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Gelation of ι-Carrageenan and Micellar Casein Mixtures under High Hydrostatic Pressure

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Effects of high-pressure treatment (HPT) on the rheological parameters and gelation of ι-carrageenan (ι-Car) and mixtures of micellar casein (MC) and ι-Car have been investigated under neutral pH conditions. It was established that HPT showed no significant effect, in the presence or absence of ionic calcium, with or without initial thermal processing, on the rheology or gelation/melting temperatures of the pure ι-Car solution. However, in mixed systems containing varying concentrations of ι-Car (up to 1 wt %) and MC (up to 8 wt %), considerable changes were detected. At the higher molar ratios of MC to ι-Car, and especially at the higher pressures, the dispersions were not thermoreversible in gelation, presumably due to the strong interactions of disrupted casein micelles with ι-Car molecules, as well as due to the formation of a dominant proteinaceous network at higher concentrations of MC. The associative protein−polysaccharide interactions in these systems are highly dependent on the ionic calcium content.

KEYWORDS: High pressure; ι-carrageenan; casein micelles; gelation; rheology; protein−polysaccharide interactions

INTRODUCTION

Carrageenans, the highly sulfated polysaccharides extracted from red seaweed, are widely used as functional ingredients in the dairy industry for stabilizing, thickening, and gelling (*1*). They exist in three main forms—κ, ι, and λ—respectively, having one, two, and three sulfate groups per repeating unit. Because of the positions of the sulfate groups, only κ- and ι-carrageenans have the ability to form gels under certain conditions. ι-Carrageenan (ι-Car) undergoes a temperature-dependent coil-to-helix transition upon cooling, leading to the formation of thermoreversible gels (*2*).

The casein micelle is a supramolecular association of individual casein subunits: α_{s1}-, α_{s2}-, β-, and κ-casein. These fractions are organized within the casein micelle according to a balance of interactions involving their hydrophobic and hydrophilic groups. The casein micelle is held together by colloidal calcium phosphate (CCP) (*3*).

The nature of the interaction between protein and polysaccharide components is important for understanding the behavior of ι-Car-containing dairy systems. Over the past few years, the effect of ι-Car on casein dispersions has been studied (*2*, *4*−*8*). The functionality of carrageenans in dairy systems has been mainly attributed to a highly specific interaction between the free sulfate groups of the carrageenans and a short positively charged region of κ-casein, which is located on the surface of the native casein micelles. No interaction was detected between the carrageenans and the other main casein monomers (α_{S1} and β) in the absence of free calcium ions. Lynch and Mulvihill (*3*) recently suggested some kind of physical interaction between the hydrophobic submicelles and ι-Car at neutral pH.

We have demonstrated previously (*9*−*11*) that high-pressure treatment (HPT) at several thousand atmospheres has the potential as a novel processing method for forming gellike textures based on micellar casein (MC) in the presence of sugars and polysaccharides. The new microstructures arise because of the influence of the high hydrostatic pressure in dissociating native casein micelles and releasing calcium ions, which can become available for interacting with polysaccharides and forming mixed biopolymer gels on reducing the pressure back to atmospheric. In particular, we have found (*9*) complex rheological effects of HPT on mixed dispersions of low-methoxyl pectin (LMP) and MC. In this paper, we apply the same approach to ι-Car and MC systems, with particular emphasis on the thermoreversible aspects of the gelation. An overview of the findings was briefly presented elsewhere (*12*).

MATERIALS AND METHODS

Materials. The skim milk powder (SMP) (St. Ivel, Wootton Bassett, U.K.) contained approximately 35 wt % protein (including 30 wt % MC, 5 wt % whey protein, and minor proteins), 55 wt % anhydrous lactose, 1 wt % fat, 5 wt % salts, and 4 wt % water. Commercial grade ι-Car (water content, 8.8 wt %; residue on ignition, 26.5 wt %) was

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obtained from the Fluka Chemie GmbH (Switzerland) and used without any further purification. Imidazole, dihydrate calcium chloride ($CaCl_2 \cdot 2H_2O$, 99%), sodium azide, and hydrochloric acid (35%) were supplied by Sigma Chemical Co. (St. Louis, MO). Imidazole was used because it is a pressure insensitive buffer.

Preparation of ι-Car Gel. Different concentrations (0.1−3 wt %) of ι-Car powder, as well as $CaCl_2 \cdot 2H_2O$ (0−100 mM), were dissolved in the imidazole buffer (5 mM, pH 6.7) at room temperature and then heated in a water bath at 70 °C for 10 min. One part of each sample was transferred into the CP 4/40 geometry (initial temperature, 80 °C) of the rheometer (see below) and cooled to 20 °C at a rate of 1 °C min^{-1} in order to determine the gelation temperature (T_g) and then reheated at the same rate to determine the gel melting temperature (T_m). Another part of each sample (∼25 mL) was transferred to a Cryovac plastic bag (Cryovac-W. R. Grace Ltd., Germany), sealed with an impulse sealer, and subjected to HPT. To investigate the effect of high pressure on gelation of cold mixed samples, some samples were pressurized without initial heating or with combined heating−pressurizing (800 MPa for 5 min at 45 °C).

Preparation of Mixed SMP and ι-Car Dispersions. Double-distilled water containing 5 mM imidazole (pH adjusted at 6.7) was used for preparation of the samples. To inhibit microbial growth, 0.4 g dm^{-3} sodium azide was added. To ensure complete hydration of the SMP, it was dissolved in the aqueous buffer, at a level corresponding to 10 wt % MC, by stirring overnight at ambient temperature. This was then used as a stock solution to make all other dilutions.

Solutions of ι-Car (0.1−1.0 wt %) were made by dissolving the powder in imidazole buffer at room temperature, heating in a water bath at 70 °C for 10 min, and then cooling to ambient temperature. An appropriate amount of stock SMP dispersion (containing up to 8 wt %, on the basis of MC) was added, followed by heating again in a water bath at 70 °C for 5 min. Around 3 mL of the well-mixed hot sample was transferred into the CP 4/40 geometry (initial temperature, 80 °C) of the rheometer (see below) and cooled (1 °C min^{-1}) to 20 °C to carry out the rheological analysis. The rest of the sample was divided into two equal parts: one part (ca. 20 mL) was transferred into a glass test tube (height 10 cm) to monitor the macroscopic appearance (phase separation, syneresis), and the other was sealed in a Cryovac bag for pressure treatment.

HPT. The sealed samples (∼20 mL) were subjected to HPT at up to 950 MPa for 1, 5, or 20 min using a Stansted Food-Lab Scale high-pressure machine (Stansted Fluid Power, Essex, U.K.) (*9*−*12*). The rates of compression and decompression were controlled and kept constant to avoid any significant temperature changes arising from adiabatic heating/cooling during the treating period. The temperature during HPT was maintained at 20 °C. Combined temperature−pressure treatment was applied in some of the cases. The samples were used immediately after treatment.

Macroscopic Observations. Visible changes in the appearance (color, uniformity, etc.) of the pressurized and nonpressurized samples over the storage period (3 weeks) were recorded. In cases where a definite phase boundary could be detected by eye, the rate of phase separation was measured by monitoring the height of the supernatant phase and expressing it as a percentage of the total height.

Rheological Measurements. Small deformation dynamic viscoelastic measurements of ι-Car and $CaCl_2$ and ι-Car and SMP mixtures were made using a controlled stress CVO rheometer (Bohlin Instruments, Gloucs, U.K.) fitted with cone and plate geometry (cone angle, 4°; plate diameter, 40 mm). The samples were transferred at ∼70 °C and cooled to 20 °C (T_g measurement) and then heated to 50−90 °C (T_m measurement). Storage and loss moduli, G' and G'', were measured in the linear viscoelastic domain for heating and cooling rates of 1 °C min^{-1}. The crossing temperatures of G' and G'' (at 0.2 Hz) during the cooling and heating cycles were taken, respectively, as measures of the gelation and melting temperatures (T_g and T_m). Some frequency sweeps (0.1−10 Hz) were performed at a constant temperature of 20 °C. Viscosity measurements of the ι-Car solutions (1−3 wt %) without any added $CaCl_2$ were carried out at 80 °C in the CVO rheometer over a wide range of shear stresses.

Table 1. Gelation and Melting Temperatures (T_g and T_m) for Solutions of ι-Car of Various Concentrations in 5 mM Imidazole Buffer (pH 6.7) in the Presence and Absence of Ca^{2+}[a]

ι-Car content (wt %)	T_g (°C)	T_m (°C)
3	30 (± 1.5)	36 (± 1.5)
2	24 (± 1)	31 (± 1)
1.5	21 (± 2)	27 (± 2)
1	(sol)	(sol)
1 + 5 mM Ca^{2+}	31 (± 1.5)	38 (± 1.5)
1 + 12.5 mM Ca^{2+}	48 (± 2)	54 (± 1.5)
1 + 25 mM Ca^{2+}	71 (± 2)	77 (± 2)
1 + 37.5 mM Ca^{2+}	72 (± 3)	79 (± 4)

[a] Numbers in brackets are standard deviations (SD).

RESULTS AND DISCUSSION

The purpose of the investigation is to explore the opportunity of using high-pressure processing to generate new biopolymer textures from mixed systems of MC and ι-Car, with particular emphasis on calcium ion redistribution during processing. We begin by considering the behavior of the polysaccharide in the absence of casein.

Influence of Ionic Calcium on Rheology of ι-Car Solutions. At low concentrations (∼1 wt %) without any added calcium ions, solutions of ι-Car at ∼80 °C are very nearly Newtonian, showing slight shear-thinning behavior. At higher concentrations (2−3 wt %), the solutions become substantially shear-thinning. **Table 1** lists values of the gelation and melting temperatures (T_g and T_m) estimated from crossovers of G' and G'' (at 0.2 Hz) on cooling and heating. We see that these parameters depend sensitively on the concentration of the hydrocolloid. No gelation/melting point could be detected at low ι-Car concentrations (≤1 wt %), but at higher concentrations, a clear difference between T_g and T_m could be perceived (T_m was typically ca. 5 °C higher than T_g). We can conclude that our ι-Car sample does not gel at <1 wt % in the absence of calcium ions.

The mechanism of ι-Car gelation depends on the concentration of the polysaccharide as well as on the presence of cations. Yuguchi et al. (*13*) have shown that ι-Car in aqueous solution undergoes a reversible sol−gel transition on cooling or on increasing the concentration. They have suggested that the gelation of ι-Car takes place mostly because of transition from single chain to double helix without association. On the contrary, κ-Car normally forms two or three associated double helices during gelation. Morris and Belton (*14*) investigated the effect of three commonly occurring cations (Na^+, K^+, and Ca^{2+}) on the gelation of ι-Car. It was found that the effectiveness of the different ions for gelation is in the order $Ca^{2+} > K^+ > Na^+$. They suggested furthermore that the selective efficiency of certain cations for gelling the polymer is linked to their immobilization at the junction zones of the gels. Moreover, the major effect of cation type is in controlling the gelation temperature and the setting time of the gel and in moderating the kinetics of network structure formation. Chronakis, Piculell, and Borgström (*15*) reported that for two carrageenan types (κ and ι), the gel−sol transition temperature increases with increasing polymer concentration. In general, the transition onset temperature determined on cooling (T_g) is lower than the melting temperature (T_m).

In this study, we are interested in how a relatively small amount of added hydrocolloid affects the viscoelastic behavior of a milk protein system. Therefore, we prefer to focus our attention on the effect of added calcium ions on gelation of ι-Car solutions of low concentration. **Figure 1** illustrates the effect of various concentrations of calcium ions on the rheology

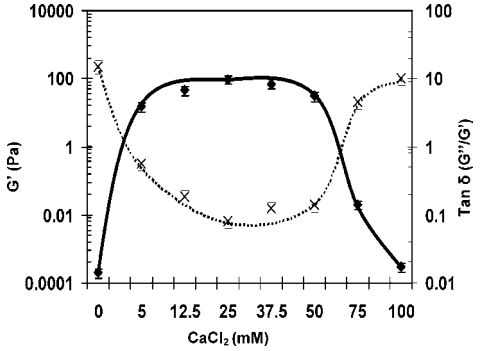

Figure 1. Influence of $CaCl_2$ concentration on storage modulus G' (—) and loss tangent tan δ (• • •) at 1 Hz of a solution of 1 wt % ι-Car in 5 mM imidazole buffer (pH 6.7). Error bars represent average standard deviations.

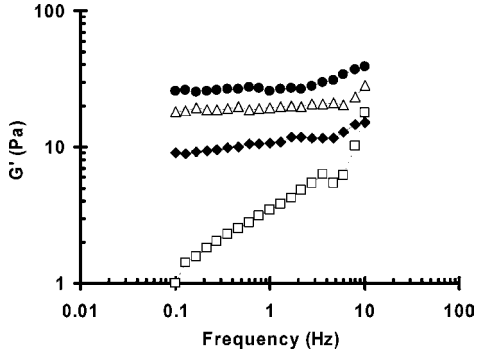

Figure 2. Frequency dependence of storage modulus G' of ι-Car gels (0.5 wt %, pH 6.7) in the presence of various Ca^{2+} concentrations: □, 2.5 mM; ♦, 4.1 mM; △, 6.3 mM; and ●, 12.5 mM.

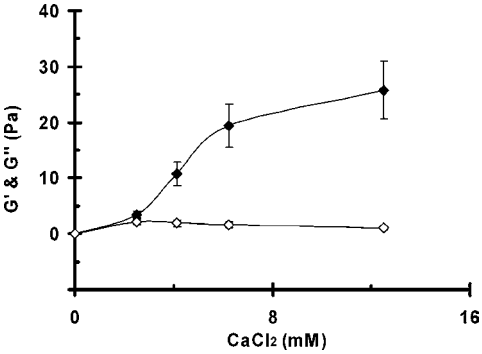

Figure 3. Effect of $CaCl_2$ concentration on the shear viscoelasticity of ι-Car gels (0.5 wt %, pH 6.7) at 1 Hz. Filled and open symbols represent G' and G'', respectively. Error bars represent average standard deviations.

of a system of 1 wt % ι-Car. In the absence of calcium ions, the solution behaves as a sollike system ($G'' > G'$), whereas it is gellike ($G' > G''$) in the presence of ionic calcium. A progressive increase in the rigidity of gels can be seen with increasing calcium ions concentration (up to 5 mM) and then a gradual increase (5−12.5 mM), reaching a maximum at ∼25 mM. The modulus levels off around 25−40 mM of Ca^{2+}, followed by a sharp reduction (>40 mM) and finally a massive change in G', which leads to the sol state again. A similar trend occurs over a wide range of ι-Car solution concentrations (up to 1 wt %) at pH 6.7. High concentrations of Ca^{2+} (>25 mM) were found to produce gels of high turbidity as compared with those made at low Ca^{2+} contents. These findings confirm that the maximum gel strength tends to occur around stoichiometric equivalence between cations and sulfate groups of the carrageenan, consistent with the Ca^{2+} promoting gelation via site binding between helices.

Michel, Mestdagh, and Axelos (*16*) found essentially the same behavior as reported here. They showed that a solution of ι-Car (up to 2.5 wt %) is liquidlike at low concentrations of calcium ions (up to 0.5 mM) but shows gellike behavior at moderate Ca^{2+} concentrations (0.5−100 mM). The polymer precipitates at high calcium ion contents (>100 mM). It was suggested (*15*) that the addition of cations beyond a certain critical value increases only the local heterogeneity of the system and contributes to phase separation without affecting the global viscoelastic properties. Piculell (*17*) has distinguished three different types of salt effects on the conformational transition of a polysaccharide: (i) lyotropic effects, which are ion specific but rather insensitive to the structural details of the macromolecule; (ii) generic or electrostatic effects, mostly for charged macromolecules; and (iii) effects due to site specific binding of certain ions onto the macromolecule. He concluded that ι-Car contains no specific ion binding sites and is influenced only by nonspecific electrostatic interactions. Any ion specific effects seen in conventionally prepared ι-Car samples are likely to be due to small levels of impurities of κ-Car.

Figure 2 shows the frequency dependence of the storage modulus of 0.5 wt % ι-Car gels in the presence of varying amounts of Ca^{2+}. At low Ca^{2+} concentrations, the storage modulus is very much dependent on frequency, but at higher concentrations (≥4 mM), it is essentially constant over the range of 0.1−10 Hz. **Figure 3** shows the influence of added Ca^{2+} on the shear moduli at 1 Hz. There is a large difference between G' and G'' in the presence of the "optimum" Ca^{2+} concentration (∼25 mM per gram of ι-Car). According to Watase and Nishinari (*18*), the added cations shield the electrostatic repulsion of the sulfate groups, thereby preventing tight binding and aggregation of the helices. Therefore, the addition of electrolyte extends the crystalline region of the carrageenan gel and hence increases the elastic modulus. For the same reason, it is expected that the elastic modulus is increased far more by divalent ions than by monovalent ions. Lynch and Mulvihill (*2*) showed that a 1 wt % ι-Car system containing 10 mM $CaCl_2$ has a low G', but its mechanical spectrum has definite gellike characteristics, with G' being virtually independent of frequency. They also mentioned that 10 mM $CaCl_2$ was insufficient to induce optimum gelation of 1 wt % ι-Car. In the presence of 20 mM $CaCl_2$, the values of G' and G'' were considerably higher and were less dependent on the frequency.

The small deformation rheology of a solution containing 0.5 wt % ι-Car and 12.5 mM Ca^{2+} over the cooling/heating cycle 70 → 20 → 70 °C is shown in **Figure 4**. It can be seen that for both G' and G'', the values are almost the same, with only as much as 2−5 °C difference in the data points between the cooling and the heating stages. In addition, this plot clearly shows the thermoreversible character of ι-Car gels. It needs to be emphasized here that in the pure ι-Car system (no protein present), only one distinct structural change occurs, thereby leading to a single step in the temperature-dependent rheology plot. This feature becomes significant when comparing systems with added MC (see below).

Figure 5 shows how the effective melting temperature (T_m) varies for 0.5 wt % ι-Car gels in the presence of increasing concentrations of Ca^{2+}. Storage and loss moduli are plotted as a function of increasing temperature. We see that T_m can change from ca. 25 to >65 °C on increasing the Ca^{2+} content from 2.5 up to 12.5 mM. This is assumed to be due to the stronger attractive electrostatic interactions. It is not surprising that with increasing gel rigidity and stronger bonds, more thermal energy is required to melt the gels, and hence, T_m increases. It is

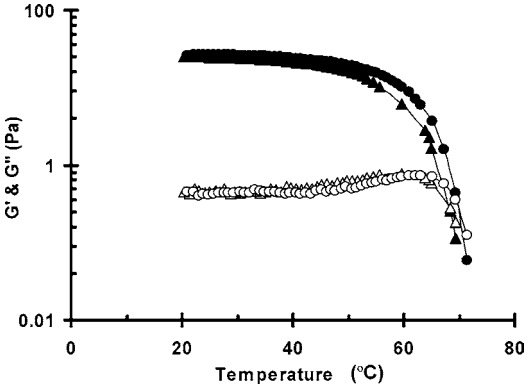

Figure 4. Variation in viscoelastic parameters (0.2 Hz) during the cooling (▲, △) and heating (●, ○) stages of a temperature cycle for a 0.5 wt % ι-Car solution (5 mM imidazole buffer, pH 6.7) containing 12.5 mM $CaCl_2$. Filled and open symbols represent *G′* and *G″*, respectively.

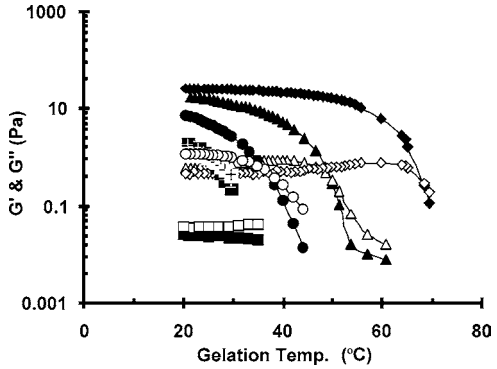

Figure 5. Variation in viscoelastic parameters (0.2 Hz) during the melting of a 0.5 wt % ι-Car gel (pH 6.7) containing various Ca^{2+} concentrations: ■, □, 0; ●, ○, 4.1 mM; ▲, △, 6.3 mM; ◊, ♦, 12.5 mM. Filled and open symbols represent *G′* and *G″*, respectively.

Table 2. Gelation and Melting Temperatures (T_g and T_m) of 0.5 wt % ι-Car Solutions in the Presence of Varying Concentrations of Ca^{2+}[a]

Ca^{2+} content (mM)	T_g (°C)	T_m (°C)
2.50	25 (± 1.0)	28 (± 1.0)
4.13	37 (± 1.1)	43 (± 1.2)
6.25	49 (± 1.6)	54 (± 1.5)
12.50	63 (± 1.9)	66 (± 1.5)

[a] Numbers in brackets are SD.

noteworthy, however, that despite these large changes in melting temperature, the difference between T_m and T_g remains essentially constant for widely varying Ca^{2+} contents (**Table 2**). This is consistent with the thermoreversibility of the sol−gel transition.

Effect of HPT on Rheology of ι-Car Solutions. Let us now consider pressurization of the polysaccharide in the absence of the protein. Pressurization of ι-Car in pH 6.7 aqueous buffer solutions at up to 950 MPa for different lengths of time has shown no significant effect on the rheological parameters or on the sol−gel transition temperatures. The only significant change was a slight increase in brightness and decrease in "stickiness" of the pressurized samples in comparison with the nonpressurized ones. In addition, when a nonheated mixed ι-Car and Ca^{2+} system was pressurized under harsh conditions (950 MPa for 30 min at ambient temperature), no gel was observed. A combined temperature/pressure treatment protocol (900 MPa, 20 min, 45 °C) was also unable to convert the solution into the gel state. According to the literature (*1*, *2*), ι-Car gelation is

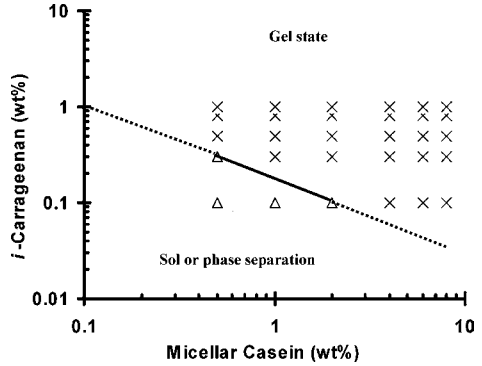

Figure 6. Phase diagram of untreated ι-Car and SMP mixtures at pH 6.7 and ambient temperature: △, sol or phase separated states; ×, gel state.

composed of two distinct stages, i.e., a coil-to-helix transition followed by electrostatic interaction between helices via calcium ions. It would seem, from the absence of rheological change in our experiments, that pressure alone (without initial heating) is insufficient to induce the coil−helix transformation.

Our findings are highly reproducible and in agreement with a short report by Suzuki (*19*) mentioning no significant effect on the rheology of carrageenan solutions following pressurization. The position must remain somewhat uncertain, however, because other studies have suggested significant effects of pressurization on carrageenan systems. In particular, there is a report in the literature (*20*) indicating gelation of a 1 wt % κ-Car solution following treatment at 600 MPa for 10 min. In addition, Steyer et al. (*21*) reported that a 3 wt % ι-Car solution, subjected to different temperatures, pressures, and treatment duration, can demonstrate different textural properties as compared to the counterparts studied at atmospheric pressure, particularly when processed at higher temperatures for longer times. Gekko and Kasuya (*22*) had also suggested earlier that under pressures up to 300 MPa, the melting temperature of carrageenan gels decreases due to the destabilization and replacement of polymer−water hydrogen bonds by polymer−polymer hydrogen bonds. This may have implications for the behavior of the mixed protein and polysaccharide systems, to which we now turn our attention.

Phase State Diagram of Mixed ι-Car and SMP Dispersions. Figure 6 shows the phase behavior of mixed ι-Car and SMP systems at room temperature without pressure treatment. In the presence of 0.1 wt % ι-Car, all mixtures containing >4 wt % MC were gellike ($G' > G''$). For 1 wt % MC, there was extensive phase separation, with almost 70% appearing as supernatant within 2 weeks. For 2 and 4 wt % MC, only 10−20% supernatant was observed, this appearing more like syneresis than complete phase separation. However, no syneresis occurred at >4 wt % MC. At a constant concentration of ι-Car, the storage modulus was found to increase for higher contents of SMP (**Figure 7**).

In mixtures containing 0.3 wt % ι-Car, no phase separation or syneresis was observed except at 4 wt % MC (maximum 10% serum layer). At higher ι-Car concentrations (>0.3 wt %), all of the mixtures behaved as gels and no macroscopic changes were perceived. As has been explained already, there was no gelation observed in ι-Car dispersions up to 1 wt % polysaccharide in the absence as well as in the presence of fairly low concentrations of MC. The main reason is that, aside from considering the role of any interaction between two biopolymers, there were not enough cations present in the system under these conditions to promote the gelation of ι-Car alone.

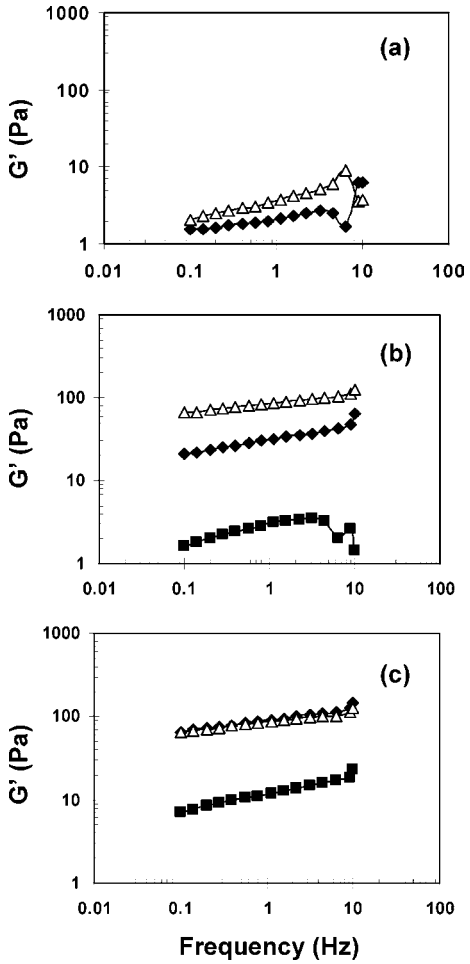

Figure 7. Frequency dependence of storage modulus G' at 20 °C for ι-Car and SMP systems (pH 6.7) containing varying contents of MC (■, 1 wt %; ♦, 4 wt %; and △, 8 wt %) and ι-Car: (**a**) 0.1, (**b**) 0.3, and (**c**) 0.5 wt %.

According to **Figure 6** then, the critical concentration to induce gelation in the presence of SMP (>2 wt % on the basis of MC) is ca. 0.1 wt %. However, other researchers (*4*) have reported that this limit can be rather lower, down to ca. 0.05 wt %, in the presence of higher concentrations of milk proteins (ca. 4 wt % of MC). Accordingly, we have plotted the sol−gel boundary as a dotted line in **Figure 6** for concentrations <0.1 wt %. Furthermore, we have found that pure ι-Car does not form any gel in the absence of Ca^{2+} at concentrations lower than 1 wt % (see **Table 1**). Therefore, we have extended the imaginary sol−gel boundary line up to 1 wt % of ι-Car. It is noteworthy to record that all of the above-mentioned mixed gels were thermoreversible even after long storage periods (>3 weeks). That is, on heating to 70−90 °C for a while (up to 30 min), they melted (became sollike), and on cooling again, they reverted back to the gel form. Additionally, almost all of the original macroscopic appearance (phase separation, syneresis) appeared again on storage.

Langendorff et al. (*5*) reported an essentially similar phase diagram for the ι-Car and SMP system at 25 °C. That is, depending on the concentrations of ι-Car and MC, the mixtures were either liquid (sollike), with or without phase separation, or gelled, with or without syneresis. There were three distinct domains: (i) a one phase gel, at a high enough concentration of ι-Car and in the presence of a reasonable amount of MC, (ii) a one phase liquid, at very low concentrations of ι-Car (up to 0.02 wt %) and moderate concentrations of MC (>2.8 wt %), or (iii) a two phase liquid, at low concentrations of ι-Car (up to 0.15 wt %) and MC (up to 2.8 wt %). The main difference between our phase diagram and that of Langendorff et al. (*5*) is that they reported that their system gelled even in the absence of SMP at quite low concentrations of ι-Car (0.15 wt %), whereas ours did not gel up to 1 wt %. This might be attributable to a difference in the purity of the ι-Car samples.

Schorsch, Jones, and Norton (*3*) have reported that the turbidity characteristics of mixed dispersions of MC (up to 12 wt %) and κ-Car (up to 0.02 wt %) dramatically changes at low protein concentrations (<4 wt %). It was proposed that carrageenan induces the flocculation of casein micelles leading finally to a bulk phase separation. The MC content at which this process occurs is dependent on the carrageenan concentration. On plotting the protein concentration at which flocculation occurs against the carrageenan concentration, a linear relation was found, suggesting that as polysaccharide is added it initially binds to the micelle surface but then at higher concentrations it bridges and causes instability. They concluded (*3*) that the same phenomenon occurs irrespective of the carrageenan concentration; at high protein concentration, the carrageenan chains have no effect on casein micelles in terms of stability. Moreover, they reported a very similar phase diagram as reported here for the aforementioned mixed system at 5 °C. They proposed that depending on the relative amounts of each component, the system may be stable or unstable, an excess of carrageenan leading to flocculation of carrageenan-coated casein micelles. This instability occurs at a very high concentration of carrageenan. When the MC and carrageenan system gels, it is probable that a casein/carrageenan network is formed with carrageenan acting as a bridge between casein particles, for which the helical conformation is required. They speculated (*3*) about two ways to stabilize incompatible systems of this type—either by preventing adsorption or by arresting the incompatibility between the species by gelation of one of the phases.

Figure 7 shows the frequency dependence of the storage modulus of the mixed ι-Car and MC dispersions and gels. For 0.1 wt % ι-Car and 1 wt % MC, there is no gel. The rheology of the gels composed of 0.1 wt % ι-Car and 4 or 8 wt % of MC is strongly dependent on the frequency over the range studied (0.1−10 Hz). Moreover, the values of the storage modulus at 8 wt % are significantly higher than that at 4 wt %. Interestingly, when the concentration of ι-Car is increased up to 0.3 wt %, gels are observed even at low concentrations of MC (1 wt %). Nevertheless, the frequency dependence of the gels was found to be slightly different at different concentrations of MC. However, surprisingly, on adding a little more ι-Car (to 0.5 wt %) at 1 and 4 wt % of MC, G' was found to increase, whereas it did not change at 8 wt %. As we reported previously for the LMP and MC system (*9*), it would seem that above a certain concentration of MC (or at high MC/ι-Car ratios) a different mechanism governs the gelation process. In other words, it can be supposed that at lower ratios, it is the polysaccharide network that entraps the protein particles. However, at higher ratios, the protein plays the main role in gelation and traps the polysaccharide molecules within the protein network. Between these extremes, there is probably some sort of interpenetration of the individual biopolymer networks.

Langendorff et al. (*5*) showed that the presence of casein micelles reduces the critical carrageenan concentration required for gelling. They found that the viscoelastic spectrum at 25 °C for 0.05 wt % ι-Car was sollike in the presence of permeate and gellike in the presence of milk (on the basis of 2.6 wt % MC). This could be because of bridging of casein micelles by

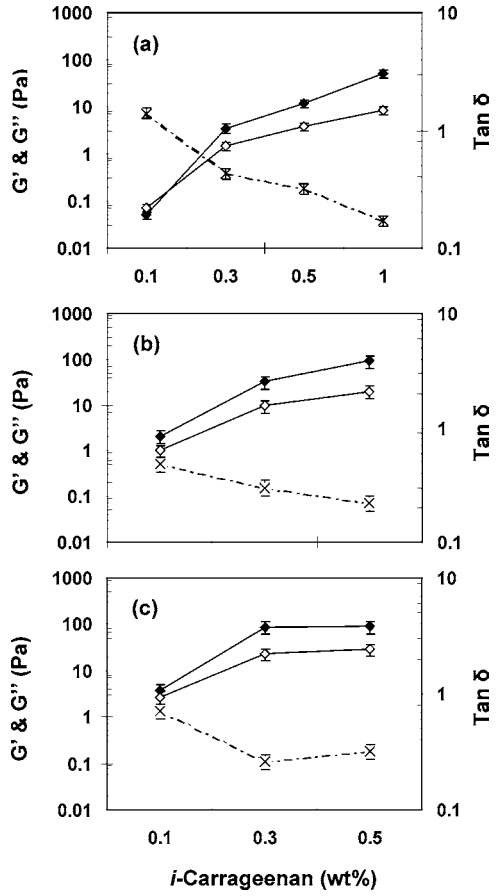

Figure 8. Effect of ι-Car concentration on the viscoelastic parameters (20 °C, 1 Hz) of ι-Car and SMP systems (pH 6.7) containing (**a**) 1, (**b**) 4, and (**c**) 8 wt % MC: ♦, storage modulus, G'; ◇, loss modulus, G''; ×, tan $\delta = G''/G'$. Error bars represent average standard deviations.

carrageenan chains. Moreover, they showed (*6*) that at 45 °C the viscoelastic spectrum of systems of 0.1 and 0.05 wt % ι-Car in the presence of milk is typical of that of a gel. Interestingly, the excess ι-Car did not seem significantly to affect the values of G' and G''. In another study (*7*), they reported essentially the same results for κ- and ι-Car, where at constant polysaccharide concentration the change from permeate to milk had a significant effect on the values of the viscoelastic parameters, as well as on their frequency dependency. Schorsch et al. (*3*) reported similar results for the κ-Car and casein micelle system.

Lynch and Mulvihill (*2*) reported that the addition of 1 wt % sodium caseinate alters the mechanical spectrum of a 1 wt % ι-Car gel containing 10 mM $CaCl_2$, i.e., by increasing G' and G'' and reducing the frequency dependence of G'' and tan δ. They mentioned that addition of higher concentrations of sodium caseinate changes the mechanical spectrum even more. They concluded that the addition of higher concentrations of sodium caseinate induces a transition from weak gel properties to true gel characteristics. Hemar et al. (*23*) showed that addition of SMP (up to 5 wt % MC) to a solution of 0.1 wt % κ-Car at neutral pH changes its flow behavior from Newtonian to pseudoplastic. Moreover, at higher polysaccharide concentrations (e.g., 1 wt %), the addition of SMP enhanced its viscoelastic parameters dramatically. For example, in the presence of 2.5 wt % MC, the value of G' was found to be 20 times higher than for the polysaccharide alone.

Figure 8 shows how increasing the MC concentration affects the storage and loss moduli in the presence of varying amounts of ι-Car. It can be seen that, on raising the ι-Car concentration

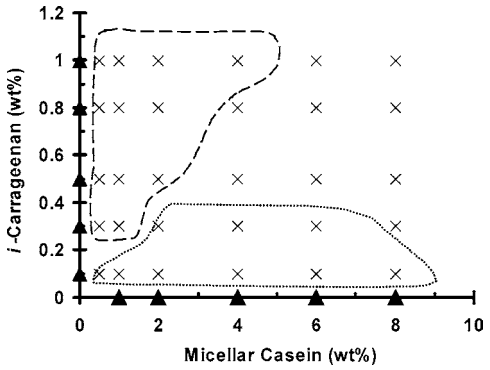

Figure 9. Phase diagram of ι-Car and SMP mixtures (pH 6.7) subjected to HPT (800 MPa, 5 min, 20 °C): ▲, sol states; ×, gel state. The dashed (- - -) and dotted (• • •) boundary lines enclose regions of thermoreversibility and syneresis, respectively.

from 0.1 to 0.3 wt %, the values of G' increase by up to 20 times, in comparison to the initial values at various concentrations of MC. In contrast, for 0.5 wt % ι-Car, the G' value was found to increase for the 1 and 4 wt % MC mixtures, but it stayed roughly the same at 8 wt %. It seems from **Figure 8** that adding progressive amounts of ι-Car to the MC system at neutral pH can increase the rigidity of the gels up to a certain concentration but beyond that point (0.3 wt %), there is not such a large change. The most pronounced effects are achieved at up to 0.3 wt % over the range of MC contents. We attribute this to a saturation of the mixed network.

Michon et al. (*8*) investigated the effects of native MC on the properties of ι-Car dispersions. Their confocal microscopy indicated remarkably different microstructures on varying the MC content, and rheological measurements and differential scanning calorimetry confirmed the changes. Consequently, they suggested (*8*) that at lower concentrations the continuous phase belongs to the carrageenan, whereas with progressive MC addition this dominant phase transforms into two interpenetrating networks.

High-Pressure-Induced Rheological Changes in Mixed ι-Car and SMP Systems. Let us now consider the effect of HPT (800 MPa for 5 min) on the macroscopic appearance and rheological properties of ι-Car and SMP systems. The phase diagram in **Figure 9** indicates that almost all sample compositions, excepting pure ι-Car (no MC) and pure SMP dispersions (no ι-Car), become gelled as a result of pressure treatment. Moreover, all these gels were more rigid, more transparent, more brittle, and more stable than their nontreated counterparts, although some of the samples did exhibit syneresis either immediately after decompression or a few hours afterward (depending on their composition). More importantly, when these pressure-induced gels were heated (to 60−90 °C), we found that some were thermoreversible, and others were not. These findings strongly imply, first of all, that solubilization of CCP under pressure can promote and accelerate the gelation process. In addition, because of CCP solubilization and subsequent micellar dissociation into nanoscale casein aggregates ("submicelles"), there is enhanced opportunity for interaction between the hydrocolloid and the sub-MC. Therefore, we have to include within the proposed mechanism a pressure-induced entanglement of both kinds of biopolymers in a mixed network, as well as electrostatic interactions among the biopolymers involving the role of released calcium ions.

It seems that the main source of thermoreversibility in the pressure-induced gels over a certain range of hydrocolloid concentration is the dominant presence of polysaccharide in the

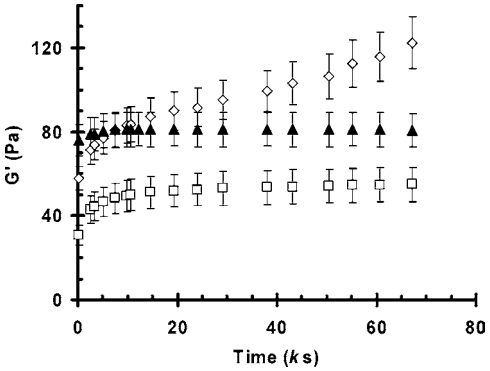

Figure 10. Time-dependent storage modulus G' (1 Hz, 20 °C) of untreated gels formed from ι-Car mixed with MC or $CaCl_2$ (□, 0.5 wt % ι-Car and 1 wt % MC; ◇, 1 wt % ι-Car and 1 wt % MC; ▲, 1 wt % ι-Car and 25 mM Ca^{2+}). Error bars represent average standard deviations.

gel network after pressurization. From the phase state diagram (**Figure 9**), we note that the range of MC content giving thermoreversibility in the presence of 0.3 wt % ι-Car is almost one-third that for 1 wt % ι-Car. As has been discussed already, the pressure-induced pure SMP gels (no added ι-Car) are not thermoreversible. Therefore, when the dominant gel network is made from casein, there should be no expectation of reversibility toward heating. This is indeed what was found.

Let us now consider in more detail the properties of untreated and HPT mixtures. For the case of untreated systems, **Figure 10** indicates how very different are the time-dependent G' values for a mixed ι-Car (1 wt %) and MC (1 wt %) system and a pure ι-Car system (1 wt %) containing the optimum content of Ca^{2+} (25 mM). It seems that there is no significant change with time for the pure ι-Car solution, whereas there is a nearly 100% increase in G' for the mixed biopolymer system. In addition, it can be seen that in the presence of a reduced MC concentration (0.5 wt %), the value of G' is significantly lower than for 1 wt % MC or pure ι-Car. Therefore, it is apparent that due to the putative interaction between the two biopolymers, as well as involvement of calcium ions in the promotion of gelation, the final gel is much stronger (higher G') than the one without casein micelles. Moreover, these results place stress on the importance of the quantity of casein micelles in determining the rheological properties of final gels. The curves in **Figure 10** imply the presence of a gradually developing interaction between hydrocolloid and casein components under ambient conditions. The findings are in agreement with those of Langendorff et al. (*5*), who reported that the elastic modulus increased with time at ambient temperature for an ι-Car system (0.56 wt %) in the presence of either permeate or milk. However, for milk, the increase was more pronounced (22% as compared to 10%), and it took much longer to reach the plateau (>500 min as compared to ~100 min).

Here in **Figure 11**, we compare the temperature-dependent rheological curves of the mixed biopolymer systems to observe how differently they behave over the cooling/heating cycle. We see that the curve for the nontreated gels shows only one step in both cooling and heating stages (**Figure 11a**), whereas the curve for the pressurized ones has two steps in the heating stage (**Figure 11c**). This suggests the existence of two kinds of structural contributions, one possibly due to the involvement of Ca^{2+} in the gelation process, and the other one due to the strong interaction between the two biopolymers, which needs more thermal energy to be destroyed. As a result, the shear modulus of the pressure-induced gel is substantially higher than

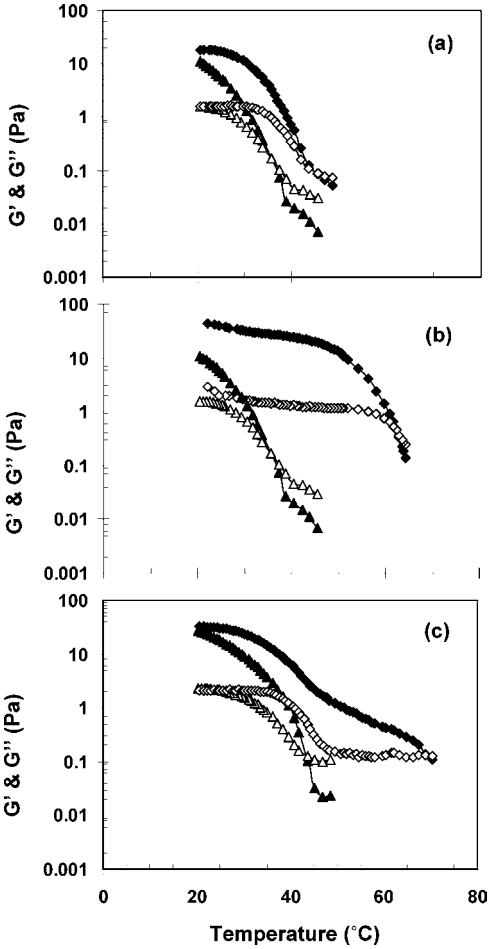

Figure 11. Variation in viscoelastic parameters (0.2 Hz) during the cooling (▲, △) and heating (♦, ◇) stages of a temperature cycle for ι-Car (0.5 wt %) and SMP (1 wt % MC) (pH 6.7): (**a**) no added $CaCl_2$, no HPT; (**b**) 12.5 mM added $CaCl_2$, no HPT; and (**c**) no added $CaCl_2$, HPT (800 MPa, 5 min, 20 °C). Filled and open symbols represent G' and G'', respectively.

that of the equivalent untreated one, and the recorded T_m value is significantly higher.

It might reasonably be assumed that this behavior is simply due to release of CCP from casein micelles on pressurization. To investigate this hypothesis, we added some extra Ca^{2+}, and we carried out the gelation/melting analysis without pressure treatment. The results show (**Figure 11b**) that while adding Ca^{2+} does significantly increase the T_m, it does not change the characteristic shape of the heating curve (from a one step curve to a two step one). Therefore, it can be inferred that the influence of HPT is to enhance the electrostatic interactions between carrageenan and submicelles, as well as to promote the gelation process through the provision of excess ionic calcium released upon pressurization. The behavior in **Figure 11a** (i.e., existence of a one step heating curve) does not imply, however, that there is no interaction between the biopolymers in the untreated samples. Indeed, we have already shown above that there is such evidence for the existence of electrostatic interaction between the two biopolymers. Nevertheless, the calcium ion effect probably masks the influence of the biopolymer interaction in the untreated samples. However, when samples are pressurized, due to the exposure of the nanoscale submicelles, there is more opportunity for both biopolymers to interact with one other, and the role of electrostatic associative protein−polysaccharide interaction becomes more obvious and dominant. This then is the likely reason we see a definite two step curve

for the pressurized gel but a one step curve for the nonpressurized gel of the same composition.

Langendorff et al. (*5*−*6*) reported a nearly 10 °C increase in polysaccharide gelation temperature in the presence of casein micelles for mixed ι-Car and permeate or ι-Car and milk systems. Moreover, the viscoelastic parameters were always higher in the presence of milk as compared to permeate. They also showed (*6*) that the gaps between T_g and T_m for permeate and milk were substantially different (2 and 22 °C, respectively). Moreover, the melting curve, in the presence of milk, showed two steps. They reported that during the heating cycle, the values of G' and G'' begin to decrease steadily and then to flatten out. However, at a higher concentration of ι-Car, the G' and G'' only decreased slightly as the temperature increased and the gel had still not melted. Therefore, they suggested that there are two structures or networks, one of which is thermally reversible and the other much more thermally stable. In addition, for the first time, they suggested (*6*, *7*) that ι-Car adsorbs onto casein micelles only when it is in the helix form. If the casein micelle concentration is sufficiently high, this attraction can induce the formation of a mixed network. As a result, the gels melt at higher temperatures than the pure or permeate-based carrageenan gels. They also proposed that at low concentrations of carrageenan, the rheology of the network is mostly governed by the casein micelles bridged by the partially helical carrageenan chains. However, at higher polymer concentrations, there is also formation of a second carrageenan network.

In terms of the involvement of the whey proteins and their interactions with carrageenan, there is no evidence in the literature for the case of ι-Car. However, Hemar et al. (*23*) studied the rheological behavior of commercial milk protein and κ-Car mixtures at neutral pH. Their confocal microscopy indicated phase separation in the presence of SMP but no phase separation with just whey proteins. Moreover, their rheological measurements showed substantial enhancement in the strength of the resulting gels with SMP but no effect with whey protein isolate. Therefore, there was no evidence of significant interaction between whey protein isolate and κ-Car, confirming earlier results for β-lactoglobulin and κ-Car (*24*, *25*). However, in a separate study by Tziboula and Horne (*26*), a synergistic effect was reported between κ-Car and whey proteins, with the rigidity of the mixed κ-Car/MC gels being apparently much greater when whey proteins were present, suggesting a specific interaction between the whey proteins and the κ-Car. Furthermore, the same authors stated (*27*) that increasing the concentration of whey proteins in recombined milks had a positive effect on gel strength.

We found that the gelation/melting temperatures of the mixtures significantly increased immediately after pressurization. **Figure 12** shows the effect of the applied pressure (for 5 or 20 min) on T_g and T_m. We note that the melting temperature is considerably more dependent on the treatment pressure than is the gelling temperature. In the untreated system, the difference between T_g and T_m is quite small (around 2−5 °C), but it increases to ca. 25 °C following treatment at 200 MPa for 5 min. There is then little further change on increasing the treatment pressure up to 800 MPa.

In a separate series of experiments, devised to test further for evidence of a possible interaction between two biopolymers, we increased the concentration of MC (to ca. three times as much) and kept the concentration of the hydrocolloid constant (**Figure 13**). What we found was essentially what we were expecting. On increasing the amount of MC, the number density of positively charged groups on κ-casein should be higher as

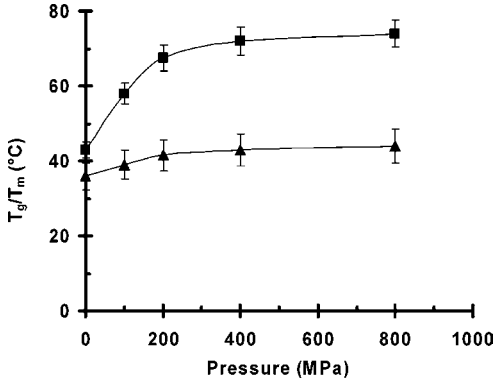

Figure 12. Effect of treatment pressure (for 5–20 min) on gelling temperature (T_g) and melting temperature (T_m) of mixed systems of ι-Car (0.5 wt %) and SMP (1 wt % MC) (pH 6.7) based on crossover of G' and G'' at 1 Hz: ▲, T_g; ■, T_m. Error bars represent average standard deviations.

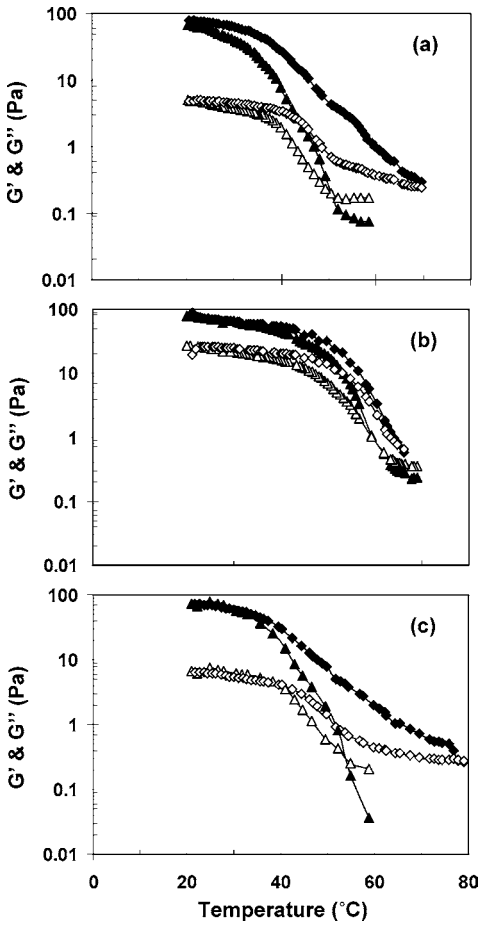

Figure 13. Variation in viscoelastic parameters (0.2 Hz) during the cooling (▲, △) and heating (♦, ◇) stages of a temperature cycle for ι-Car (0.5 wt %) and SMP (3 wt % MC) (pH 6.7): (**a**) no added $CaCl_2$, no HPT; (**b**) 12.5 mM added $CaCl_2$, no HPT; and (**c**) no added $CaCl_2$, HPT (100 MPa, 5 min, 20 °C). Filled and open symbols represent G' and G'', respectively.

compared with the conditions of **Figure 11**; therefore, the number density of electrostatic bonds should be greater. The resulting cooling/heating curves reflected these expectations rather well. It is apparent that the curve in **Figure 13a** is composed of two steps whereas that in **Figure 11a** shows only one step in the heating stage. Moreover, because of the higher concentration of free Ca^{2+} as well as the larger protein content, the gelation/melting temperatures are significantly higher. In

addition, we wanted to confirm that these effects were not just the consequence of the extra Ca^{2+}; therefore, we added some extra calcium ions and repeated the analyses. Surprisingly, the results (**Figure 13b**) showed that adding extra ionic calcium could mask the effect of protein−polysaccharide intermolecular interaction and consequently the curves reverted to the one step form. Additionally, there was found to be no big difference between T_g and T_m as reported earlier in **Figure 11b**. On the other hand, following pressurization (**Figure 13c**), the value of T_m was remarkably increased. Moreover, the appearance of two (or maybe even three) steps in the melting curves confirms the occurrence of new structural elements or additional interactions among the biopolymers.

Snoeren et al. (*28*) have suggested that the main reason for the lack of interaction with the major caseins (namely, α_s- and β-caseins) is the fairly even distribution of positively and negatively charged residues along most of their polypeptide chains and the lack of a positively charged region like the one in κ-casein. However, interactions between all types of caseins and carrageenans have been reported frequently in the presence of calcium ions (*29*−*31*). Lynch and Mulvihill (*4*) reported that the addition of α_s-, β-, or κ-caseins increases the gellike character of ι-Car gels in the presence of 10 mM $CaCl_2$. They mentioned that the relative effects of these caseins on the rheology of the gels are in the order $\kappa > \beta > \alpha_s$. In another study (*2*), it was found that, even in the absence of calcium ions, α_s- and β-caseins can increase the storage modulus of ι-Car gels via physical interaction. It was explained (*2*) that at around neutral pH values, both α_s- and β-caseins as well as ι-Car have net negative charges, implying little expectation of attractive protein−polysaccharide electrostatic interaction. Nevertheless, such interactions cannot be ruled out, because proteins can certainly form soluble electrostatic complexes with sulfated polysaccharides around neutral pH, as demonstrated for the case of bovine serum albumin and ι-Car (*32*, *33*). In addition, it is likely that repulsive interactions occur in the mixed gels, with the polysaccharide being excluded from the volume occupied by the protein and vice versa. Such contributory effects, resulting in a high local concentration of ι-Car, may also explain the ability of α_s- and β-caseins to modify the rheology of calcium free carrageenan gels.

Taking into account the above-mentioned evidence, as well as the disruption of casein micelles upon HPT, it seems obvious that due to enhanced interactions between κ-casein and ι-Car, as well as strong interactions between exposed α_s- and β-caseins and the mediatory role of pressure-released calcium ions, the rheological parameters of pressure-induced gels should be significantly higher than their untreated counterparts. In addition, the melting temperature should depend directly on the intensity of interactions between the biopolymers. Moreover, as well as the two types of structural contributions that have been proposed so far for the untreated samples, one can propose another type of pressure-induced structural contribution, which is much stronger than the other two. Consequently, one might even envisage a three step melting curve that is somehow different in shape from the two step version.

Treatment of ι-Car (0.5 wt %) and MC (3 wt %) at the moderately low pressure of 100 MPa for 5 min does not change the rheological parameters in comparison with its untreated counterpart. Nevertheless, it has been shown that such a treatment does remarkably influence the rheology of a similar mixed system containing a lower concentration of MC (see **Figure 12**). In contrast, treatment at higher pressures (≥300 MPa, 5 min) shows a considerable change in G' and in the frequency dependence. On the other hand, in the presence of excess calcium ions, there is very complicated behavior over the frequency range studied. Around 0.1−0.5 Hz, the behavior is independent of frequency, whereas it is very dependent at higher frequencies. Moreover, there is significant increase in G' in the presence of calcium ions.

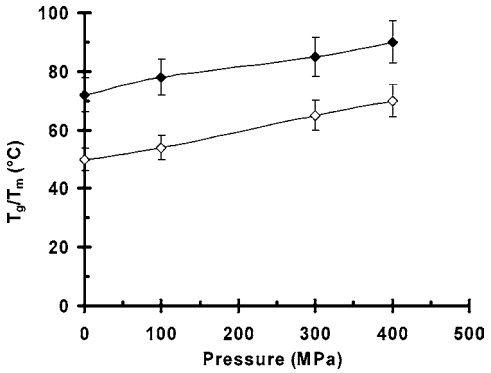

Figure 14. Effect of treatment pressure (for 5 min) on gelling temperature (T_g) and melting temperature (T_m) of mixed systems of ι-Car (0.5 wt %) and SMP (3 wt % MC) (pH 6.7) based on crossover of G' and G'' at 1 Hz: ◇, T_g; ♦, T_m. Error bars represent average standard deviations.

Figure 14 shows how treatment pressure influences the gelation/melting behavior at the higher MC concentration (3 wt %). As explained above, because of the nonthermoreversibility of the pressure-induced gels, performing these experiments at the highest pressures was practically impossible. Therefore, we just went up to 400 MPa. It can be seen that whereas the difference between gelation and melting temperatures is essentially independent of the applied pressure, there is a gradual increase in both T_g and T_m. Comparing these findings with the results shown in **Figure 12** indicates the importance of both biopolymers in determining the rheological characteristics of the mixed gels.

CONCLUDING REMARKS

Results for the influence of Ca^{2+} on the rheology of ι-Car solutions have shown that the maximum gel rigidity can be achieved over a certain range of added $CaCl_2$. In agreement with previous workers, we have also found relationships between the concentration of ionic calcium, the ι-Car content, and the melting/gelation temperatures. These findings confirm the idea that the maximum gel strength tends to occur around stoichiometric equivalence between calcium ions and sulfate groups and indicates that the cations probably promote gelation via site-binding between helices.

In terms of the high-pressure effect on ι-Car alone, no significant change has been found for either the rheological parameters or the sol−gel transition temperature. This suggests that hydrostatic pressure by itself (at ambient temperature) is incapable of converting a nonheated solution of ι-Car into the gel form.

With respect to the interaction of casein micelles and ι-Car, we have established state diagrams for untreated and pressurized mixed systems. It is established that untreated samples become gelled for high concentrations of ι-Car and MC, whereas at low biopolymer concentrations the system exhibits sollike behavior or in certain cases there is phase separation. In contrast, almost all of the pressurized samples were found to gel, irrespective of the concentration of their major components. However, only some of the gels were thermoreversible, and those that contained lower concentrations of ι-Car showed significant syneresis.

Overall, our results strongly confirm the existence of an associative electrostatic interaction between these two biopolymers. This protein−polysaccharide interaction is highly dependent on the ionic calcium content. Because of the disintegration of the casein micelle structure, the consequent increase in concentration of free calcium ions, and the interpenetration of the two biopolymer components upon pressurization, the rheological properties of the pressure-induced gels are significantly different from those of the untreated ones.

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