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Effect of addition of κ-carrageenan on the mechanical and structural properties of β-lactoglobulin gels

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Abstract

The effect of the addition of a polysaccharide, κ-carrageenan (KCG), on the properties of heat induced gels of a globular protein, β-lactoglobulin (BLG), was studied at pH 7 and 0.1 M NaCl The shear modulus was measured as a function of heating time. At a given heating time and BLG concentration, the shear modulus has a maximum as a function of the KCG concentration. The initial increase can be attributed to the increase of the kinetics of the aggregation of BLG induced by KCG. The subsequent decrease can be attributed to the effect of micro phase separation. Macroscopic phase separation is not observed, but can be easily induced by centrifugation. Transmission electron microscopy of gels with and without the addition of KCG demonstrates the increased micro phase separation induced by KCG. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: κ-carrageenan; β-lactoglobulin; Gel point

1. Introduction

The study of mixed biopolymer gels is important both from a fundamental and applied point of view. Desirable gel properties can be obtained with mixed gels that are difficult to attain with pure systems. Here we report a study of the effect of the addition of a polysaccharide, κ-carrageenan (KCG), on the heat induced gelation of a globular protein, β-lactoglobulin (BLG), at pH 7 and 0.1 M NaCl. BLG is a whey protein with a molar mass of 18 400 g/mol and a radius of 2 nm (Mc Kenzie, 1971). When the temperature is increased above about 50°C the proteins partially denature, aggregate and finally form an opalescent white gel (Mc Kenzie, 1971). KCG is an anionic polysaccharide issued from red algae. KCG undergoes a coil–helix transition below a temperature that depends on the type and concentration of ions present (Rees, 1969). BLG was chosen because heat set BLG gels have been studied extensively in the past. We use KCG as the second component because it does not show specific interactions with BLG at room temperature. At 0.1 M NaCl the coil–helix transition of KCG occurs below 11°C. At room temperature only electrostatic repulsion is important because the two components are both negatively charged. Another motivation is that both products are widely used in the food industry.

In previous papers (Gimel, Durand, & Nicolai, 1994; Aymard, Gimel, Nicolai, & Durand, 1996) we have reported a static and dynamic light scattering study of the heat induced aggregation process of BLG. We have shown that at pH7 and 0.1 M salt BLG aggregates in the first step to form small well-defined particles of about 90 monomers. In the second step these particles associate to form self similar aggregates that grow in size until a gel is formed. The structure of the aggregates was found to be independent of the temperature of heating and the concentration, which however, strongly modify the kinetics of the aggregation. More recently, we have reported the preliminary results of a static and dynamic light scattering study of the influence of KCG on the aggregation process (Capron, Nicolai, & Durand, 1999). The principle conclusion of this work was that the structure of the aggregates is not influenced by the presence of KCG, but that the second step of the aggregation process is accelerated if KCG is added. The time needed to reach the gel point (t_g) decreases with increasing KCG concentration. The formation of the well-defined particles in the first step is apparently not influenced by the presence of KCG.

In this paper we report the results of rheology and transmission electron microscopy (TEM) on the systems after the

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gel point. We will show that the system is not stable close to and beyond the gel point but tends to phase separate. Macroscopic phase separation is inhibited by the gel formation. However, microscopic phase separation occurs and is more important, more the KCG is present. The acceleration of the aggregation process is confirmed qualitatively by turbidity measurements.

2. Material and methods

2.1. Sample preparation

BLG was supplied by BBRD (lot no. 754) and contained equal quantities of the genetic variants A and B. The solutions were dialysed against the solvent and filtered through 0.1 μm pore size Anotop filters. Concentrations were determined both by UV spectrometry at 278 nm ($\epsilon = 0.96$ l/cm) and refractometry ($dn/dc = 0.189$ ml/g).

The sodium KCG was supplied by SKW Biosystems. The powder was dispersed in water after which the ionic strength was adjusted to 0.1 M by the addition of 0.2 M NaCl at 40°C. The solution was then filtered through 0.45μm pore size Millex filters. The concentrations were determined by refractometry ($dn/dc = 0.15$ ml/g).

All the samples were adjusted to pH 7 and 200 ppm NaN_3 was added to avoid bacterial growth. Mixed solutions of BLG and KCG were stable at room temperature. During the heating the samples were covered with paraffin oil to prevent evaporation.

2.2. Experimental techniques

2.2.1. Turbidimetry

A Shimadzu UV–VIS spectrophotometer was used to measure the transmitted intensity (I) in the wavelength range 400–800 nm. The temperature was controlled within 0.1°C by a thermostated bath. The optical length (l) of the cells used was 0.5 cm. The turbidity τ is calculated as $\tau = (2.303/l)\log(I/I_0)$, where I_0 is the transmitted intensity of the solvent.

2.2.2. Rheology

Oscillatory shear measurements were done with a Carri-Med CSL500 (TA instruments) controlled stress rheometer. The temperature was controlled within 0.1°C by a thermostated bath. The samples were covered with a thin layer of paraffin oil to prevent evaporation of the solvent. The storage (G') and the loss (G'') moduli were determined at a constant applied strain of 0.5%. Frequency sweeps were carried out over the range 0.1–10 Hz. A special coaxial cylinder measuring geometry (Normand & Ravey, 1997) was used in order to avoid slippage effects. The large gap used in this geometry (3 mm) means that the possible interface effects are much reduced. The samples were put in the rheometer at room temperature and then connected to the thermostated bath preheated at 75°C. The temperature stabilised in less than 2 min.

2.2.3. Transmission electron microscopy

For fixation, small cubes of samples ($\cong 4\ \mathrm{mm}^3$) were placed in 1% paraformaldehyde + 0.05% glutaraldehyde in phosphate buffered saline, pH7.6, for 4 h at 4°C. The samples were then washed overnight in the PB5 buffer at 4°C and dehydrated through alcohol (50, 70, 90% alcohol 15 min, absolute alcohol 2 × 30 min). Subsequently, the samples were placed in resin [6 parts LR Gold (London Resin Co) + four parts GMA [low acid] (Polysciences) + 0.1% Benzoin Ethyl Ether [initiation] (Polysciences). Following several changes of resin over a period of 3 days, the samples were finally embedded in gelatin capsules, using freshly prepared resin and polymerised for 24 h at room temperature using UV polymerisation (360 nm).

The procedure for immuno staining was as follows:

1. Ultrathin sections (≅60 nm) of the samples were collected on collodion coated (2% collodion in amyl-acetate) nickel grids.
2. Sections were incubated on 20 μl aliquots of Tris/BSA (Tris buffered saline (TBS) 20 mM Tris + 225 mM NaCl pH7.6 + 1% Bovine Serum Albumin (BSA)) for 20 min. at room temperature.
3. Sections were transferred to 20 μl aliquots of the first antibody (rabbit a β-lactoglobulin 1:3000 dilution (Bethyl Labs); rabbit a-κ-carrageenan 1:400 dilution) diluted in TBS/BSA/TW (TBS + 1% BSA + 0.1% Tween 20) and kept overnight at room temperature (≅18 h).
4. The grids were washed in several changes of TBS and transferred to 20 μl aliquots of secondary antibody (goat a-rabbit IgG/Gold 5 nm diameter, 1:200 dilution (British Biocell International) diluted in the TBS/BSA buffer and kept for 60 min at room temperature.
5. The grids were washed using TBS followed by deionised water.
6. The labelled sections were silver enhanced (silver enhancement Kit LI (nanoprobes)) for 2 × 5 min, followed by washing in deionised water.
7. The grids were finally post stained using 2% aqueous Uranyl acetate, followed by lead citrate.

The samples were examined using a JEOL 100 CX II transmission electron microscope.

3. Results

Samples with BLG concentrations of 40 g/l and various concentrations of KCG were heated at 78°C in a thermostat bath. After 75 min the samples were rapidly cooled to room temperature which stops the reaction, and subsequently centrifuged for 40 min at 1000 g. After centrifugation the

Table 1
The characteristics of the lower gel phase and the upper liquid phase obtained after centrifugation of BLG gels with different amounts of KCG added for 40 min at 1000 g. The BLG concentration was 40 g/l at pH7 and 0.1 M NaCl. The solutions were heated at 78°C during 75 min

Concentration of KCG (g/l)	Weight fraction of the lower phase	Fraction BLG in the upper phase	Fraction KCG in the upper phase
0	0.21	0.02	–
2	0.63	0.11	0.63
4	0.60	0.06	0.97
7.5	0.57	0.04	0.97

systems are separated in a white gel phase at the bottom and a clear liquid upper phase. The gel fraction was weighed and the concentration of each component in the upper phase was determined. The concentration of BLG was measured by UV absorption and the concentration of KCG was calculated from the RI signal after subtraction of the protein contribution. The results are summarized in Table 1. Table 1 shows that the BLG gels formed in the presence of KCG expel water much more easily. If more than 2 g/l KCG is added almost all KCG is expelled from the gel. In all cases only a small fraction BLG is found in the upper phase.

3.1. Turbidity

We measured the increase of the turbidity upon heating solutions containing 20 g/l BLG and various amounts of KCG between 0 and 4 g/l. In Fig. 1 the turbidity at 800 nm is plotted as a function of heating time at 75°C. The increase in the turbidity is due to the formation of large aggregates. Fig. 1 shows qualitatively that more the KCG is present, faster is the aggregation. A more quantitative analysis is difficult because the turbidity depends on the structure and polydispersity of the aggregates. In addition, the effects of interactions and multiple scattering have to be considered.

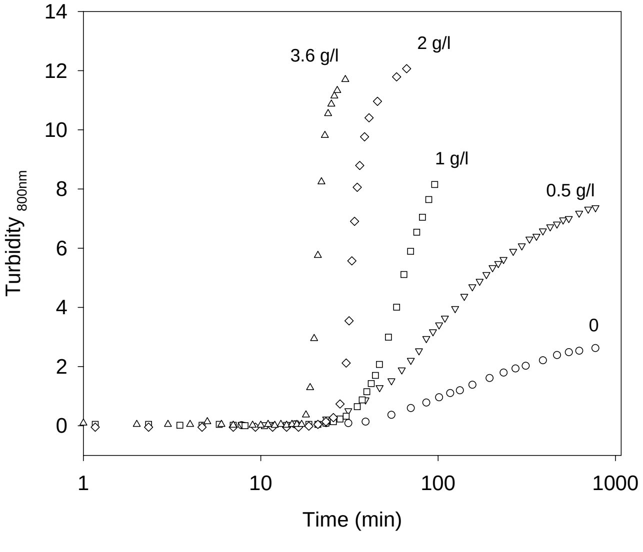

Fig. 1. Turbidity at 800 nm as a function of heating time at 75°C of a solution containing 20 g/l BLG and various amounts of KCG as indicated in the figure.

3.2. Rheology

The shear modulus was measured upon heating solutions containing 50 g/l BLG and various amounts of KCG between 0 and 6 g/l. In Fig. 2 we plotted the storage modulus (G') at 1 Hz as a function of heating time at 75°C. With the set-up used in these experiments, the very first stages of the gel formation cannot be investigated because the shear modulus is weak. As soon as G' is measurable, the frequency dependence of G' and G'' is small over the range 0.1–10 Hz.

The initial increase in G' occurs at times that are shorter if more KCG is present. This observation is in agreement with the decrease of the gel time with increasing KCG concentration previously reported (Capron et al., 1999) and is due to the increase of the aggregation kinetics. For all solutions we observe a continuous increase in G' with heating time. G'does not stabilise even after 2 h of heating. In one case we measured up to 13 h and still found no stabilisation of G'. This very slow stabilisation was observed earlier for pure BLG gels (Renard, 1994; Stading, Langton, & Hermansson, 1992; Verheul, Roefs, Mellema, and de Kruif, 1998).

In Fig. 3 we show the dependence of G' on the KCG concentration after 1 and 2 h of heating. For a given heating time G' increases first rapidly with the KCG concentration, reaches a maximum at about 1 g/l and subsequently decreases at higher concentrations. When comparing the shear modulus at different concentrations at a given heating time we have to consider both the concentration dependence of the kinetics and that of the gel structure. The initial increase of G' at low concentrations can be attributed to the increasing rate of aggregation, although we cannot exclude an effect on the gel structure. However, the subsequent decrease has to be attributed to a variation of the gel structure as the rate of aggregation continues to increase.

Fig. 4 shows the frequency dependence of G' after 2 h of heating for pure BLG and for mixed systems containing 0.5, 2 and 6 g/l KCG. The frequency dependence is weak and independent of the amount of KCG added.

To investigate the effect of cooling on the shear modulus we have cooled the gels to 10°C at a rate of 1°/min. As can be seen in Fig. 5 the effect is that G' is increased by a factor

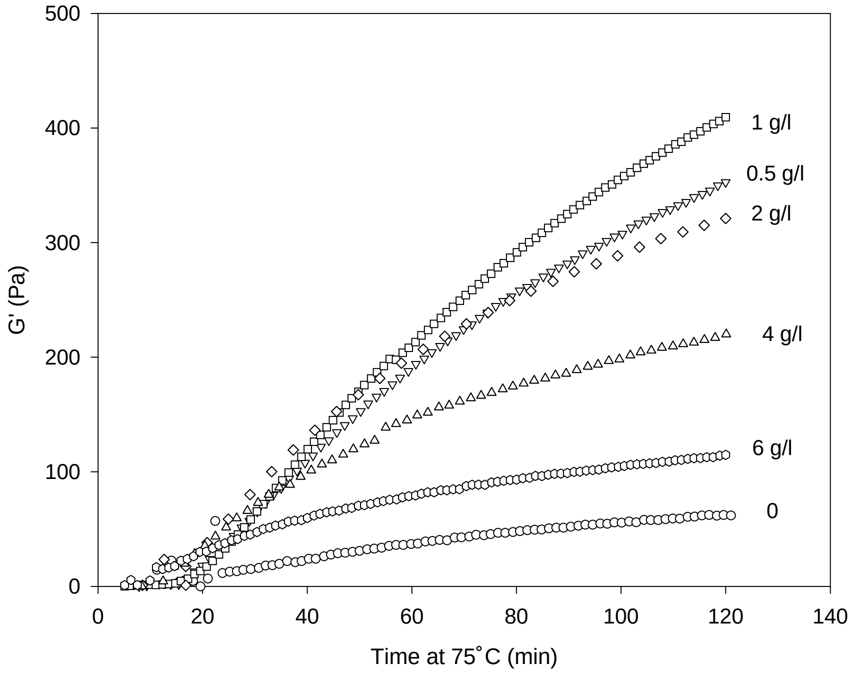

Fig. 2. Evolution of G' at 1 Hz as a function of the heating time at 75°C of a solution containing 50 g/l BLG and various amounts of KCG as indicated in the figure.

of about 4.3 and that the reaction stops. The frequency dependence is not influenced by cooling, see Fig. 4. The increase of G' can be attributed to a strengthening of the interactions between the proteins leading to an increased rigidity of the protein aggregates (Fernandes, 1994; McClements & Keogh, 1995). Both hydrogen bonding and van der Waals forces increase in strength as the temperature is lowered. The coil–helix transition of KCG occurs at 11°C, but its effect on G' is not visible as the increase of G' is independent of the amount of KCG present.

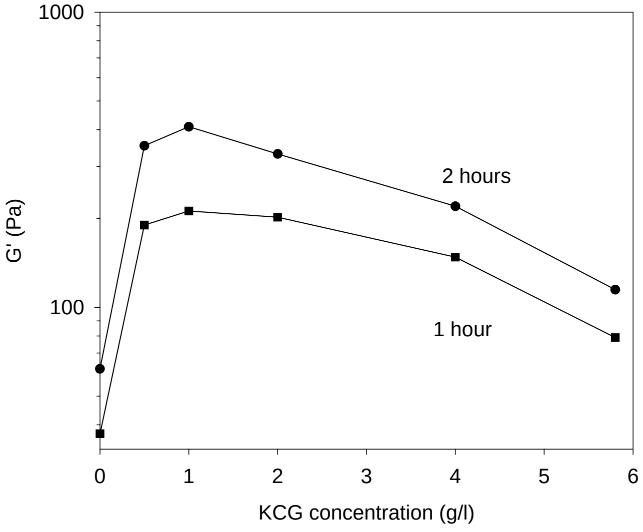

Fig. 3. Dependence of G' at 1 Hz on the KCG concentration after heating for 1 or 2 h at 75°C.

3.3. Transmission electron microscopy

In Fig. 6 we show a micrograph of a gel formed by heating a solution containing 60 g/l BLG during 2 h at 78°C. Fig. 7 shows a micrograph of the same system with 4 g/l KCG added. In both cases microphase separation has occurred, but the phase separation has been developed further in the presence of KCG. KCG is excluded from the protein rich

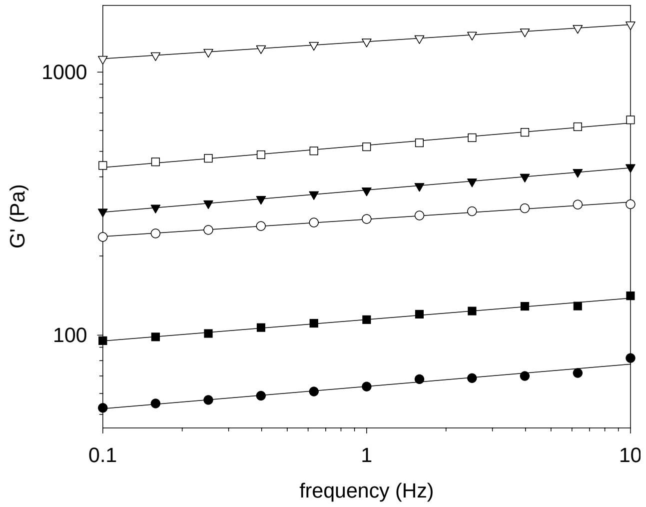

Fig. 4. Frequency dependence of G' of solutions containing 50 g/l BLG with various amounts of KCG after heating for 2 h at 75°C. The closed symbols show the results at 75°C and the open symbols show the results after cooling to 10°C. The KCG concentrations were: 0 g/l (○), 2 g/l (▽) and 6 g/l (□).

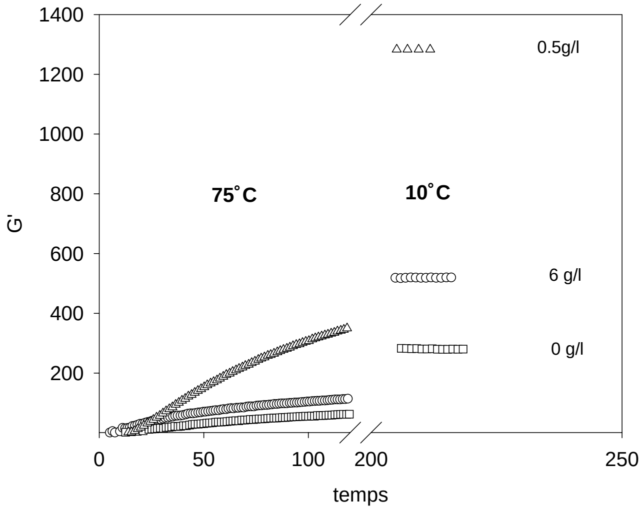

Fig. 5. Evolution of G' at 1 Hz as a function of the heating time at 75°C of a solution containing 50 g/l BLG and various amounts of KCG as indicated in the figure. After 2 h. the solutions were cooled to 10°C at a rate of 1°/min. The KCG concentrations were: 0 g/l (□), 0.5 g/l (△) and 6 g/l (○).

microdomains, which explains why KCG is easily expelled from the gel phase by centrifugation.

As explained in Section 2, KCG can also be visualised using specific antibodies. The resulting micrographs at two magnifications are shown in Fig. 8. Surprisingly, KCG rich domains can be seen together with solvent rich and protein rich domains implying that the KCG phase separates from the solvent inside the gel. This result is unexpected because pure KCG does not phase separate from the solvent. However, when we study pure KCG using the same method we sometimes also observe a phase separation so we cannot exclude that the phase separation of KCG from the solvent is an artefact of the preparation.

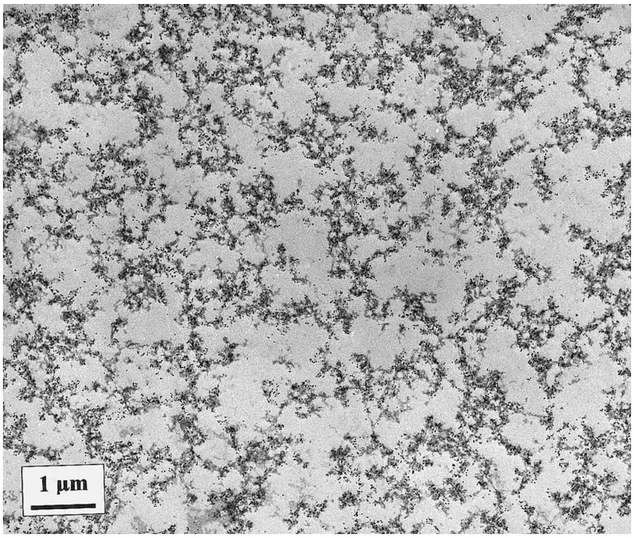

Fig. 6. The TEM micrograph of a gel formed by heating a solution containing 60 g/l BLG during 2 h at 78°C with BLG staining.

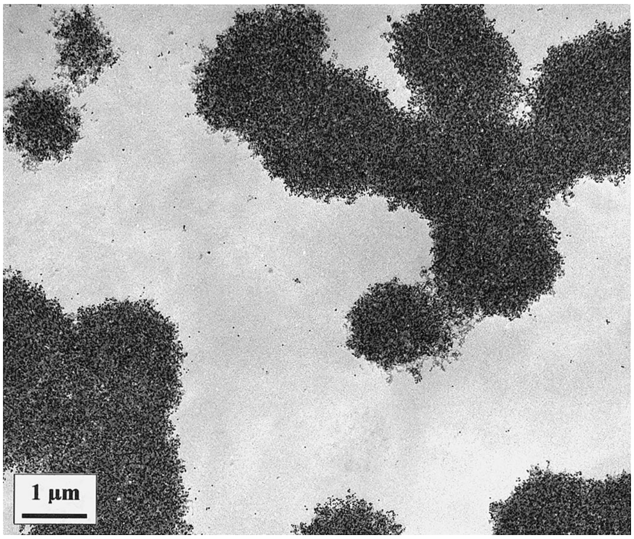

Fig. 7. The TEM micrograph of a gel formed by heating a solution containing 60 g/l BLG and 4 g/l KCG during 2 h at 78°C with BLG staining.

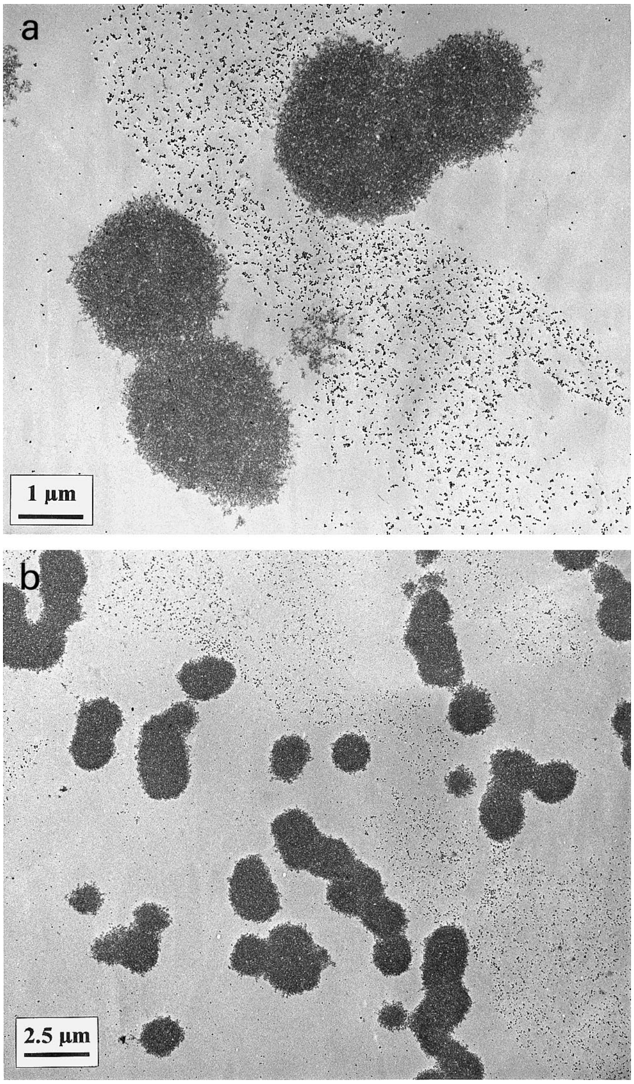

Fig. 8. (a) and (b): TEM micrographs of the same system as shown in Fig. 7, but here with KCG staining at two magnifications.

4. Discussion and conclusions

Two features are clear from the present results on the effect of KCG on heat induced gelation of BLG. The rate of the gel formation is increased with increasing KCG concentration and at a given heating time the phase separation is further developed if KCG is present. From light scattering measurements on pre-gel BLG aggregates we know that the structure of the aggregates is not modified by the presence of KCG (Capron et al., 1999). Therefore we believe that the influence on the mechanical properties is solely due to the more developed microphase separation in mixed gels.

There is an interesting parallel between the effect of KCG addition and the effect of ionic strength in the case of pure BLG gels studied by Verheul (1998) and Verheul et al. (1998). They observed that the shear modulus at a given heating time decreases with increasing ionic strength. This decrease correlated with a more open structure of the gels at higher ionic strengths. They also measured the permeability of the gels which is related to the size of the pores in the gel. When comparing the shear modulus and the permeability as a function of time they observed that while G' continues to increase, the permeability stabilises soon after the gel point. They concluded that the large scale structure of the gel is fixed close to the gel point. At this point, non-aggregated protein still present in the solution continues to strengthen the structure without modifying the porosity. The more open is the structure formed and fixed just after the gel point the weaker is the gel.

The authors suggest that the structure at the gel point is a fractal network based on the observation that the pre-gel aggregates have a fractal structure. We believe that the structure of the gel is not fractal for two reasons. First of all a fractal structure implies that the pore size varies from about 20 nm up to macroscopic values. The lower limit is determined by the size of the elementary unit of the fractal structure. Secondly, the size distribution of the aggregates is broad which means that smaller aggregates fill the pores of larger aggregates. Micrographs of the gels show on the one hand a single characteristic pore size and on the other hand a clear separation between protein rich and protein poor domains. We believe that the structure of the gel is in fact determined by a competition of two effects. The first effect is the increasing connectivity that leads to gel formation and the second effect is the tendency of phase separation. The large scale structure is fixed just after the gel point because the phase separation is frustrated by the gel formation. The stronger the tendency to phase separation the coarser will be the structure at the gel point. A good illustration of the competition of these two effects is given by Tromp, Rennie, & Jones (1995) for the ternary system of gelatin and dextran in water.

An interesting question is whether the tendency to phase separation appears only when the system is very close to the gel point or already for smaller aggregates. When the reaction is quenched by cooling to room temperature before the gel point the solutions are stable even close to the gel point. This means that if phase separation occurs before the gel point it happens only at elevated temperatures where the effect cannot be separated from the increasing aggregation.

We are currently conducting a more systematic study with the hope of resolving some of the remaining questions. We will also investigate whether the observed effects of the addition of KCG are the same for other types of polysaccharides.

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