaming and emulsifying properties (Tolstoguzov, 1991). It has been found that the stabilization of emulsions can be increased substantially on transition from the protein only system to that of the protein–polysaccharide where covalent complexes are formed by dry heating (Dickinson, 1991). In contrast destabilization has been reported when low levels of polysaccharide are added to protein stabilized emulsions (Cao *et al.*, 1991).

Here we employ dynamic light scattering — used in a very approximate way — sedimentation velocity analytical ultracentrifugation and relative viscosity to further characterize BSA–alginate complexes and compare the properties of these with the products formed in pectin–BSA solutions. We would like to stress that all

measurements were performed at a fixed concentration (each component at 2 mg/ml in every case), and, for the dynamic light scattering, a fixed angle (90°) was used. Although this must mean that values for molecular size and weight are not absolute, the relative comparisons we consider are nonetheless useful.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA) was supplied in a highly purified form by Sigma Chemical Co. (Poole, UK) and was used without any further purification. Sodium alginate was supplied by Protan Ltd (Drammen, Norway) in a highly purified and fractionated form. This material had a guluronate/mannuronate ratio of 7·2:2·8 and a weight average molecular weight, M_w of (210 000 ± 10 000) g/mol as determined by Zimm plot classical light scattering and related procedures (Martinsen *et al.*, 1991) or (201 000 ± 11 000) g/mol using low speed sedimentation equilibrium in the analytical ultracentrifuge (Horton *et al.*, 1991). Our own preparation was checked by Dr G. Berth and Mrs E. Luck (Deutsches Institut für Ernährungsforschung, Germany) using a Zimm procedure from classical light scattering and an acceptable value of 250 000 was obtained. The pectin used was a highly purified pectin supplied by Copenhagen Pectin Fabrik (Denmark) with a degree of esterification (DE) of 70%. Its weight average molecular weight, as determined by low speed sedimentation equilibrium was (130 000 ± 15 000) g/mol.

Methods

Preparation of solutions

Stock concentrations were made up to 2·0 mg/ml for each macromolecular component in phosphate chloride buffer (pH 6·5; $I = 0{\cdot}1$ M) (Green, 1933). Care was taken to ensure full dissolution of the solute, and for this purpose, gentle magnetic stirring over a 2 h period was employed. Since BSA preparations (monomer molecular weight = 67 000 g/mol) are known to self-associate under certain conditions our own material was checked by the Zimm method and the molecular weight of (131 000 ± 5000) g/mol we found does indeed suggest most, if not all, in the dimerized state. Reagents were Analar grade.

Mixed solutions of protein–polysaccharide were prepared on a 1:1 volume basis, sealed in a 10 ml boiling tube and heated for 60 min at the required temperature in a thermostatted water bath.

Dynamic light scattering

A volume of 2–3 ml of sample was removed from the boiling tube and filtered through a 0·8 μm pore disposable filter head attachment on a syringe into a sterile eppendorf, refiltered and injected into the 1 cm cuvette via capillary tubing attachments and filter head. The cuvette had been adapted to avoid dust contamination (see, for example, Sanders & Cannel, 1980). All work was performed in a laminar air flow cabinet to further minimize dust contamination.

The fluorimeter cuvette was then placed in the goniometer of the DLS equipment (Malvern Instruments, UK, model: 4700 C). A Siemens 25 mW helium/neon laser ($\lambda = 632{\cdot}8$ nm) was focused onto the centre of the cuvette and a fixed scattering angle of 90° employed. Measurements were made at 25·0°C. The system was programmed to perform a total of 50 runs for each sample and the mean values then calculated for an apparent z-average translational diffusion coefficient, D_z, a guide to the polydispersity of the preparation via the so-called 'Polydispersity factor', PF, which is the z-averaged normalized variance of the diffusion coefficients (Pusey, 1974), and the effective or apparent hydrodynamic or 'Stokes' diameter, d_H. It should be stressed that no correction to zero angle, zero sample time or zero concentration of D_z or d_H was employed. Estimates for the effective hydrodynamic diameter were only used as a relative guide to the average size within a given mixture as a function of conditions.

Sedimentation velocity in the analytical ultracentrifuge

An MSE (Crawley, UK) Mk II analytical ultracentrifuge equipped with Schlieren optics was used. A 20 mm optical path length cell with loading volumes of 720 μl was employed and samples run at 34–35 000 rpm for 4–5 h. The temperature was maintained at 25·0°C. A camera attached to the eyepiece took photographs every 30 min and the sedimentation coefficient, s, calculated from these using a computer graphics digitizing tablet interfaced to an Apple IIE microcomputer. Again, because only relative changes in size were of interest, no attempt was made to correct the sedimentation coefficient, s, to standard solvent conditions or to correct for concentration effects.

Capilliary viscometry

The relative 'kinematic' viscosity, η_r, (i.e. not density corrected) of the samples was measured using a 2 ml capillary viscometer in a Comark No. 2 thermostated water bath at 25·0°C. A series of 10 measurements of the time taken for sample to fall between two electronically sensitive marks on the U-tube was made automatically for each sample and provided an accurate mean value. Because of the low concentrations employed (≈2 mg/ml), no correction for density was deemed necessary (Yang, 1961), and hence the relative viscosity was then calculated from the approximate relation by $\eta_r = t/t_o$ where t and t_o are the flow times of the sample and solvent respectively.

RESULTS

The results obtained for the effective hydrodynamic diameter, d_H, (from the dynamic light scattering measurements) as a function of heat treatment temperature are shown in Figs 1 and 2 for BSA treated with alginate and pectin, respectively. There are interesting differences between the two systems.

Polysaccharide controls

The values for d_H for the 'native' pectin at the lower temperatures are considerably higher than those obtained for the alginate. We interpret this in terms of the presence of a significant proportion of large aggregates in solution since the effective size, considering the approximations we have made and the well reported problems of light scattering on these substances (Berth, 1992), is greater than would be expected for an isolated pectin molecule. Heat treatment reduces the size when subsequently measured at 25·0°C, presumably as a result of degradation by beta-elimination (Albersheim *et al.*, 1960). The decrease in pectin molecular size on heating under these conditions has also been confirmed by low speed sedimentation equilibrium (Gudo, Dhami & Harding, unpublished results). In contrast alginate alone possesses a smaller size and heat treatment has no measurable effect.

Protein–polysaccharide mixtures

The two polysaccharides also show very different behaviour in the presence of BSA. If the apparent particle size of the filtered material is used as a criterion for

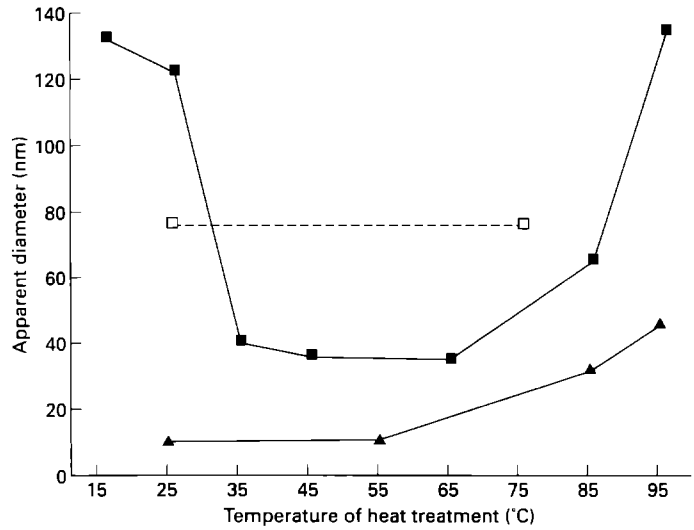

Fig. 1. Effective hydrodynamic diameter as a function of heat treatment temperature for the BSA–alginate mixed system (■) compared to BSA (▲) and alginate (□) alone as estimated by dynamic light scattering.

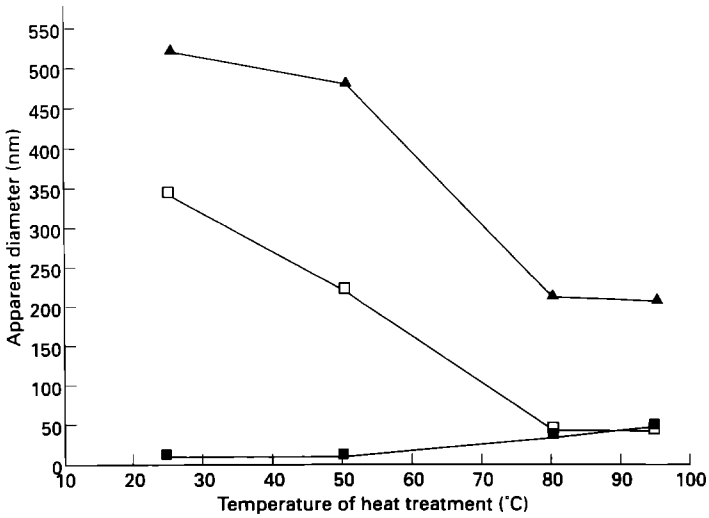

Fig. 2. Effective hydrodynamic diameter as a function of heat treatment temperature for the BSA–pectin mixed system (□) compared to BSA (■) and pectin (▲) alone as estimated by dynamic light scattering.

characterizing macromolecular interaction, then at first sight the data suggests that BSA and alginate (Fig. 1) undergo an interaction at temperatures >85°C whereas BSA and pectin do not (Fig. 2). However, in the presence of the protein, the effective diameter of the particles in the mixture containing pectin is lower than would be expected on the basis of the sizes found for the separate components, particularly after heating at high temperatures.

In the case of alginate and BSA mixtures (Fig. 1), a significant increase in size is observed for the interacted species. There is a minimum diameter at intermediate heating temperatures.

To get a guide to the apparent molecular weight of the alginate–BSA complexes—bearing in mind the approximations we have mentioned above—the (weight average) apparent sedimentation coefficient, s, was obtained and combined with the (z-average) apparent translational diffusion coefficient, D_z, using the Svedberg equation (see, for example, Tanford, 1961) to give a very approximate idea of the weight average (see Pusey, 1974) molecular weight, M_w.

$$M_w = \frac{sRT}{D_z(1 - \bar{v}\rho_o)}$$

where ρ_o is the density of the solvent and $\bar{v}$ the partial specific volume of the solute. For $\bar{v}$, we took a crude mean of the values for the BSA (0·734 ml/g, van Holde, 1985) and the alginate (0·450 ml/g, Ball, 1989).

Figure 3 shows the results obtained for the apparent s value and M_w as a function of heating temperature for the alginate–BSA mixture. At the highest level of heat treatment, it can be seen that very high molecular weight complexes are formed. It appears also that high molecular weight entities are formed at ambient temperatures with the preparation examined, but since they are destroyed by mild heat treatment (Figs 1–3), this would suggest these interactions are relatively weak. The complexes formed at high heat treatment are less polydisperse (Table 1). After mild heat treatment they reform very slowly or not at all. The existence of these associates is consistent with the findings of Imeson *et al.* (1977), who found that chromatography experiments on non-heated mixtures of BSA and alginate gave two separate peaks, whereas both components eluted together after heat treatment.

Table 1. Estimates for the apparent translational diffusion coefficients and corrected polydispersity factors (Pusey, 1974) of the mixed solution of BSA and alginate from dynamic light scattering

Temperature of heat treatment (°C)	$10^7 \times$ apparent diffusion coefficient, D (cm^2/s)	Polydispersity factor (PF)
No heat	0·48 ± 0·04	3·34 ± 0·11
25·0	0·35 ± 0·03	3·36 ± 0·13
35·0	1·81 ± 0·20	1·04 ± 0·04
45·0	1·68 ± 0·20	0·99 ± 0·03
65·0	1·44 ± 0·03	0·68 ± 0·02
80·0	0·75 ± 0·01	0·59 ± 0·07
95·0	0·36 ± 0·01	0·87 ± 0·04

The final piece of evidence for this strong dependence of treatment temperature on the interaction between alginate and BSA is shown through viscometry studies. The relative viscosity of the mixture (Fig. 4) appears also to follow the same trend as the other figures, apparently confirming that there is a minimum in hydrodynamic volume at intermediate heating temperatures, at least for this particular preparation.

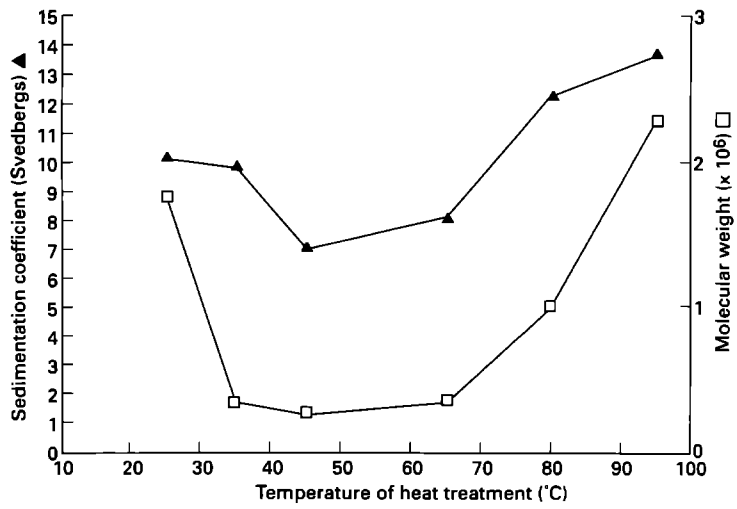

Fig. 3. Apparent sedimentation coefficient, s, and corresponding estimates for the apparent weight average molecular weight (from the Svedberg equation) M_w, as a function of treatment temperature for the BSA–alginate mixed system.

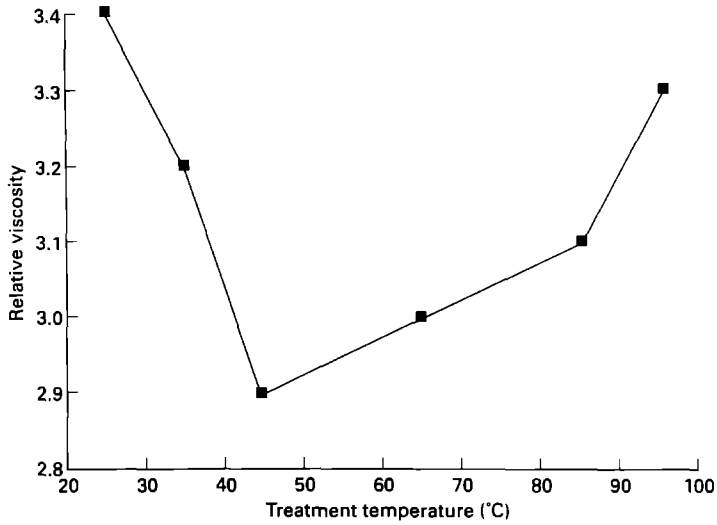

Fig. 4. Relative viscosity of the BSA–alginate mixed solution as a function of heat treatment temperature.

DISCUSSION

From the very first observations as the heating vessels were removed from the water bath, it became obvious that the two polysaccharides behaved very differently in the presence of the protein. The clear presence of a small proportion of supramolecular interaction products (white 'fibrous' aggregates) in the 95°C heated BSA–alginate system was not seen in the pectin–BSA solution which merely showed an increase in turbidity corresponding closely to that of the protein control alone under the same heat treatment. Measurements were made on filtered solutions thus the very large aggregates would not have been present.

Absolute molecular weights for complexes

As explained above, the results in Fig. 3 for molecular weight are only relative because of all the assumptions involved. It might have been better to measure the molecular weights using some absolute tehnique such as classical light scattering (Zimm plot etc.) or sedimentation equilibrium, both of which are readily at our disposal, but we would like to point out that even these have problems here. (i) Even with these latter methods, a concentration extrapolation to zero is necessary since the value measured at a finite concentration (in these experiments 2 mg/ml of each component) is only an apparent one because of thermodynamic non-ideality. However, if the interaction is concentration sensitive (which is indeed possible) this procedure would lead to severe problems in interpretation. (ii) Since the complex is a 'copolymer' of carbohydrate and protein, assumptions would have to be made concerning the 'refractive increment' (dn/dc) for classical light scattering or equivalently the 'partial specific volume' $\bar{v}$ for sedimentation equilibrium.

Differences in behaviour of the alginate–BSA and pectin–BSA mixtures

Pectin is employed in heat processed foods to maintain proteins in solution, at pHs close to or below the isoelectric point of the solution (Rolin, 1993). The relatively low molecular size of the BSA–pectin mixture at high temperatures is consistent with the reported stabilizing ability although, of course, in our case measurements were made above the protein isoelectric point.

One possible reason for the lower than expected size of the heated pectin is that in mixed polymer systems that do not phase separate, the degree of coil expansion of the individual components can be substantially reduced. However, it seems unlikely that this is sufficient to explain the very small hydrodynamic volume obtained for the mixture, although it could be a contributing factor.

It seems probable that the major factor is that at high temperatures the large pectin aggregates dissociate. On cooling in the absence of protein they reassociate, resulting in a large hydrodynamic volume even for the degraded material. In the presence of the denatured protein there is a pectin–protein association. We would speculate that it is the smooth region of the pectin chain which interacts electrostatically with the positively charged groups on the protein, although hydrophobic interactions involving the pectin methoxyl groups may also be significant. Denaturation of the protein facilitates this by rendering the molecule more flexible, allowing the optimum configuration for interactions as well as exposing the non-polar regions of the molecule. The degree of association, and hence the complex size, is limited by the low molecular weight of the degraded pectin, which reduces the probability of bridging between protein molecules and the low charge density on the smooth regions of the high DE pectin. Another

factor that may be operating is steric stabilization involving the hairy, neutral sugar-rich regions of the pectin chain.

In contrast, alginate, which after heat treatment will have a much higher molecular weight than pectin and possess a higher homogeneous charge density, can interact with many protein molecules. It has been suggested that these complexes consist of a central polysaccharide onto which macro-ions gradually attach (Wajnermann *et al.*, 1974). If this were the case then the maximum apparent molecular weight ($\approx 2{\cdot}6 \times 10^6$) found for the alginate would suggest that of the order of 50 moles of BSA associate with 1 mole of alginate.

Recent microelectrophoresis studies on oil droplets in the presence of alginate–BSA mixtures suggest that the alginate is adsorbed onto the protein–water surface (Ward-Smith, Hey & Mitchell, in preparation). Since there are some analogies between heat and surface denaturation, this is supporting evidence for complex formation between alginate and denatured BSA under conditions where the latter has a net negative charge. The destabilization of emulsions by low concentrations of added anionic polysaccharides (Cao *et al.*, 1991) could be the result of bridging due to protein–polysaccharide interactions of the type observed here. If this hypothesis is correct then high methoxyl pectin, at least after heat treatment, would not destabilize emulsions.

ACKNOWLEDGEMENTS

Useful discussions with Dr G. Berth and the practical assistance of E. Luck (Deutsches Institut für Ernährungsforschung, 0-1505 Bergholz-Rehbrücke, Germany) are gratefully acknowledged.

REFERENCES

Albersheim, P., Neukom, H. & Deuel, H. (1960). *Arch. Biophys.*, **90**, 4–6.

Ball, A. (1989). Molecular weights of industrial polysaccharides. PLD dissertation, University of Nottingham, UK.

Berth, G. (1992). *Carbohydr. Polym.*, **19**, 1–9.

Cao, Y., Dickinson, E. & Wedlock, D.J. (1991). *Food Hydrocoll.*, **5**, 443–54.

Dickinson, E. (1991). *Food Hydrocolloids*, **5**, 281–96.

Dickinson, E. & Euston, S (1991). In *Food Polymers, Gels and Colloids*, ed. E. Dickinson. RSC Publications, pp. 132–46.

Green, A.A. (1933). *J. Am. Chem. Soc.*, **55**, 2331–6.

Horton, J.C., Harding, S.E., Mitchell, J.R. & Morton-Holmes, D.F. (1991). *Food Hydrocoll.*, **5**, 125–7.

Imeson, A.R., Watson, P.R., Mitchell, J.R. & Ledward, D.A. (1977). *J. Food. Technol.*, **13**, 661–8.

Ledward, D.A. (1979). In *Polysaccharides in Foods*, eds J.M.V. Blanshard & J.R. Mitchell. Butterworths, London, pp. 205–16.

Martinsen, A., Skjåk-Braek, G., Smidsrod, O., Zanetti, F. & Paoletti, S. (1991). *Carbohydr. Polym.*, **15**(2), 171–94.

Morris, E. (1986). Mixed polymer gels. In *Food Gels*, ed. P. Harris. Elsevier, Amsterdam, pp. 291–359.

Pusey, P. (1974). In *Photon Correlation and Light Beating Spectroscopy*, eds . Cummins & E.R. Pike. Plenum Press, New York, pp. 387–428.

Rees, D.A. & Wright, A.W. (1971). *J. Chem. Soc.*, **13**, 1366–72.

Rolin, C. (1993) In *Industrial Gums*, eds R.C. Whistler & J.N. BeMiller. Academic Press, San Diego, CA, USA, pp. 257–93.

Sanders, A.H. & Cannel, D.S. (1980). In *Light Scattering in Liquids and Macromolecular Solutions*, eds V. Degiorgio, M. Corti & M. Giglio. Plenum Press, New York, pp. 173–82.

Semenova, M.G., Boltina, V.B., Dmitrochenko, A.P, Leontiev, A.L., Polyakov, V.I., Braudo, E.E. & Tolstoguzov, V.B. (1991). *Carbohydr. Polym.*, **15**, 367–85.

Tanford, C. (1961). In *Physical Chemistry of Food Macromolecules*, John Wiley, New York, p. 408.

Tolstoguzov, V.B. (1986). In *Functional Properties of Food Macromolecules*, eds J.R. Mitchell & D.A. Ledward, Ch.9. Elsevier, Amsterdam.

Tolstoguzov, V.B. (1991). In *Gums and Stabilisers for the Food Industry 6*, eds G.O. Philips, P.A. Williams & D.J. Wedlock, Oxford University Press, Oxford, UK, pp. 241–67.

van Holde, K.E. (1985). In *Physical Chemistry*. Prentice Hall, Englewood Cliffs, NJ, USA, p. 117

Wajnermann, E.S., Grinberg, V. & Tolstoguzov, V.B. (1974). *Coll. Polym. Sci.*, **252**, 234.

Yang, J.T. (1961). *Adv. Prot. Chem.*, **16**, 323–400.