Casein Interactions: Casting Light on the Black Boxes, the Structure in Dairy Products

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Casein Interactions: Casting Light on the *Black Boxes*, the Structure in Dairy Products

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ABSTRACT

This paper reviews the literature on the interactions of the caseins and suggests that the state of association of these proteins is governed by a balance of attractive hydrophobic interactions and electrostatic repulsion. The effects of temperature, pH and ionic strength on the self-association and calcium-induced aggregation of the individual caseins are rationalized in terms of their influence on this balance of forces. The discussion is then extended to the nature of the interactions prevailing in the casein micelle and a dual-bonding model of the casein micelle is formulated, reflecting the need for two different forms of bonding in the network. This model plausibly accounts for the effects of temperature, pH, ionic strength, micellar dissociating agents and solvent on the integrity of the micelle in terms of their influence on the hydrophobic/electrostatic balance of forces. Finally, it is postulated that the same type of bonding prevails in casein gels produced by renneting or acidification. The model is then used to explain the influence of temperature on rennet gel strength, the effects of forewarming on rennet and acid coagulation properties of milk, and the effect of temperature on the viscoelastic properties of a concentrated micellar suspension. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

It is now widely accepted that fermented milk gels and rennet curds are particle gels, networks built up of casein micelles or marginally modified micelles. These networks are a result of aggregation reactions brought about by modifying or reducing the repulsive interaction energy between the stable micelles. We believe that the measured viscoelastic properties of the network are an expression of the textural properties of the gelled product as perceived by the consumer. Since texture is a 'quality' attribute governing consumer acceptability, research institutes throughout the world have extensive programmes pursuing different aspects of this problem of understanding how these networks are structured and held together.

If we are to consider the rheological properties of our gel network, a very simple expression relates the complex shear modulus to the number of stress carrying bonds and the strength of those individual bonds. In recent publications, the nature of the particles, the casein micelles, and the morphological properties of the gel, the geometrical distribution of the network in space, have been reviewed (Holt and Horne, 1996; Horne, 1998). DeKruif (1998) has summarized an elegant approach using the colloidal theories of the adhesive sphere to treat the micellar protein surface as a polymer brush and to derive scaling relationships to link the various destabilization modes of the casein micelle—renneting, acidification, ethanol, ionic conditions. In this contribution, I will be concentrating on the nature of the bonds that are formed in the networks, raising some questions, puzzles and paradoxes, which will have to be considered before

we can readily tailor textural properties of dairy products to consumer requirements.

Among the puzzles are:

- The exact nature of the bonds within the casein micelle, those bonds controlling its internal structure.
- The temperature dependence of the bond strength.
- The beneficial influence of pre-heat treatment on the elastic properties of acid gels and the detrimental effect of the same treatment on rennet curd strengths.
- The viscoelastic properties of casein micelle suspensions and their temperature dependence.

Whether we talk of individual caseins and their association properties, or casein in stable casein micelles, or casein micelles in their destabilized situations in rennet or acid gels, we are still dealing with largely the same casein molecules. We are not considering situations where the molecule has undergone excessive heat treatment and hence possibly suffered some chemical modifications to its residues. If it is still the same molecule, then its interactions must also be the same, the same capacity for hydrophobic interaction, for hydrogen bonding or electrostatic repulsion. So, our proposal, in this paper, is to review the considerable literature on casein interactions and to attempt to explain the puzzles and paradoxes listed above by application of that knowledge.

CASEIN INTERACTIONS

Recently, Dickinson *et al.* (1997) have been applying the self-consistent-field theory of Scheutjens and Fleer

(1979, 1980) to calculate the conformation of β-casein and α_{s1}-casein adsorbed at a planar hydrophobic interface. Whilst the calculations have provided extensive predictions of layer properties and conformation borne out by many experimental results, the most important conclusion from the point of view of the behaviour of the caseins as emulsion stabilisers is the prediction of a tail-train structure for the adsorbed β-casein molecule and a train-loop-train structure for α_{s1}-casein with anchor points at both ends of the molecule (Dickinson *et al.*, 1997). Schematic representations of these structures are given in Fig. 1.

This result is introduced here because the hydrophobic surface imitating the emulsion droplet air/water interface in the model calculations could equally be the hydrophobic region of another casein molecule. Self-association of β-casein molecules could then produce the hedgehog like micellar polymer of Fig. 2b with their hydrophobic trains arrayed in a parallel/anti-parallel sequence. Such assembly would require a degree of cooperativity characterized by a monomer $\rightleftharpoons$ micelle equilibrium of the type observed for this system by Payens and co-workers (Payens and Van Markwijk, 1963; Payens *et al.*, 1969). With its central core and less dense coating of repellent tails, such a model structure is also similar to the ellipsoid structure envisaged by Kajiwara *et al.* (1988) with a hairy outer layer giving it the required larger hydrodynamic volume than its radius of gyration would merit on a uniform density basis.

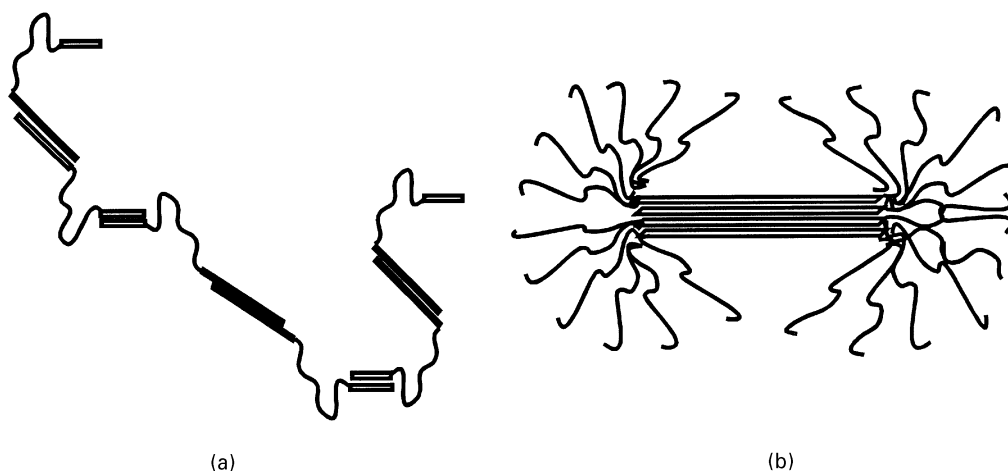Similarly, in a solution environment, it is unlikely that the two hydrophobic ends of the α_{s1}-casein molecule would link intra molecularly to form a ring but rather that such linkages would form intermolecularly to produce the worm-like polymeric chain of Fig. 2a, again as observed by Payens and Schmidt (Payens and Schmidt, 1966; Schmidt, 1970a, b). Though we have no further evidence based on self-consistent-field calculations to substantiate the model, we would anticipate that α_{s2}-casein with a similar distribution of charge and hydrophobicity would show a similar pattern of self-association to α_{s1}-casein. κ-Casein is the mirror image of β-casein in the distribution of its hydrophobic and charged residues with a predominantly hydrophobic neutral *N*-terminal region and a highly charged *C*-terminal peptide. Hence it too exhibits a monomer $\rightleftharpoons$ micelle equilibrium at moderate protein concentrations without the marked temperature dependence in the transition found with β-casein (Vreeman *et al.*, 1977; Vreeman, 1979).

Self-association of the caseins is thus driven by hydrophobic interactions but electrostatic repulsive interactions are also important because these define the degree of polymerisation and limit further growth. Thus increasing pH, which increases protein charge, decreases the polymer size for both α_{s1}-casein and β-casein whereas increasing ionic strength, which decreases the range of the electrostatic repulsion component, allows the formation of larger polymers of both casein species (Payens *et al.*, 1969; Schmidt, 1970a, b).

It is important to note that the shielding of electrostatic charge is a non-specific effect of ionic strength which moderates the range of the electrostatic interaction

Fig. 1. Schematic diagrams, based on predictions of self-consistent-field calculations, depicting typical conformations of (a) α_{s1}-casein and (b) β-casein adsorbed onto planar hydrophobic interface. Bars are simply used to denote hydrophobic regions of molecules and do not imply rigidity.

Fig. 2. Diagrammatic representations of polymeric structures of (a) α_{s1}-casein and (b) β-casein, showing linkages through interaction of hydrophobic regions of the molecules (rectangular bars), with α_{s1}-casein forming a linear polymer and β-casein giving a surfactant-like micellar structure.

but does nothing to change the magnitude of the charge that is the source of the interaction. Such changes in charge and the overcoming of these electrostatic repulsions can be accomplished in two ways, by lowering pH and titrating away sufficient of the charge of the phosphoseryl and carboxyl groups to induce acid precipitation of the caseins at their isoelectric points, or, in the case of the highly phosphorylated members, by precipitating these in the presence of ionic calcium, the order of sensitivity being $\alpha_{s2} > \alpha_{s1} > \beta$ (Holt, 1992). In the case of α_{s1}-casein, the aggregation reaction shows a lag-phase with little change in molecular weight with time until a critical time beyond which a rapid increase in molecular weight occurs. Horne and Dalgleish (1980) demonstrated that the logarithm of this critical coagulation time was a linear function of Q^2, where Q is the net negative charge of the protein taking into account the number of calcium ions bound to the protein at the level of calcium employed. Thus $Q = -22 + 2n$ where -22 is the protein charge at pH 7 and n is the number of calcium ions bound. Moreover, this equation held quantitatively when the charge on the protein was modified by reaction of positively charged lysyl residues with dansyl chloride or fluorescamine (Horne, 1983). Effectively this model treats the casein as a hydrophobic colloid where the energy of interaction between molecules is calculated as the sum of electrostatic repulsion and hydrophobic attraction as

$$\begin{matrix} \text{Interaction} \\ \text{energy} \end{matrix} = \begin{matrix} \text{Electrostatic} \\ \text{repulsion} \end{matrix} + \begin{matrix} \text{Hydrophobic} \\ \text{interaction} \end{matrix} . \quad (1)$$

The level of calcium binding is thus crucial to the aggregative stability of the protein. Dalgleish and Parker (1980) found binding to α_{s1}-casein to increase with increasing pH and temperature but to decrease as ionic strength was increased. However, in using this behaviour to estimate stability, the effects of pH on protein charge *per se* or of ionic strength on the range of electrostatic interaction should also be taken into account. Though no similar studies have been carried out for α_{s2}-casein, the pronounced calcium sensitivity of this protein would indicate that a similar mechanism would operate.

The aggregation of β-casein induced by Ca^{2+} ions shows a marked temperature dependence with no precipitation seen at $4°C$ (Parker and Dalgleish, 1981). This is similar behaviour to the self-association of this protein where individual molecules are dominant at low temperatures and micellar aggregates at $T \geq 20°C$ (Payens *et al.*, 1969). The presence of the lag phase in α_{s1}-casein aggregation by calcium has been interpreted as due to the existence of a nucleation and growth mechanism (Dalgleish *et al.*, 1981). We would postulate that the driving force for this nucleation is hydrophobic but that completion of this stage of the reaction is only achievable in β-casein solutions at higher temperatures. κ-Casein does not bind calcium to any great extent, hence it is not possible to overcome the electrostatic repulsion and induce aggregation of this protein in the manner postulated for the α_s- and β-caseins.

CASEIN MICELLE STRUCTURE

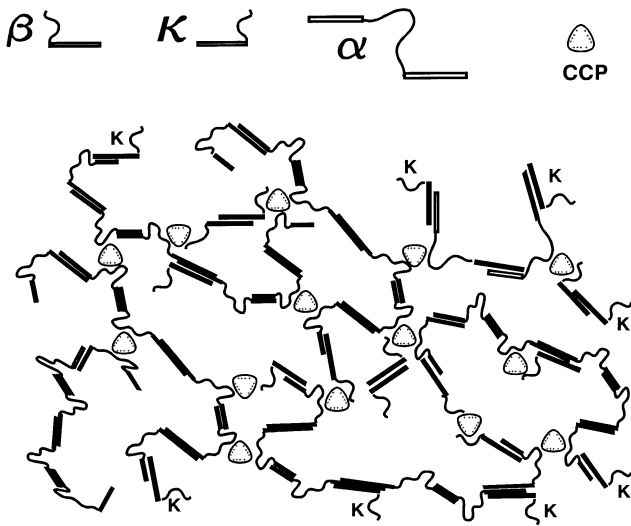Casein micelle structure and casein micelle models have been extensively reviewed (Schmidt, 1982; Walstra, 1990, 1998; Holt, 1992; Horne, 1992) and views of the subject have become deeply entrenched, particularly those on the existence of a sub-micellar structure. This section is not intended to be a thorough review of the background to this argument but rather an attempt to test the ability of the preceding ideas on casein interactions to explain the properties and behaviour of the caseins in micelle formation and disassembly.

In the sub-micellar models of Schmidt (1982) and Walstra (1990), where the individual caseins come together in their appropriate proportions to form internal submicelles, if depleted in κ-casein, or external sub-units rich in κ-casein, colloidal calcium phosphate is regarded as the cement which links these discrete sub-units together. In the model of Holt (1992) which regards the micelle as a mineralized, cross-linked protein gel, the colloidal calcium phosphate nanoclusters are the agents responsible for cross-linking the proteins and holding the network together.

Here we view the micellar calcium phosphate not just as cross-links but also as neutralizing agents which, being positively charged (Schmidt, 1982), bind to negatively charged phosphoserine clusters to reduce the protein charge to the level where the attractive interactions between the hydrophobic regions of the caseins can be allowed to dominate. That these latter interactions are essential for micellar integrity is demonstrated by the observations of McGann and Fox (1974) that the micelles can be extensively dissociated by urea, broken down by an agent which does not rupture the calcium phosphate linkages. Further, micellar integrity is largely maintained when the calcium phosphate is dissolved out by acidification (Dalgleish and Law, 1989), pointing to another casein binding interaction which we recognize here as hydrophobic. That the micelle is not dissociated by the loss of the colloidal calcium phosphate is due to the concurrent neutralization of the phosphoserine charge by the acid, thus maintaining an attractive interaction balance in favour of the hydrophobic interaction. If the milk is dialysed and the pH is then restored to that of the original milk, dissociation of the micelle complex is observed (Lucey *et al.*, 1997) as electrostatic repulsion becomes dominant in our interaction equation. The same dissociation is observed at natural pH when the calcium is removed by sequestration with EDTA (Griffin *et al.*, 1988) or following loss of the calcium phosphate by dialysis against a phosphate-free buffer (Holt *et al.*, 1986). The latter treatments could be interpreted as simple removal of the calcium phosphate cross-links, but fit equally well into the scenario where they reinstate phosphoserine charge and allow dominant electrostatic effects to loosen the hold of hydrophobic bonding between the casein molecules.

Hydrophobic interactions must also be invoked to link the κ-casein to the micelle. The only phosphoseryl residue in this member of the casein family lies in the macropeptide which forms the putative hairy layer deemed essential for micellar stability in all accepted models. This residue is not involved in any cross-linking via colloidal calcium phosphate. The alternative, overlooked in the Holt (1992) model, but satisfied by the orientation of the molecule, sees a strong hydrophobic bonding of the *N*-terminal region of κ-casein to the rest of the micelle.

The growth of the micelle is thus envisaged as depicted in Fig. 3. Two types of linkage between protein molecules

Fig. 3. Dual bonding model of structure of casein micelle, with α, β and κ-casein depicted as indicated. Bonding occurs between the hydrophobic regions, shown as rectangular bars, and by linkage of hydrophilic regions containing phosphoserine clusters to colloidal calcium phosphate clusters (CCP). Molecules of κ-casein limit further growth, as explained in text, and are labelled with letter 'K'.

are postulated. The first is hydrophobic where two or more hydrophobic regions from different molecules form a bonded cluster. Growth of these polymers is inhibited by the protein charged residues whose repulsion pushes up the interaction free energy. Neutralization of the phosphoserine clusters by incorporation into the colloidal calcium phosphate diminishes that free energy as well as producing the second type of cross-linking bridge, since it is considered that up to four or more phosphoserine clusters from different casein molecules can be accommodated at each calcium phosphate nanocluster (Holt, 1992). Though the κ-casein molecules can interact via their hydrophobic domains with the hydrophobic regions of the other caseins, further growth beyond the κ-casein is not possible because it possesses neither a phosphoserine cluster for linkage via colloidal calcium phosphate, nor another hydrophobic anchor point to extend the chain via this route. κ-Casein acts as a terminator for both types of growth and, unless circumvented by the growing network, will become part of the surface structure of the micelle. Hence its surface location arises naturally in this model.

Maintenance of micellar integrity is, however, still a balancing act. Increasing pH from the natural value in milk leads to dissociation of the micelles. Whether this is due to conversion of the phosphoseryl residues from singly to doubly negatively charged units which are no longer capable of linking to the colloidal calcium phosphate nanoclusters or whether the increase in charge itself is sufficient, the balance of eqn (1) swings in favour of electrostatic repulsion and the micelles dissociate, as is evidenced by the translucent appearance of the milk at pH values greater than 8. Increasing ionic strength also leads to micellar dissociation (Saito, 1973), possibly because it decreases the pK values of the acidic groups and hence increases protein charge but with possible other influences on the calcium phosphate equilibria about which we could only speculate.

MICELLAR INTERACTIONS

The concept of the casein micelle electrosterically stabilized by a 'hairy layer' coat of κ-casein (Holt, 1975; Walstra, 1979) seems to enjoy universal acceptance. In the picture being developed herein, the highly charged macropeptide tail physically prevents the approach and interaction of hydrophobic regions of the casein molecules which would allow aggregation and gelation to take place. Direct removal of the macropeptide by cleavage with chymosin, neutralization of its charge and any remaining charge on the caseins and its collapse in the non-solvent ethanol are all methods for reducing its effectiveness. That hydrophobic interactions then play a part in the ensuing micellar aggregation and network growth is evidenced by the fact that neither fully renneted micelles nor acidified milk systems at their isoelectric point show any signs of aggregation at low temperatures ($<10°C$).

The destabilization of casein micelles by ethanol has previously been rationalized in a very similar scheme to that outlined here (Horne and Muir, 1990; Horne, 1992). Light scattering studies (Horne, 1984, 1986; Horne and Davidson, 1986) revealed the collapse of the hairy layer and the consequent loss of the stabilizing steric component with subcritical concentrations of ethanol but there are numerous effects pointing to the involvement of electrostatic influences in the remaining micellar interactions. Amongst these are the influence of ionic calcium levels and pH on the ethanol stability (Horne and Parker, 1981a, b); the demonstration using a range of alcohols that destabilization is achieved at a critical dielectric constant value (Horne and Parker, 1981c); the observation that the effects of chemical modification of the protein by a variety of agents can be quantitatively explained by the change in net protein charge (Horne and Parker, 1982); the effects of forewarming and its influence on calcium phosphate solubility increasing protein charge and ethanol stability (Horne and Parker, 1981d). All of these effects can be reconciled within this model of a balance of forces which has to swing in favour of hydrophobic interaction to allow aggregation to proceed. Given the success of the model in explaining ethanol stability effects, our hypothesis is that the bonding in acid and rennet gel networks is the same balance of electrostatic repulsion and hydrophobic interaction. The remainder of the paper is devoted to rationalizing the response of these gels to changes in temperature, pH and ionic strength in terms of the effects such changes have produced in casein self-association or micelle formation and thus validating our model.

MICELLAR GELS

Horne (1995, 1996) recently developed a mean field theory of gel strength based partly on his observation of scaling behaviour in the kinetic profiles of gel formation during renneting or acidification of skim milk. The scaling parameters were found to be the gelation time, t_g, defined as the time at which a measurable complex shear modulus becomes detectable above instrumental noise, and G_∞, the shear modulus value at infinite time. A consequence of the scaling behaviour, however, is that the shear modulus at any chosen multiple of the coagulation time is a fixed fraction of that shear modulus at $t = \infty$

(Horne, 1995). Thus, the behaviour of the shear modulus, G^*, at say $2t_g$, will mimic in all respects the behaviour of the ultimate gel strength, G_∞, its dependence on composition, temperature, pH, etc.

Temperature dependence of gel strength in rennet curds

As part of an extensive study of the factors influencing the values of the scaling parameters, measurements were made of the kinetics of rennet curd formation as a function of temperature, maintaining all other reaction and instrumental conditions constant. The resulting plot of shear modulus at $2t_g$ as a function of reaction temperatures between 20 and 45°C is shown in Fig. 4. As can be seen, the gel strength increases strongly and linearly to reach a maximum between 35 and 40°C and thereafter declines to a value at 45°C little greater than that at 20°C. Can this maximum in gel strength as a function of incubation temperature be reconciled within the hypothesized framework of molecular interactions?

The linear increase with temperature on the low side of the maximum, we would anticipate from the increasing strength of hydrophobic interactions with temperature. An increase in temperature, however, also promotes calcium binding (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981) which would decrease protein charge and electrostatic repulsion, further swinging the interaction energy balance in favour of attraction and greater bond strength. Yet another effect of temperature rise in this system is a decrease in the solubility of calcium phosphate. Anyone who has prepared a milk ultrafiltrate permeate will know that raising the temperature will cause it to lose its initial clarity and become turbid due to the precipitation of calcium phosphate. A consequence of this behaviour in a renneted milk gel could be a change in the status or nature of the colloidal calcium phosphate, a withdrawal of calcium bound to casein and a consequent increase in the protein charge. The latter effect, even if it did not lead to dissociation of the micelles would cause a weakening of the bonds, measurable as the decrease we see in gel strength. Loosening of the internal
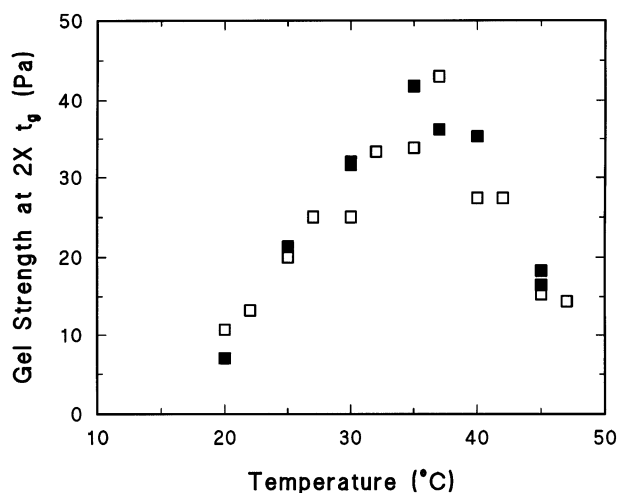
structure of the micelle as temperature is increased has also been observed by nmr measurements as an increase in the mobility of protons on amino acid side chains of the caseins (Rollema and Branches, 1989), pointing to a 'melting' of the structure.

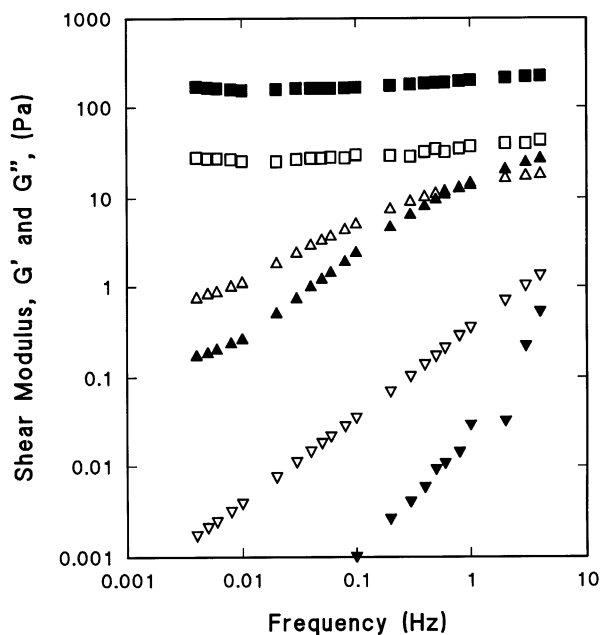## Effect of forewarming on acid and rennet gels

One of the paradoxes of forewarming a milk (by heating it at 90°C for 10 min or more) is that it produces stronger gels in fermented milks (Dannenberg and Kessler, 1988; Horne and Davidson, 1993) but inhibits curd formation on renneting (Marshall, 1986; Van Hooydonk *et al.*, 1987; Dalgleish, 1990). Forewarming also has a beneficial effect in increasing ethanol stability at natural pH (Horne and Parker, 1981d). Such heat treatment leads to the formation of a disulphide bridged complex between κ-casein and β-lactoglobulin (Snoeren and Van der Spek, 1977; Smits and Van Brouwershaven, 1980; Van Hooydonk *et al.*, 1987; Law *et al.*, 1994).

Since both cysteine residues in κ-casein are located in the hydrophobic N-terminal para-κ-casein which remains with the micelle on renneting, the covalently linked β-lactoglobulin will also remain with the micelle. The negative charges on this molecule will shift the balance of the intermicellar interaction following renneting, if not away from aggregation entirely, at least in a manner which will reduce the rate of aggregation. Singh and Fox (1985) have also found that, at pH values greater than 6.7, the β-lactoglobulin—κ-casein complex dissociates from the micelle. Presumably the increase in pH has brought about an increase in charge on the molecules constituting the sub-κ-casein layer near the surface of the micelle. This increase in electrostatic repulsion between the complex and the sub-surface layer would then produce the observed dissociation.

In the acid gel situation forewarmed milks have been observed to gel at a critical pH higher than that found with an non-heat-treated milk (Horne and Davidson, 1993). The forewarmed and non-forewarmed milks also follow different master curves in their scaling behaviour (Horne, 1998), indicating that the heat treatment has brought about a change in the mechanism of the aggregation/gelation reaction. The transition between the two limiting conditions has been linked to the denaturation reaction of the whey proteins (Horne and Davidson, 1993). A further factor implicating the whey proteins is the realization that the critical pH in forewarmed milks is close to their isoelectric point. From the point of view of the interaction balance hypothesis, the isoelectric point is the point of net zero charge and hence of minimum electrostatic repulsion. Hydrophobic interaction is allowed to dominate and aggregation and gelation follow.

Separated concentrated casein micelles

For the final example, we consider the interactions within the concentrated casein micelle suspensions prepared by high-speed centrifugation of milk, a method commonly employed to separate casein micelles from other milk constituents. Depending on the length of drainage time allowed, the casein pellets contain between 17 and 22% protein. We have examined the rheological properties of this pellet as a function of oscillation frequency and the temperature to which the pellet is subsequently adjusted.

Fig. 4. Gel strength in rennet gels as a function of temperature of formation. Plots are shown for two levels of chymosin concentration, demonstrating that the effect is independent of enzyme activity. Gel strength is taken as the shear modulus value reached at twice the measured coagulation time.

Fig. 5. Viscoelastic behaviour of pelleted fraction of casein micelles, measured at selected temperatures as a function of frequency. Micelles were pelleted from skim milk by centrifugation at 19,000 rpm for 60 min in the SS34 rotor of a Sorvall RC-5B centrifuge. Measurements were performed using double-gap geometry on a Bohlin CVO rheometer, with an applied stress of 0.05 Pa. Open symbols denote viscous modulus, G'', in all cases and solid symbols, the elastic modulus, G''. Measuring temperatures were 5°C (■), 15°C (▲) and 30°C (▼).

At high temperature (40°C) the pellet flows freely. Its viscosity is Newtonian and independent of frequency. At low temperature (5 and 10°C), this micellar suspension shows all the properties of a classical viscoelastic gel with elastic moduli independent of frequency and phase angle less than 45° (Fig. 5).

These suspensions are largely free from soluble calcium phosphate and other milk salts. The temperature dependent properties to be considered are those of the caseins and the micelles themselves, in particular the temperature dependence of the hydrophobic interactions. At low temperatures, the strength of such interactions is low. Both β-casein and κ-casein are known to depart from the micelle under such conditions (Dalgleish and Law, 1988) but in the close packed conditions prevailing in the micelle pellet they are liable to link across to neighbouring micelles or become entangled with protein loosened from these neighbours. As temperature is increased, the strength of the hydrophobic interactions increases, the micelles are tightened up, as it were, and become more compact, allowing the suspension to flow freely.

CONCLUSIONS

Our working hypothesis in this paper is that the bonding in various casein systems of industrial and commercial interest is no different from that prevailing between self-associating individual caseins, namely a balance of electrostatic repulsion and attractive hydrophobic interactions. In a series of examples ranging from the internal

structure of casein micelles to casein gel systems, we have plausibly rationalized various aspects of the behaviour of these systems in terms of the known effects of pH, temperature and ionic strength on the same basic interactions between the individual proteins themselves. If we stand back and apply the knowledge we already have, we may find that we know more than we realize about how casein gel structures are put together or how we may manipulate the textural properties of products based on these.

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