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Milk protein–carrageenan interactions

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Abstract

Factorial experimental designs were employed to study the major factors governing the gelation of milk/κ-carrageenan mixtures. The relative effects of carrageenan concentration, pH, ionic strength, casein proteins, whey proteins and κ-casein genotype on the mechanical properties of the gels were studied using dynamic rheological techniques. Gel transition temperature was governed mainly by ionic strength and carrageenan concentration whilst gel strength was influenced by carrageenan concentration and pH. It was found that the relative importance of the milk proteins to gelation varied with carrageenan concentration. It appeared that at high levels of κ-carrageenan (i.e. 0.1% w/w) gelation was predominately the result of association of κ-carrageenan helices to produce self-supporting gels. Milk proteins appeared to have little effect at high levels of carrageenan. However, milk proteins interfered with gel formation at carrageenan concentrations lower than 0.018% w/w, thus increasing the amount of micellar casein by twofold inhibited gelation. Serum proteins also suppressed gelation, albeit at lower κ-carrageenan concentrations. These results suggested that, in the presence of milk proteins, gel formation involved mainly carrageenan–carrageenan cross-linkages and not carrageenan–casein or casein–casein linkages. Nevertheless, the possibility of a strong interaction between κ-carrageenan and κ-casein on the micelles which has to be satisfied first, leaving insufficient carrageenan to provide network forming capability, cannot be ruled out.

Introduction

Polysaccharides are widely used in the dairy industry as stabilizing, thickening and gelling agents. Some polysaccharides provide stability by modifying the rheological properties of the continuous aqueous phase while others, such as κ- and ι-carrageenans, are thought also to interact directly with the milk proteins.

The carrageenans are cell wall polysaccharides of the red seaweeds (Rhodophycae). They are linear polymers, with a backbone structure of alternating α-1,4 and β-1,3-linked galactose residues and varying proportions of sulphate half ester groups, which impart a negative charge to the molecules and influence its functionality.

In aqueous solutions and in the presence of cations carrageenans form thermoreversible gels. At temperatures above the sol–gel transition temperature the carrageenans exist as random coils. Gelation occurs on cooling and has been attributed to a two-stage reaction involving helix formation followed by aggregation (1). The exact mechanism of this reaction is not fully elucidated. The domain model proposed by Morris *et al.* postulated the conformational transition from random coils to intertwined helices (domains) which, in the presence of K^+ and other cations, aggregate to form a three-dimensional network (2). However, this model has been called into question because the data were derived from a heavily contaminated carrageenan sample (3). Recent work has showed that the gelation of κ-carrageenan occurs at a supramolecular level. Initially, helical dimers associate into fine rigid rods which then aggregate into long supermolecular assemblies where two or more superstrands are aligned in parallel or are tightly packed in some periodic manner (4–6).

In the presence of milk proteins, gelation occurs at relatively low carrageenan concentrations and at temperatures below the carrageenan helix-to-coil transition temperature. From work to date, mainly on separated proteins, it appears that carrageenans interact specifically with κ-casein to form a complex which aggregates into a three-dimensional network. This interaction has been ascribed to electrostatic attraction between the sulphate groups of carrageenan and a predominately positive region on the κ-casein molecule (7–10). However, evidence for a similar interaction between κ-carrageenan and micellar

caseins in milk is less clear-cut. Cations such as K^+ and Ca^{2+}, which are important for the gelation of carrageenans, are present in milk and it is possible that the casein micelles act as a filler to impart strength and rigidity to the gel.

Most of the studies on carrageenan/milk protein interactions have been carried out in model systems that bear little resemblance to complex foods. In this work the path of carrageenan gel formation is followed in real milk systems using dynamic rheology. It is apparent that gelation of κ-carrageenan in the presence of milk proteins is influenced by many experimental variables. Among the putatively important factors are the κ-carrageenan concentration, protein level, ionic environment, pH, presence of casein micelles and inclusion of serum proteins. In order to elucidate the relative importance of these to gelation properties, a fractional factorial experimental design involving seven variables has been applied. In addition, it was also recognized that gelation and gel strength could be affected by κ-casein genotype through its influence on the levels of κ-casein in milk (κ-casein BB genotype is associated with higher κ-casein content). As most of the κ-casein is on the surface of the casein micelles and carrageenans are known to interact with κ-casein, it is anticipated that an increase in κ-casein content should influence gelation.

Materials and methods

Factorial experimental designs

The two experimental designs employed were selected from the library of fractional factorial designs implemented in Minitab version 9.2. The first design was a quarter factorial design, chosen to allow the main effects of seven variables, each set at two levels, to be resolved free from second order interactions in 32 plots. The second design was a full factorial of four variables each set at two levels in 16 plots. In both designs some of the second order interactions were confounded with each other.

Milk fractions

Bulk milk from the institute herd or milk from individual cows homozygous with respect to κ-casein A or B genetic variant was skimmed by centrifugation at 1200 *g* for 30 min at 4°C (Mistral 6000 centrifuge; Sanyo Gallenkamp plc., Leicester, UK) and filtered (GFA; Whatman, Maidstone, Kent, UK). The non-fat solids (TS) content of the milk was 9% w/w (9% milk).

Concentrated milk was prepared using a rotary evaporator (Büchi rotavapor R; Büchi Labortechnik AG, Flawil, Switzerland). The non-fat solids content of the concentrate was determined by a rapid method (11), and adjusted to 22% w/w (22% milk).

Skim milk ultrafiltrate (SMUF) was prepared from skim milk (9% SMUF) or concentrated milk (22% SMUF) by filtration on a pressure filter equipped with a membrane of molecular weight cut-off of 30kDa (Amicon Ltd, Danvers, MA).

Micellar caseins (MC) were pelleted from skim milk (9% MC) or concentrated milk (22% MC) by centrifugation at 43 000 *g* for 120 min and 48 000*g* for 210 min at 20°C respectively (Sorvall RC 5B; Du Pont Instruments, Newtown, CT). The micellar caseins were resuspended in SMUF by stirring overnight at 4°C.

Serum fraction. The supernatant layer obtained during the preparation of micellar caseins was designated as the serum fraction. This comprised mainly the serum proteins and the soluble phase of milk (9 or 22% serum). When required the serum fraction was concentrated twofold by ultrafiltration using a membrane of molecular weight cut-off 30 kDa; this resulted in an increase in the concentration of the serum proteins whilst the ionic environment was kept constant.

Milk protein/κ-carrageenan mixtures

κ-Carrageenan from *Eucheuma cottonoii* (Sigma Aldrich Co., Poole, UK) was added to the appropriate milk fraction while stirring at room temperature. The carrageenan molecules were allowed to hydrate by heating at 70°C for 20 min.

Rheological studies

Dynamic rheological studies were performed on a constant stress rheometer (CVO; Bohlin Instruments, Gloucestershire, UK), using the double gap measuring geometry. This geometry is the most sensitive available system for dynamic rheology studies in low viscosity media.

After transfer to the rheometer, an oscillating applied stress (5 mPa, frequency 0.08 Hz) was used to determine the storage modulus (G'), viscous modulus (G''), complex viscosity (η^*) and phase angle (δ) of the samples as they were cooled from 70 to 20°C at a rate of 1.3°C/min. After reaching 20°C, development of gel strength in the samples was studied for a further 20 min. The stress of 5 mPa was the minimum applicable for the chosen geometry. Although some of the gels were extremely weak the applied stress was within their linear viscoelastic regions . The transition temperature (T_{TR}) of the gels was taken as the point where $\tan\delta = (G'/G'') = 1$. Preliminary work ensured that the above experimental protocol gave reproducible data.

Statistical analysis

Analysis of variance was performed using the statistical package Minitab version 9.2 (Minitab Inc., State College, Philadelphia, PA).

Results and discussion

Identification of key factors influencing the gelation of κ-carrageenan in milk systems

Each variable studied was set at two levels representing the

extremes of treatments (Table 1). The different combinations of the levels corresponding to ionic concentration, presence or absence of casein and/or serum proteins as well as κ-casein genotype gave rise to media to which κ-carrageenan was added. An example of treatment combinations and the resulting media is shown in Table 2. Skim milk contained both the casein and serum proteins; SMUF consisted of the soluble phase of milk; micellar caseins resuspended in SMUF consisted of the casein fraction and the soluble fraction of milk (the serum protein fraction was removed); serum contained the soluble fraction and serum proteins. Manipulation of the protein level was achieved by preparing micellar caseins or serum proteins from concentrated milk. The relative importance of ionic concentration was studied by dispersing carrageenan in SMUF prepared from skim milk or concentrated milk. The latter has an ionic content 2.5 times higher than the former.

Typical profiles obtained during cooling of κ-carrageenan solutions are shown in Figure 1, with decreasing temperature running from right to left. Moving from low to high carrageenan concentration resulted in an increase in T_{TR} from 28 to 35°C and an increase in G' by more than two orders of magnitude (compare Fig. 1a and b). G' values correspond to elasticity at 20°C. The presence of the milk proteins resulted in a further shift in T_{TR} to 38°C but had little effect on G' (compare Fig. 1b and d). However, the most dramatic increase in T_{TR} (i.e. to 47°C) was observed when the ionic strength and protein level in the system was increased 2.5-fold (compare Fig. 1d and c); note that in Figure 1c carrageenan is at the lower level. This effect was largely the result of an increase in the ionic strength rather than in milk protein level because similar large changes in gelation temperature were also observed when carrageenan was dispersed in SMUF prepared from concentrated milk (results not shown). It is also noteworthy that the rate of cooling of the milk carrageenan mixtures had a significant and complex effect on gelation. This effect is currently being investigated and the results will be discussed in a future publication.

Table 1 Description of the factors and specification of the levels for the first design

Factor	Low level	High level
κ-Carrageenan conc. (% w/w)	0.02	0.1
Protein level and ionic environment	Milk fractions from 9% TS milk	Milk fractions from 22% TS milk
pH	6.7	7.3
Casein micelles	absent	present
Serum proteins	absent	present
κ-casein genotype	milk from κ-casein AA cow	milk from κ-casein BB cow
Gel development time	40 min at 20°C	20 min at 20°C

The effects of ionic environment and carrageenan concentration on the gelation temperature are well understood (12,13). The gelation temperature can be predicted by calculating the cation activity contributions from the solution and the carrageenan itself, reduced by their activity coefficient of ~0.5. This calculation alone explains why the carrageenan concentration affects the gelation temperature at low ionic concentrations but has no effect at high added salt concentrations.

Plots of the main effects—the differences between the means of treatment combinations at the highest and lowest levels of the variables—are shown in Figure 2a–c. Apart from the ionic effects already mentioned, other factors such as carrageenan concentration ($P < 0.005$) and the presence of serum proteins ($P < 0.01$) also influenced T_{TR}, albeit to a lesser extent (Fig. 2a). The presence of casein micelles, pH and κ-casein genotype had little effect on gelation temperature. Furthermore, it was observed that at high ionic concentration (for example, when carrageenan was dispersed

Table 2 Example of factor levels and resulting treatment combinations

Run	Carrageenan conc.	Protein level and ion conc.	Casein micelles	Serum protein	κ-casein genotype	pH	Gel development time (20°C)	Medium
1	high	low	high	high	high	low	high	skim milk of κ-casein AA genotype (9%TS)
2	low	low	low	high	low	low	high	9% serum from skim milk of κ-casein BB genotype
3	high	high	low	low	high	low	low	22% SMUF; κ-casein AA milk
4	low	high	high	low	low	high	low	22% MC resuspended in 22% SMUF; κ-casein BB milk
5	high	high	low	high	low	low	high	22% serum; κ-casein BB milk
6	high	high	high	high	low	high	low	concentrated milk (22% TS) of κ-casein BB genotype
7	low	low	high	low	high	high	low	9% MC resuspended in 9% SMUF from κ-casein
8	low	low	low	low	high	high	high	9% SMUF from skim milk of κ-casein AA genotype

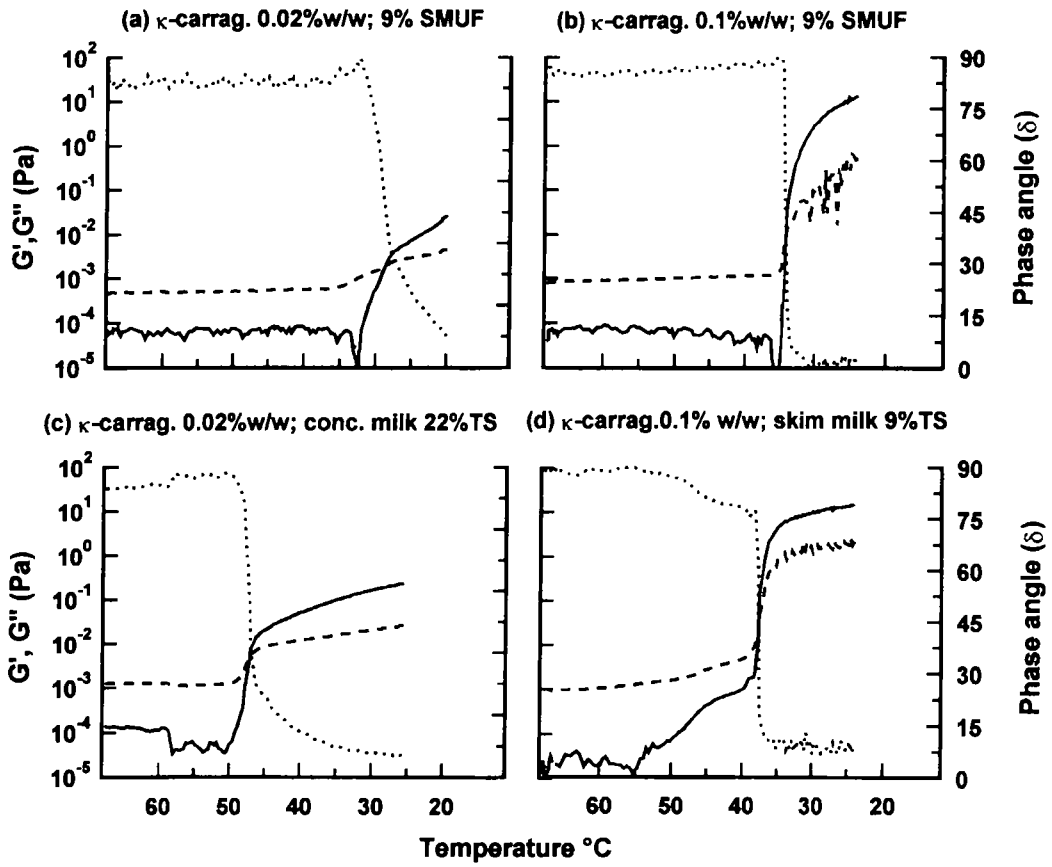

Figure 1 Effect of ionic environment and κ-carrageenan concentration on development of rheological parameters, G' —, G'' - - -, and δ on cooling. (a) 0.02% carrageenan in 9% SMUF; (b) 0.10% carrageenan in 9% SMUF; (c) 0.02% carrageenan in skim milk (22%TS); (d) 0.10% carrageenan in skim milk (9% TS).

in concentrated milk, serum or SMUF prepared from concentrated milk), T_{TR} was independent of the κ-carrageenan concentration. Similar observations were made by Nilsson *et al.* (14), who found that at high concentration of added NaCl the onset of helix formation was independent of the polyelectrolyte concentration, whilst the polyelectrolyte concentration dependence at low salt concentration was attributed to electrostatic interactions only. The same authors also showed that the coil–helix transition of carrageenans was very sensitive to electrostatic effects (14).

The strength of the gels, however, depended strongly on κ-carrageenan concentration and pH whilst the contribution to gel properties from ionic concentration and milk proteins was insignificant (Fig. 2b and c). This is in accord with the well-known dependence of the elastic modulus of κ-carrageenan gels on carrageenan concentration (12). The effect of pH on gel properties is a complex one. Increasing the pH of milk results in an increase of the charge of the caseins and influences the solubility of the colloidal calcium phosphate. Preliminary work has indicated that increasing the pH of milk from 6.7 to 7.3 results in the breakdown of the casein micelles and the release of the micellar calcium phosphate. These changes in the electrostatic balance of the κ-carrageenan/ milk protein systems, brought about by changes in pH, are expected to influence gel properties and require further study.

These results indicated that gelation of milk–carrageenan mixtures, with carrageenan concentrations 0.02% and greater, was dominated by the coil–helix transition of κ-carrageenan. At high levels of carrageenan, milk proteins appear to have little effect on gelation. Gelation is predominately the result of association of κ-carrageenan helices to produce strong self-supporting gels. The mechanism of gelation and mechanical properties of the gels are influenced mainly by the concentration of carrageenan, the ionic environment and the pH. An interesting feature of the gelation profiles was a dip in G' (and a corresponding peak in δ values) observed just before the gelation point. These features were associated with instrument inertia effects. Below the gelation point the lack of structure in the material is reflected by G' values (i.e. 10^{-4}–10^{-5} Pa). These values are extremely low and possibly outside the sensitivity limit of the geometry used. Just before the gelation point, at the onset of structure, there is a sharp increase in the phase angle which reflects the first 'real' measurement of the contributions from the viscous and elastic components to the structure of the system.

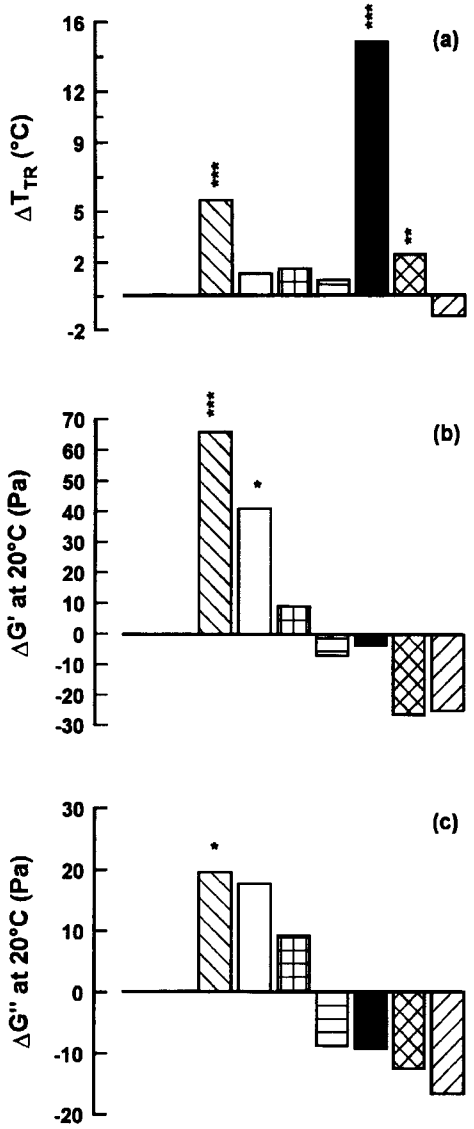

Figure 2 Plots of the estimated main effects of κ-carrageenan concentration ▨; pH □; casein micelles ▣; κ-casein genotype ▤; protein level and ionic concentration ■; serum proteins ▩; and gel development time ▨ on: (a) shift in mean transition temperature (ΔT_{TR}) attributable to change in variable indicated; (b) shift in mean value of G' at 20°C ($\Delta G'$); (c) shift in mean value of G''at 20°C ($\Delta G''$) during gelation of κ-carrageenan–milk protein mixtures. Data are the results of analysis of variance of measured parameters.

Influence of milk proteins on gelation at low κ-carrageenan levels

Whilst milk proteins appear to have little effect on gelation at high levels of κ-carrageenan, they seem to become important at low κ-carrageenan concentration. We employed a full factorial design of four variables set at two levels each to study the relative contributions of the milk protein fractions (casein or serum proteins) to gelation properties, at carrageenan concentrations of 0.012–0.018% w/w and at constant ionic strength (Table 3). Under these conditions the impact of carrageenan–carrageenan interactions on the mechanical properties of the gel is weak. Therefore, any changes in gelation properties may be attributed to changes in the protein content in the system.

Table 3 Description of factors and specification of the levels for the second design

Variable	Low level	High level
κ-Carrageenan	0.012 % w/w	0.016 % w/w
κ-Carrageenan	0.014 % w/w	0.018 % w/w
Protein type	caseins	serum proteins
Protein level	as in skim milk (×1)	twofold (×2)

Casein micelles or the serum proteins were redispersed in SMUF; at low levels the concentration of the protein fraction was similar to that of skim milk whilst at high levels the protein content was increased twofold. In all cases the ionic environment was kept constant, equivalent to the soluble phase of skim milk at its normal concentration.

As can be seen in Figures 3 and 4, an increase in the milk protein content of the system prevented gel formation. This effect was more pronounced with the micellar caseins where doubling the concentration of casein micelles also suppressed gelation at the higher carrageenan level (compare Fig. 3a and c). Whey proteins were effective only at low carrageenan concentration (i.e. 0.012% w/w), where doubling their concentration inhibited gelation (Figs 3b and d). When gelation did occur, the gel transition temperature was negatively correlated with the protein level. Again this effect was more pronounced with the micellar caseins, so that at 0.018% w/w κ-carrageenan and high casein level, gelation occurred at 21.9°C. For the remaining gels, gelation occurred at a temperature between 29.7 and 34.8°C. These values were in agreement with T_{TR} values observed previously in milk–carrageenan mixtures of low ionic strength. Analysis of variance confirmed that at low κ-carrageenan concentrations the contributions to the mechanical properties of the gels from the polysaccharide component were insignificant (Fig. 5a).

Analysis of variance also showed that the protein level was positively related to G' whilst the protein type had a negative effect (Fig. 5a). This merely reflects the observation that relatively stronger gels were produced when serum proteins were present, relative to the situation where gel formation was inhibited by the presence of the micellar caseins (Fig. 4b and d).

Conclusions

Milk protein/κ-carrageenan interactions are multifactorial. For the variables studied, their relative importance to gelation varies with carrageenan concentration. It appears that at high levels of κ-carrageenan (i.e. 0.1% w/w) gelation is predominately the result of association of κ-carrageenan helices to produce strong self-supporting gels. The mechanism of gelation and mechanical properties of the gels

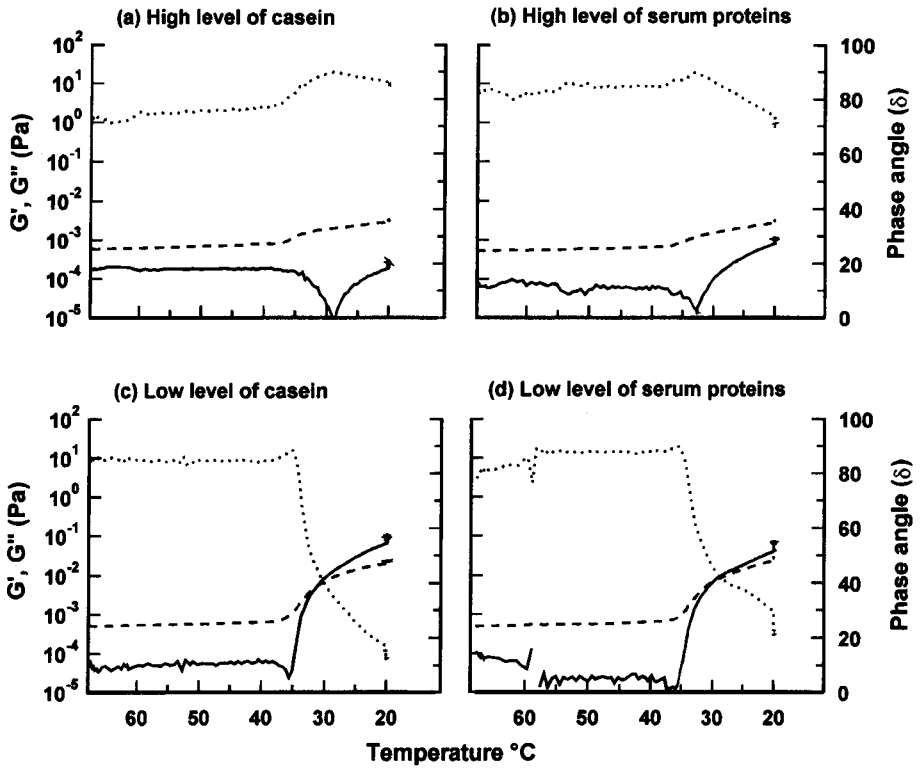

Figure 3 Effect of protein type and level on gel development kinetics measuring G' —, G'' - - - and δ · · · during cooling of κ-carrageenan (0.012% w/w) in milk ultrafiltrate containing caseins or whey proteins at the following levels: (a) high casein; (b) high whey proteins; (c) low casein; and (d) low whey proteins.

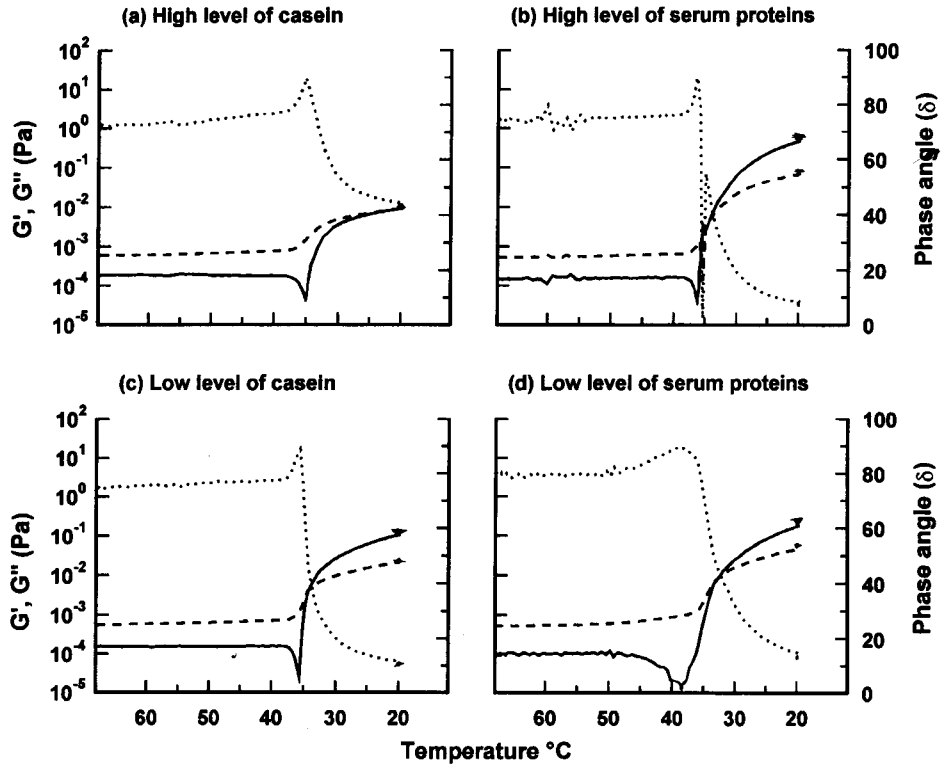

Figure 4 Effect of protein type and level on gel developing kinetics measuring G' —, G'' - - - and δ · · · during cooling of κ-carrageenan (0.018% w/w) in milk ultrafiltrate containing caseins of whey proteins at the following levels: (a) high casein; (b) high whey proteins; (c) low casein; and (d) low whey proteins.

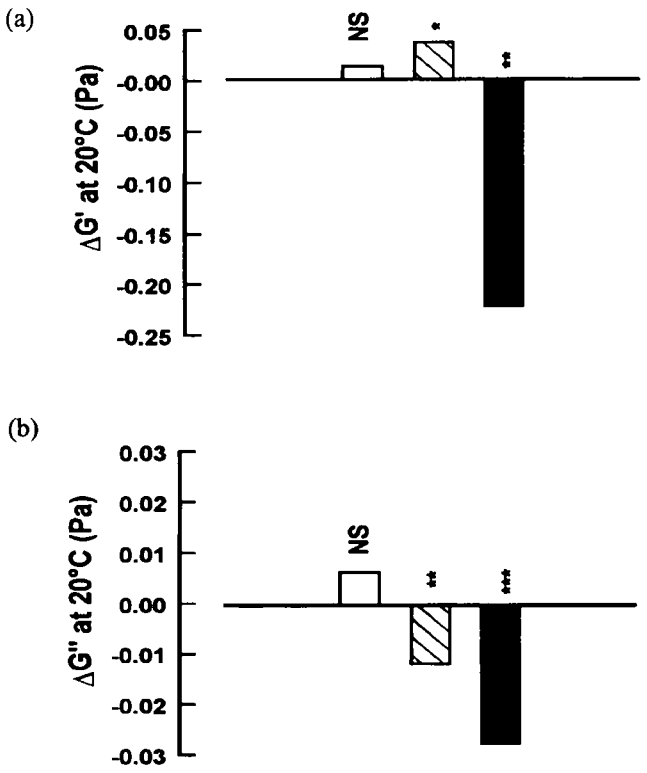

Figure 5 Plots of the estimated main effects of κ-carrageenan concentration □, protein level ▨ and protein type ■ on: (a) shift in mean value of G' at 20°C ($\Delta G'$); (b) shift in mean value of G'' at 20°C ($\Delta G''$) during gelation of κ-carrageenan–milk protein mixtures. Data are results of analysis of variance of measured parameters.

are influenced mainly by the concentration of carrageenan, ionic environment and pH. Milk proteins appear to have little effect on gelation at high levels of carrageenan. However, the role of milk proteins becomes important when κ-carrageenan concentration is <0.018% w/w. We found that at a given ionic strength and κ-carrageenan concentration, milk proteins interfere with gel formation due to the domination of protein–carrageenan interactions reducing the availability of carrageenan molecules for a gelation role. This phenomenon is non-specific, since although it is more pronounced with the micellar caseins, serum proteins also inhibit gelation, albeit at lower κ-carrageenan concentrations. Our results suggest that although interaction between κ-carrageenan and κ-casein cannot be ruled out, gel formation involves mainly carrageenan–carrageenan cross-linkages and not carrageenan–casein or casein–casein linkages.

Acknowledgements

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