Stability of Casein Micelles Cross-Linked by Transglutaminase

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ABSTRACT

In this study, caseins micelles were internally cross-linked using the enzyme transglutaminase (TGase). The integrity of the micelles was examined on solubilization of micellar calcium phosphate (MCP) or on disruption of hydrophobic interactions and breakage of hydrogen bonds. The level of monomeric caseins, determined electrophoretically, decreased with increasing time of incubation with TGase at 30°C; after incubation for 24 h, no monomeric β - or κ -caseins were detected, whereas only a small level of monomeric α_{S1} -casein remained, suggesting near complete intramicellar cross-linking. The ability of casein micelles to maintain structural integrity on disruption of hydrophobic interactions (using urea, sodium dodecyl sulfate, or heating in the presence of ethanol), solubilization of MCP (using the calcium-chelating agent trisodium citrate) or high-pressure treatment was estimated by measurement of the L^* -value of milk; i.e., the amount of back-scattered light. The amount of light scattered by casein micelles in noncross-linked milk was reduced by >95% on complete disruption of hydrophobic interactions or complete solubilization of MCP; treatment of milk with TGase increased the stability of casein micelles against disruption by all methods studied and stability increased progressively with incubation time. After 24 h of cross-linking, reductions in the extent of light scattering were still apparent in the presence of high levels of dissociating agents, possibly through citrate-induced removal of MCP nanoclusters from the micelles, or urea- or sodium dodecyl sulfate-induced increases in solvent refractive index, which reduce the extent of light-scattering.

Key words: milk, casein micelle, transglutaminase, micellar stability

INTRODUCTION

The caseins are a class of 4 phosphoproteins (α_{S1} -, α_{S2} -, β -, and κ -casein) that represent the majority of

protein in milk from most mammalian species. Although some caseins are present in the serum phase of milk, most caseins are found in association colloids called casein micelles. Such casein micelles range in diameter from 50 to 300 nm, are highly hydrated, and contain, on a DM basis, ~94% casein and ~6% inorganic materials. These inorganic materials are collectively referred to as micellar calcium phosphate (MCP) and consist primarily of calcium and phosphate, with lower levels of magnesium and citrate also present (Fox, 2003). Although only a minor constituent of casein micelles on a weight basis, MCP plays a crucial role in maintaining micellar integrity. According to the model for casein micelle structure described by De Kruif and Holt (2003), so-called nanoclusters, which contain an amorphous MCP core with a diameter of 2 to 3 nm and are surrounded by a shell of caseins, are randomly distributed throughout the casein micelle. Three of the caseins (α_{S1} -, α_{S2} -, and β -casein) contain centers of phosphorylation (at least 3 phosphoserine residues in close proximity) that can bind to the amorphous MCP cluster, thereby forming a stabilizing protein shell (De Kruif and Holt, 2003). Both α_{S1} - and α_{S2} -casein contain more than one phosphate center and can thus act as a linking agent between nanoclusters (De Kruif and Holt, 2003). Such cross-linking enables nanoclusters to associate to form casein micelles, a process that is further aided by balance between intermolecular attractive hydrophobic interactions and electrostatic interactions, which may be attractive or repulsive (De Kruif and Holt, 2003). A casein micelle of 100 nm in size contains ~800 nanoclusters (Holt et al., 2003). The surface of the micelles is covered primarily with κ -casein, which provides intermicellar electrostatic and steric repulsion, according to De Kruif and Zhulina (1996) in a manner similar to a polyelectrolyte brush.

The stability of casein micelles may be divided into 2 categories—intermicellar and intramicellar stability. The intermicellar, or colloidal, stability of casein micelles, denotes the stability of casein micelles against aggregation; for example, under the influence of heat, ethanol, acid, or rennet. Such stabilities are well characterized and, in some cases, form the basis of the conversion of milk into other dairy products; for example,

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rennet- or acid-induced coagulation of milk forms the basis of the manufacture of cheese or yogurt, respectively. The intramicellar stability; that is, the ability of the casein micelle to maintain its internal structural integrity under the influence of environmental changes, is of considerable influence for properties of products derived from milk. As described above, MCP and hydrophobic interactions are primary features in maintaining micellar integrity. The integrity and stability of casein micelles is very good from a colloidal point of view, because micelles remain stable on drying, freezing, and addition of salts. Solubilization of MCP, most easily achieved through addition of a calcium-chelating agent, results in disintegration of the casein micelles (Odagiri and Nickerson, 1964; Morr, 1967; De Kruif and Holt, 2003), probably into small, hydrophobically bound, casein aggregates. Furthermore, treatment of milk at high hydrostatic pressure can result in considerable disruption of casein micelles (Huppertz et al., 2004a,b, 2006; Anema et al., 2005), presumably also through solubilization of MCP (Huppertz et al., 2006). Disruption of casein micelles can also be achieved through disruption of hydrophobic interactions; for example, through addition of urea (McGann and Fox, 1974; Aoki et al., 1986; Holt, 1998; De Kruif and Holt, 2003) or SDS (Lefebvre-Cases et al., 1998, 2001), or heating milk to >60°C in the presence of >30% ethanol (O'Connell et al., 2001a,b).

As a result of dissociation of casein micelles under the influence of such environmental conditions, average particle size in skimmed milk is reduced considerably, often to a size nondetectable by many traditionally particle size analysis techniques. Because of reductions in particle size of its constituent colloids, the ability of milk (or any other colloidal suspension) to scatter light is reduced considerably, as indicated by reductions in turbidity, a measure of the total amount of scattered light, or by the L^* -value of milk, a measure of the amount of back-scattered light (see Van der Hulst, 1957; Bohren and Huffman, 2004).

Increasing the stability of casein micelles against disruption may positively affect the functional properties of the milk; for example, reducing the extent of heat-induced dissociation of κ -casein from the micelle can enhance the stability of milk against heat-induced coagulation (O'Connell and Fox, 2003). Traditionally, covalent intermolecular cross-linking of proteins was achieved through addition of glutaraldehyde (Anderson et al., 1984), but this agent is not permitted for use in food products due to its toxicity. However, over the last 10 yr, it has been established that treatment of milk with the enzyme transglutaminase (**TGase**) can result in covalent intramicellar cross-linking of caseins (Traore and Meunier, 1991, 1992; Lorenzen, 2000a,b;

Sharma et al., 2001); such treatment can indeed, through prevention of heat-induced dissociation of κ -casein, increase the heat stability of milk (O'Sullivan et al., 2001, 2002a). Furthermore, O'Sullivan et al. (2002b) reported that treatment of milk with TGase can increase the stability of casein micelles against disruption on addition of urea or citrate, heating in the presence of ethanol, or high-pressure (**HP**) treatment. These potentially significant findings by O'Sullivan et al. (2002b) were studied over a limited range of experimental conditions; hence, further study of these phenomena appears warranted. In this communication, studies on the effect of TGase-induced cross-linking on the stability of casein micelles against disruption under the influence of urea, SDS, citrate, heating in the presence of ethanol, or HP treatment, are reported. The studies cover a wide range of environmental conditions and incubation times, with the aim of providing a more complete understanding of the potential of TGase-induced cross-linking to increase micellar stability.

MATERIALS AND METHODS

Incubation of Milk with TGase

Skim milk, containing 3.21% protein, was prepared by reconstituting low-heat skim milk powder (Irish Dairy Board, Dublin, Ireland) in demineralized H_2O at a level of 9% (wt/wt); sodium azide (0.5 g/L) was added to the milk to prevent microbial growth. The milk was stored overnight at 5°C to ensure complete equilibration of minerals and hydration of casein micelles. Milk was subsequently warmed to 30°C, TGase (Activa TG, with a declared activity of ~1,000 units/g, a gift from Ajinomoto Europe Sales, Hamburg, Germany) was added at a level of 0.10 g/L, and milk was incubated at 30°C for 0 to 24 h. Following incubation, TGase was inactivated by heating at 70°C for 10 min, followed by rapid cooling to room temperature in ice-water. Incubation experiments were repeated 3 times on individual milk samples.

Estimation of the Extent of TGase-Induced Cross-Linking

The extent of TGase-induced cross-linking of milk proteins was estimated by SDS-PAGE under reducing conditions using a separating and stacking gel containing 15.0 or 4.0% acrylamide, respectively. Gels were run at 200 V, stained using 0.1% (wt/vol) Coomassie Brilliant Blue R250 in a 5:1:4 mixture of methanol, acetic acid, and deionized water, and destained in a 7:5:88 mixture of methanol, acetic acid, and deionized water; destained gels were scanned using a BioRad

GS800 calibrated densitometer (BioRad Laboratories, Hercules, CA).

Estimation of the Stability of Casein Micelles

To study the influence of added urea, SDS, or trisodium citrate on the stability of casein micelles, untreated or TGase-treated milks were mixed with an equal volume of 0.0 to 12.0 *M* urea, 0.0 to 4.0% (wt/vol) SDS, or 0 to 100 mM trisodium citrate, and left for 30 min. Subsequently, the extent of disruption was estimated as the amount of back-scattered light by determination of the L^* -value of milk using a Minolta CR300 colorimeter (Minolta Camera Co., Osaka, Japan); acquired L^* -values were normalized to values ranging from 0 to 1 using the respective values of untreated milk (i.e., milk diluted with an equal volume of demineralized water) and milk serum, according to

$$L^*_{\text{normalized}} (L^*_n) = \frac{[L^*_{\text{sample}} - L^*_{\text{serum}}]}{[L^*_{\text{untreated}} - L^*_{\text{serum}}]}$$

To study the influence of heating in the presence of ethanol on micellar stability, untreated or TGase-treated milk was mixed with an equal volume of 70% (vol/vol) ethanol, and subsequently heated at 60, 70 or 80°C for 10 min, followed by determination and normalization of the L^* -value, as described above.

The stability of milk against HP-induced disruption was estimated by determination of the L^* -value of milk immediately after treatment at 300, 400, 500, or 600 MPa for 15 min at 20°C using a Stansted Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, UK) using a 90:10 mixture of ethanol and castor oil as the pressure-transmitting medium, as described by Huppertz et al. (2004a). L^* -values were normalized as described above, using the value of milk before enzymatic cross-linking and HP treatment as the untreated sample.

RESULTS AND DISCUSSION

TGase-Induced Cross-Linking of Caseins

Incubation of milk with TGase at 30°C for 0 to 24 h resulted in a progressive reduction of the levels of monomeric α_{S1} -, β -, and κ -caseins, as determined by SDS-PAGE (Figure 1); such reductions in monomeric caseins are due to TGase-induced cross-linking of the caseins. Transglutaminase catalyzes the formation of intermolecular cross-links between protein molecules through the transfer of an acyl group between the γ -carboxyamide group of a peptide-bound glutamine residue (acyl donor) and the primary amino group of an

Figure 1. Sodium dodecyl sulfate-PAGE electrophoretogram of reconstituted low heat skim milk powder after incubation with 0.10 g/L of transglutaminase for 0 (lane 1), 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), 6 (lane 7), 7 (lane 8), 8 (lane 9), or 24 (lane 10) h at 30°C.

amine (acyl acceptor; Zhu et al., 1995). Caseins have been shown to be excellent substrates for TGase-induced cross-linking (Ikura et al., 1980; Traore and Meunier, 1991, 1992; Lorenzen, 2000a,b; Sharma et al., 2001). Transglutaminase-induced reductions in levels of monomeric caseins were accompanied by the appearance of fractions of relatively low electrophoretic mobility, presumably cross-linked casein polymers (Figure 1). The level of polymers increased with incubation time up to 2 h, but decreased on prolonged incubation, which is probably due to the fact that polymers became too large to enter the gel.

Monomeric κ - or β -caseins were reduced to nondetectable levels after incubation for 5 or 24 h, respectively, whereas a small level of monomeric α_{S1} -casein remained after 24 h of incubation (Figure 1). A similar order of rate of cross-linking: κ - > β - > α_{S1} -casein, was reported by Sharma et al. (2001); differences among caseins in their susceptibility to TGase-induced cross-linking in milk are probably related to their specific location in the micelle, rather than differences in specificity between caseins, because Ikura et al. (1980) showed that, in isolated form, κ -casein was less susceptible to TGase-induced cross-linking than was α_{S1} - or β -casein. In milk, caseins exist primarily in the form of casein micelles. The surface of the casein micelle is covered primarily with κ -casein as well as some β -casein, whereas α_{S1} -casein is found primarily in the core of the micelle, often associated with the MCP nanoclusters

Figure 2. Influence of addition of trisodium citrate to a final concentration of 0 (●), 10 (○), 20 (▼), 30 (▽), 40 (■), or 50 (□) mmol/L on the normalized L^* -value of milk incubated with 0.10 g/L of transglutaminase at 30°C for 0 to 24 h. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

(De Kruif and Holt, 2003). Thus, considering their more readily accessible locations in the casein micelle, it is not surprising that β - and κ -casein are cross-linked more readily in milk than α_{S1} -casein.

The normalized L^* -value of milk (L_n^*) was not affected by treatment with TGase (Figure 2), suggesting that such treatment has little effect on the size of the micelles.

Stability of TGase-Treated Casein Micelles Against Calcium Chelation

Addition of trisodium citrate to untreated milk (0 h) progressively reduced L_n^* to <0.1 on addition of 40 or 50 mM trisodium citrate (Figure 2); such reductions in L_n^* indicate extensive citrate-induced disruption of casein micelles, as previously noted by Odagiri and Nickerson (1964), Morr (1967), and Mizuno and Lucey (2005). Citrate-induced disruption of casein micelles is due to the calcium-chelating activity of citrate, which disrupts MCP nanoclusters and thus compromises micellar integrity. Citrate-induced reductions in L_n^* decreased progressively with increasing incubation time with TGase (Figure 2). However, even after 24 h of TGase treatment, where little residual monomeric casein was observed (Figure 1), and hence a high micellar integrity would be expected, reductions in L_n^* by up to 50% were observed; possible reasons for such discrepancies are discussed later.

Figure 3. Influence of addition of urea to a final concentration of 0 (●), 2 (○), 3 (▼), 4 (▽), or 6 (■) mol/L on the normalized L^* -value of milk incubated with 0.10 g/L of transglutaminase at 30°C for 0 to 24 h. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

Stability of TGase-Treated Casein Micelles Against Disruption of Hydrophobic Interactions

Addition of urea to untreated milk (0 h) progressively reduced L_n^* to <0.01 on addition of 6 M urea (Figure 3), indicating extensive urea-induced disruption of casein micelles. Disruption of casein micelles through addition of urea is probably due to the fact that urea is capable of breaking hydrogen bonds and disrupting hydrophobic interactions. Urea has the ability to form hydrogen bonds and may actively compete with existing inter- and intramolecular hydrogen bonds stabilizing proteins (Creighton, 1984; Damodaran, 1996; Nandel et al., 1998). Furthermore, adding urea increases the solubility of hydrophobic amino acids because urea, through its ability to form hydrogen bonds, disrupts the hydrogen-bonded structure of water, thereby increasing its solvent quality for hydrophobic residues (Creighton, 1984; Damodaran, 1996; Nandel et al., 1998). A reduction in the strength of hydrophobic interactions may result in intermolecular repulsive forces becoming dominant over attractive forces, ultimately leading to disruption of casein micelles. Based on the nanocluster model for the internal structure of casein micelles, one would expect high concentrations of urea to disrupt casein micelles into nanoclusters and free caseins in the presence of high concentrations of urea, as also strongly suggested by experimental data from Aoki et al. (1986) and Holt (1998). The sharp increase in L_n^* at 6 mol/L of urea between 5 and 6 h of incubation with TGase (Figure 3) suggests a 2-stage mechanism in the TGase-induced

Figure 4. Influence of addition of SDS to a final concentration of 0.00 (●), 0.25 (○), 0.50 (▼), 1.00 (▽), 1.50 (■), or 2.00 (□) % (wt/vol) on the normalized L^* -value of milk incubated with 0.10 g/L of transglutaminase at 30°C for 0 to 24 h. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

Figure 5. Influence of mixing milk, treated with 0.10 g/L of transglutaminase for 0 to 24 h at 30°C, with an equal volume of 70% (vol/vol) ethanol, followed by heating at 20 (●), 60 (○), 70 (▼), or 80 (▽) °C for 10 min on its normalized L^* -value. Values are means of data from triplicate experiments on individual milk samples with the standard deviation indicated by vertical error bars.

stabilization of casein micelles against disruption by 6 mol/L of urea.

Treatment of milk with TGase increased the stability of casein micelles against disruption by urea progressively with increasing treatment time (Figure 3) but, as for citrate (Figure 2), cross-linking with TGase for 24 h did not completely prevent urea-induced reductions in L_n^* .

Like urea, the anionic detergent SDS can disrupt hydrophobic interactions; the mechanism of SDS-induced disruption of hydrophobic interactions involves strong preferential binding of SDS to hydrophobic protein areas (Damodaran, 1996). Initial binding of SDS to proteins occurs via electrostatic interactions between the sulfate group of SDS and positively charged amino acid residues, but this is quickly dominated by the alkyl chain of SDS and hydrophobic amino acid side chains (Nozaki et al., 1974; Sudhindra and Prakash, 1993). As a result, addition of SDS to untreated milk results in disruption of casein micelles, as indicated in Figure 4 by reductions in L_n^* , and previously observed by Lefebvre-Cases et al. (1998, 2001). The stability of casein micelles against SDS-induced disruption increased progressively with incubation time with TGase, but complete prevention of SDS-induced reductions in L_n^* could not be established, even after 24 h of TGase treatment (Figure 4).

Addition of an equal volume of 70% (vol/vol) ethanol to milk, followed by warming to a temperature >60°C, also resulted in disruption of casein micelles (Figure

5). Ethanol-mediated, heat-induced disruption of casein micelles is presumably due to disruption of hydrophobic interactions as a result of increasing the solvent quality of the medium for hydrophobic residues, due to a reduced dielectric constant of the medium caused by added ethanol and increased temperature (O'Connell et al., 2001a,b). Treatment with TGase for 1 h greatly increased the stability of casein micelles against ethanol-mediated, heat-induced disruption, with only small further increases in micellar stability after longer treatment times with TGase (Figure 5); this suggests that only a small extent of TGase-induced cross-linking of caseins is required to increase micellar stability against ethanol-mediated heat-induced dissociation considerably, unlike micellar stability against high levels of SDS, which increased more gradually (Figure 4). Hence, it would appear that, although both SDS-induced and ethanol-mediated heat-induced disruption of casein micelles occur through disruption of hydrophobic interactions, their net effect on micelle stability differs considerably. Transglutaminase-induced increases in micellar stability against urea-induced disruption (Figure 3) increased more gradually than those in micellar stability against disruption by SDS (Figure 4) or heating in the presence of ethanol (Figure 5). This finding suggests that hydrogen bonding, which is disrupted by urea but not by SDS or heating in the presence of ethanol, is a major factor in maintaining micellar integrity.

Figure 6. Influence of high pressure treatment at 0.1 (●), 300 (○), 400 (▼), 500 (▽), or 600 (■) MPa for 10 min at 20°C on the normalized L^* -value of milk treated with 0.10 g/L of transglutaminase for 0 to 24 h at 30°C. Values are means of data from triplicate experiments on individual milk samples with the standard deviation indicated by vertical error bars.

Stability of TGase-Treated Casein Micelles Against High Pressure

Under HP treatment, considerable disruption of casein micelles occurs (Kromkamp et al., 1996; Huppertz et al., 2006), presumably as a result of HP-induced solubilization of MCP (Huppertz et al., 2006); as a result, average casein micelle size (Huppertz et al., 2004a; Anema et al., 2005) and the L^* -value (Johnston et al., 1992; Huppertz et al., 2004b) of milk treated at 300 to 800 MPa is considerably lower than in untreated milk. High-pressure treatment of untreated milk (0 h) at 300 to 600 MPa for 10 min reduced L_n^* progressively with increasing pressure, to ~0.25 at ≥500 MPa (Figure 6). The stability of casein micelles against HP-induced disruption increased progressively with incubation time with TGase, particularly during the first 5 h of incubation. After 24 h of TGase treatment, HP-induced reductions in L_n^* were <0.10 (Figure 6), indicating that the stability of extensively cross-linked casein micelles against HP-induced reductions in L_n^* is notably higher than that against reductions in L_n^* induced by, for example, citrate (Figure 3) or urea (Figure 4).

Kinetics of TGase-Induced Stabilization of Casein Micelles

From the data presented in Figures 2 to 6, it is clear that enzymatic cross-linking stabilizes the casein micelles against disruption with the degree of stability increasing with incubation time with TGase. The exper-

imental data in Figures 2 to 6 have the form of the following function:

$$L_n^*(t) = a \{1 - b \cdot e^{(-c \cdot t)}\}$$

From this function, it can be derived that

$$L_n^*(t = 0) = a(1 - b)$$

and

$$L_n^*(t = \infty) = a$$

Thus,

$$b = 1 - \{L_n^*(t = 0) / L_n^*(t = \infty)\}$$

Assuming $L_n^*(t = \infty) = L_n^*(t = 24 \text{ h})$, which is justified because increasing treatment time to >24 h or increasing TGase concentration 5-fold for milk incubated for 24 h did not further increase micelle stability (data not shown), all data can be well reproduced by adjusting the only free variable, c . Optimal data fits for milk containing 50 mmol/L of citrate, 6 mol/L of urea, or 2.0% (wt/vol) SDS, milk heated in the presence of 35% (vol/vol) ethanol to 80°C, or HP-treated at 600 MPa for 10 min were found at $c = 0.124, 0.129, 0.121, 0.524$, or 0.244, respectively (Figure 7, panels A to E). Data predicted using such values, particularly for milk containing citrate (Figure 7A) or SDS (Figure 7C) or HP-treated at 600 MPa (Figure 7E), show excellent correlation with experimentally determined values. The scattering intensity of a casein micelle dispersion as a function of scattering angle shows a gradual downward slope. Hence, in a crude approximation, L_n^* is a measure of the intensity of scattered light, similar to what could be derived from the turbidity of a sample, and hence the molar mass of the micelles. Thus, this kinetic data analysis indicates that the molar mass of caseins in the presence of a dissociating agent is primarily a function of treatment time and, hence, the degree of crosslinking, as logically expected.

Stability of Extensively Cross-Linked Casein Micelles Against Disruption

As outlined in previous sections, treatment of milk with TGase for 24 h at 30°C reduces the levels of monomeric β - and κ -caseins to nondetectable levels, and the level of monomeric α_{S1} -casein is also reduced extensively (Figure 1). Such extensive covalent intramolecular cross-linking would suggest that the casein micelles have an extremely high stability against dissociation

Figure 7. Influence of addition of 50 mmol/L trisodium citrate (A), 6 mol/L urea (B), or 20 g/L SDS (C), heating to 80°C in the presence of 35% (vol/vol) ethanol (D), or high pressure treatment at 600 MPa for 10 min (E) on the normalized L^* -value of skim milk treated with 0.1 g/L of transglutaminase for 0 to 24 h at 30°C. Data points represent experimentally determined values, whereas solid lines are best fits to the equation $L_n^*(t) = a \{1 - b \cdot e^{(-c \cdot t)}\}$.

induced by disruption of hydrophobic interactions or calcium chelation, which is actually what is observed. At first sight, the reduction of L_n^* on addition of 6 mol/L of urea (Figure 3), which was sufficient to reduce L_n^* to <0.1 in untreated milk, appears to indicate disruption of the casein micelles, but what must also be considered is that intensity of light scattering is proportional to $(dn/dc)^2$, where dn/dc is the refractive index increment; that is, the difference in refractive index between casein micelles and the continuous phase. The refractive index of casein micelles is ~1.570 (Griffin and Griffin, 1985) and that of milk serum ~1.348 (Walstra and

Jenness, 1984), the latter increasing to ~1.398 on addition of 6 mol/L of urea (Warren and Gordon, 1966); hence, the intensity of light scattered by intact casein micelles would be reduced by 40% due to the presence of 6 mol/L urea. Thus, the relatively low values for L_n^* of micelles cross-linked for 24 h in the presence of 6 mol/L of urea is largely explained by the influence of urea on the refractive index of the suspension medium. Furthermore, some monomeric casein is present in extensively cross-linked micelles (data not shown), which may be removed from the micelles by disrupting hydrophobic interactions, thus reducing the scattering in-

tensity of the micelles, and thus L_n^* . Similarly, adding 2.0% (wt/vol) SDS also increases the refractive index of the suspending medium (Tartar and Lelong, 1956; Anacker et al., 1964), which, combined with the removal of some micellar monomeric casein by SDS, may explain the reduced light scattering observed and again suggests very high micellar stability.

Addition of 50 mmol/L citrate increases the refractive index of an aqueous medium by ~ 0.003 units (Salabat et al., 2005), which would lead to a $\sim 3\%$ reduction in the intensity of light scattering; this is by no means sufficient to explain the 55% reduction in L_n^* of extensively cross-linked micelles on adding 50 mmol/L citrate (Figure 2). This large reduction in L_n^* could indicate that extensive cross-linking of casein micelles is insufficient to prevent micellar disruption by citrate but, in this view, it is perhaps surprising that HP treatment of extensively cross-linked milk at 300 to 600 MPa had little effect on L_n^* (Figure 6), despite the fact that during treatment at 400 MPa, complete disruption of noncross-linked casein micelles (presumably through solubilization of MCP) is observed, with only limited reformation on release of pressure (Huppertz et al., 2006). If the cross-linked micelles were unstable, one may have expected extensive HP-induced reductions in L_n^* for TGase-treated milk. In this context, it is perhaps important to consider that HP-induced solubilization of MCP is completely reversible on release of pressure (Hubbard et al., 2002), whereas citrate irreversibly removes the calcium phosphate from the casein micelles. On a DM basis, the calcium phosphate nanoclusters represent $\sim 6\%$ of the weight of the casein micelle. Thus, if the refractive index increment of calcium phosphate is the same as that of the caseins, scattering would be reduced by $\sim 12\%$, because scattering is a function of the concentration of scattering particles and the molecular mass of scattering particles, both of which are reduced by 6%. However, the refractive index of hydroxyapatite, a form of calcium phosphate quite similar to that found in MCP nanoclusters, is ~ 1.65 (Mitchell et al., 1943) and thus, considerably higher than that of casein micelles. Therefore, removal of MCP would also diminish light scattering of casein micelles through a reduction of the refractive index increment, dn/dc . Taking the above into account, a hypothesis for considerable citrate-induced reductions in L_n^* for casein micelles cross-linked extensively by TGase may be considered, in which TGase-induced crosslinking results in a covalently-linked micellar framework, which is stable against disruption. When MCP is chelated, this micellar framework remains intact but the MCP nanoclusters are removed from the micelles, thereby reducing the molecular mass

of the micelles and thus, the intensity of light scattering of the micellar suspension.

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