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Disruption and Reassociation of Casein Micelles under High Pressure: Influence of Milk Serum Composition and Casein Micelle Concentration

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In this study, factors influencing the disruption and aggregation of casein micelles during high-pressure (HP) treatment at 250 MPa for 40 min were studied in situ in serum protein-free casein micelle suspensions. In control milk, light transmission increased with treatment time for ~15 min, after which a progressive partial reversal of the HP-induced increase in light transmission occurred, indicating initial HP-induced disruption of casein micelles, followed by reformation of casein aggregates from micellar fragments. The extent of HP-induced micellar disruption was negatively correlated with the concentration of casein micelles, milk pH, and levels of added ethanol, calcium chloride, or sodium chloride and positively correlated with the level of added sodium phosphate. The reformation of casein aggregates during prolonged HP treatment did not occur when HP-induced disruption of casein micelles was limited (<60%) or very extensive (>95%) and was promoted by a low initial milk pH or added sodium phosphate, sodium chloride, or ethanol. On the basis of these findings, a mechanism for HP-induced disruption of casein micelles and subsequent aggregation of micellar fragments is proposed, in which the main element appears to be HP-induced solubilization of micellar calcium phosphate.

KEYWORDS: High pressure; milk; casein micelle; micellar calcium phosphate

INTRODUCTION

The caseins are a class of four phosphoproteins (α_{s1}-, α_{s2}-, β-, and κ-casein), which represent ~80% of total protein in bovine milk. Because of their relatively open conformation and considerable degree of conformational flexibility, caseins are classified as rheomorphic proteins (*1*). Most casein in bovine milk exists in highly hydrated (~3 g H_2O g^{-1} protein) association colloids (50−300 nm) called casein micelles. The dry matter of casein micelles consists of ~94% protein and ~6% inorganic materials, referred to as micellar calcium phosphate (MCP). MCP contains primarily calcium and phosphate but also small amounts of magnesium and citrate (*2*). MCP exists primarily in the form of nanometer-sized amorphous clusters, which are thermodynamically stable and sequestered by a shell of caseins containing at least one phosphate center, i.e., a cluster of at least three phosphoseryl groups in close proximity (*3*). MCP nanoclusters are randomly distributed throughout the casein micelle; a casein micelle with a radius of 100 nm contains ~800 nanoclusters (*4*). Both α_{s1}- and α_{s2}-casein contain more than one phosphate center and can hence cross-link nanoclusters (*5*). Such cross-linking enables nanoclusters to associate and form particles of colloidal dimensions, i.e., casein micelles, a process that is further aided by hydrophobic interactions between caseins (*5*). The micelles are sterically stabilized by polyelectrolyte brush, which, at least in part, is composed of the hydrophilic C-terminal region of κ-casein (*5*−*7*).

To adequately investigate high pressure (HP)-induced changes in casein micelles, it is important to first consider the basic physical and chemical effects of HP on biological systems. According to Le Chatelier's principle, application of a stress to a system in equilibrium changes the equilibrium to counteract this stress (*8*). Pressurization involves a reduction in the volume of a system, which can be counteracted by favoring reactions that involve a reduction in volume and suppressing those that involve an increase in volume. The volume changes associated with interactions of proteins are primarily due to changes in the compactness of arrangement of water around the proteins, rather than properties of the protein itself (*9*).

The association of caseins is strongly affected by pressure; the scattering intensity of β-casein micelles decreased up to ~150 MPa but increased greatly at a higher pressure (*10*). The initial decrease was confirmed to be due to micellar dissociation by also performing the experiment at 4 °C (*10*), where β-casein is in its monomeric state (*11*). Experiments on aqueous solutions of other micelle-forming molecules, e.g., sodium dodecyl sulfate

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(*12*−*14*), sodium perfluorooctanoate (*15*), pentaethylene glycol monooctyl ether (*16*), and Triton X 100 (*17*), suggest that structural arrangement of water molecules plays an important role in pressure-induced changes in micellization, since micellization is at a minimum at 100−150 MPa, regardless of the molecule that forms the micelles. We therefore think that the properties of water cause the HP-induced changes in micellization. Water exhibits a very peculiar phase behavior (*18*, *19*); at room temperature, hydrogen bridge distances involved in nearest neighbor contacts are at a distinct minimum around 200 MPa (*20*). Although, as Okhulov et al. (*20*) note, this seems odd, it must be realized that the average lifetime of a hydrogen bridge is of the order of 10^{-10}−10^{-12} s (*21*), with an energy of about 23.3 kJ/mol or about 5 kT (*22*), which makes it unrealistic to imagine a static structure of water, and the findings of Okhulov (*20*) should be considered a correlation length.

From the above, it is evident that HP affects the protein−solvent interactions. Generally, because increased pressure lowers the available volume, it tends to decrease entropy. In food science literature, this is invariably attributed to hydrophobic interactions, as a panacea for all observations, which does not contribute to understanding, since a detailed understanding of hydrophobic interactions is still lacking and involves not only entropy but also enthalpy (*23*). Hence, we cannot speculate on how the change in interactions between the water molecules affects the solvency of casein (or other macromolecules); therefore, we refrain from further speculation; we believe that this process should, pending on further clarification, be simply referred to as a solvent-mediated association. Ion pairs are also of crucial importance in maintaining the integrity of casein micelles and are influenced by pressure treatment; the level of soluble inorganic phosphate increased with pressure (*24*) suggesting solubilization of MCP.

As a result of HP-induced changes in the association of caseins and solubilization of MCP, casein micelles are affected considerably under HP, and properties of casein micelles in HP-treated milk differ considerably from those in untreated milk. While a considerable body of knowledge exists on properties of casein micelles in HP-treated milk (for review, see ref *25*), little information is available on changes in casein micelles during HP treatment. In situ measurements have shown a time-dependent increase in the light transmission of milk, which is more extensive and faster at a higher pressure, indicating HP-induced disintegration of casein micelles (*26*−*28*). At 400 MPa, the light transmission of milk is similar to that of milk serum, indicating complete disruption of casein micelles (*27*). During treatment at 250 or 300 MPa, transmission increases with treatment time up to a maximum value, followed by a sharp reduction in transmission on prolonged treatment, indicating initial disintegration of micelles, followed by the reformation of casein particles of colloidal dimensions (*27*, *28*), the extent of which increases strongly with treatment temperature (*28*). HP-induced disruption of casein micelles occurs presumably as a result of solubilization of MCP and dissociation of other, stabilizing ion pairs (*27*), whereas the reformation phenomena observed during prolonged treatment at 250 or 300 MPa may be driven by hydrophobic bonding, as increased temperature promotes the aggregation (*28*).

The objective of the studies presented in this article is to examine the influence of pH, casein micelle concentration, addition of calcium chloride, sodium phosphate or sodium chloride, or partial replacement of water with ethanol in milk on the disruption and reassociation of casein micelles during HP treatment.

MATERIALS AND METHODS

Sample Preparation. Serum protein-free (SPF) milk powder (prepared by microfiltration and ultrafiltration at NIZO Food Research, Ede, The Netherlands) was reconstituted at a level of 8.4, 10.5, or 16.8% (w/v). Sodium azide (0.5 g L^{-1}) was added to all reconstituted milk samples to prevent microbial growth. Milk serum was prepared by dialysis of 25 mL of demineralized H_2O vs 2 × 500 mL of 8.4% SPF milk for 48 h at 5 °C.

The pH of 8.4% SPF milk was adjusted to 6.2, 6.6, or 7.0 using 1 M HCl or 1 M NaOH; stock solutions of 1 M calcium chloride or 1 M sodium phosphate (pH 6.6) were added to 8.4% SPF (pH 6.6) to yield a final concentration of 2, 5, or 10 mmol L^{-1} of calcium chloride or sodium phosphate, followed by readjustment of the milk pH to 6.6. Sodium chloride was added to 8.4% SPF milk at a level of 50, 100, or 200 mmol L^{-1}, followed by readjustment of the milk pH to 6.6. To prepare milks of different casein contents, 2× concentrated milk (16.8% SPF milk) was diluted with milk serum to yield 0.5×, 0.75×, 1.0×, or 1.5× concentrated milk. Milk containing 8.4% milk solids and 0, 5, 10, or 20% (v/v) ethanol was prepared by mixing appropriate amounts of 10.5% SPF milk, demineralized H_2O, and ethanol. All experiments were repeated on three individual milk samples.

Estimation of the Light Transmission of Milk during HP Treatment. An optical-grade glass cuvette (path length, 10 mm) was filled with a milk sample, closed with a movable plunger, and placed in the HP unit. HP treatment was performed for 40 min at 250 MPa at 20 °C, using Baysilon M20 oil (Roland Chemie B. V., Amsterdam, The Netherlands) as the pressure-transmitting medium. The rate of increase of pressure was ∼100 MPa min^{-1}. These conditions were chosen based on data from a previous study (*27*), which indicated that during treatment at such conditions, an intermediate level of micellar disruption was observed, followed by considerable reformation of micellar particles on prolonged treatment, which was deemed optimal for this study. Prior to, and during HP treatment, the transmission of laser light (λ = 632.8 nm; LGK 7626 50 mW He−Ne laser, Siemens, Munich, Germany) through the sample was measured through sapphire glass windows fitted into the HP vessel. Transmission values (Tr) were normalized using the value for milk serum (Tr_{serum} = 1.00) and the dark count of the detector ($Tr_{darkcount}$ = 0.00) according to:

$$Tr_n = \frac{(Tr_{sample} - Tr_{darkcount})}{(Tr_{serum} - Tr_{darkcount})} \quad (1)$$

RESULTS

Influence of Casein Concentration on Changes in Casein Micelles during HP Treatment. The Tr_n treatment time profile of 1× concentrated SPF milk prepared by dilution of 2× concentrated SPF milk with milk serum (**Figure 1**) was similar to that of control unconcentrated SPF milk (**Figure 2**), indicating that dilution of concentrated milk with milk serum caused little alteration to milk, except for a reduction in the casein concentration. The extent of the HP-induced increase in Tr_n, which indicates the extent of HP-induced disruption of casein micelles, was inversely correlated with casein concentration, whereas the time point at which the maximum value for Tr_n was observed was similar in 0.75×, 1.0×, 1.5×, or 2.0× concentrated SPF milk but was reached considerably faster in 0.5× concentrated SPF milk (**Figure 1**). Reformation of particles resembling casein micelles (casein particles), indicated by reversal of HP-induced increases in Tr_n, was not observed in 0.5× concentrated SPF milk, but the extent of reformation increased with total solids concentration for 0.75−1.5× concentrated SPF milk; reformation of casein particles in 2× concentrated SPF milk was less extensive than in 1.5× concentrated SPF milk (**Figure 1**).

Influence of Added Minerals on Changes in Casein Micelles during HP Treatment. The extent and rate of HP-induced increases in Tr_n decreased with increasing level of added calcium chloride (2−10 mmol L^{-1}) in SPF milk (**Figure 2**).

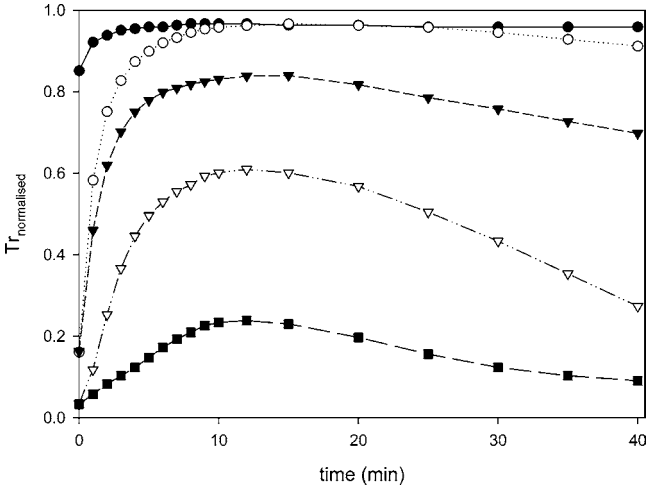

Figure 1. Normalized light transmission of 0.5 (●), 0.75 (○), 1.0 (▼), 1.5 (▽), or 2.0 (■) × concentrated reconstituted SPF skim milk powder during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

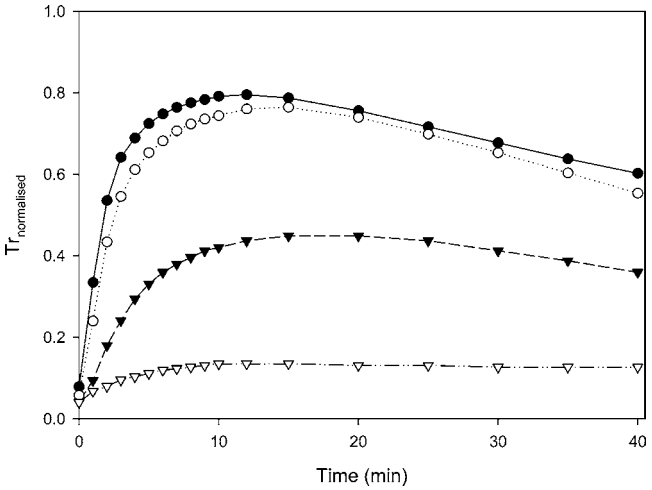

Figure 2. Normalized light transmission of 8.4% (w/v) reconstituted SPF skim milk powder, containing 0 (●), 2 (○), 5 (▼), or 10 (▽) mmol L^{-1} added $CaCl_2$, during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

Adding 2 mmol L^{-1} calcium chloride to milk had little effect on the reformation of casein particles during prolonged HP treatment, but 5 or 10 mmol L^{-1} added calcium chloride inhibited this process considerably or completely, respectively (**Figure 2**).

Adding 2 or 5 mmol L^{-1} sodium phosphate to SPF milk had little influence on the HP-induced increase in Tr_n, but adding 10 mmol L^{-1} sodium phosphate increased the susceptibility of casein micelles to HP-induced disruption considerably (**Figure 3**). Little difference in the rate of reformation of casein particles on prolonged HP treatment was observed between milk containing 0, 2, or 5 mmol L^{-1} added sodium phosphate, but the rate of reformation was greater when 10 mmol L^{-1} sodium phosphate was added (**Figure 3**).

Adding 50, 100, or 200 mmol L^{-1} sodium chloride to SPF milk prior to HP treatment caused a small, progressive, decrease in the susceptibility of casein micelles to HP-induced disruption,

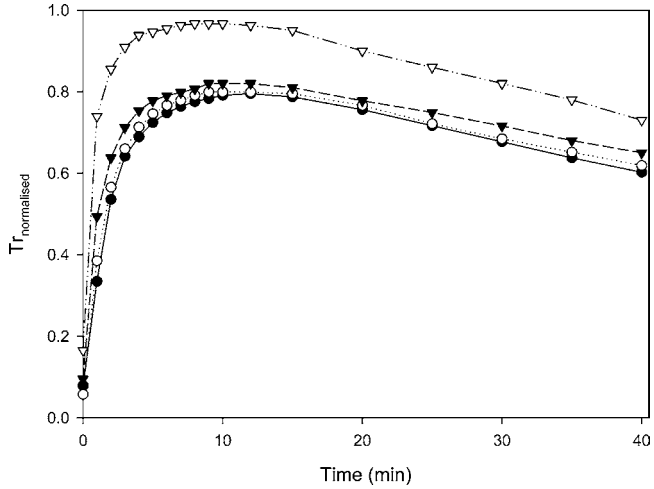

Figure 3. Normalized light transmission of 8.4% (w/v) reconstituted SPF skim milk powder, containing 0 (●), 2 (○), 5 (▼), or 10 (▽) mmol L^{-1} added Na_2HPO_4, during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

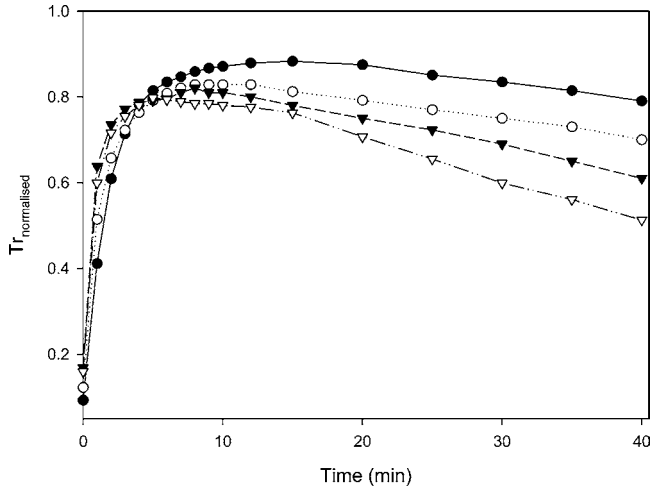

Figure 4. Normalized light transmission of 8.4% (w/v) reconstituted SPF skim milk powder, containing 0 (●), 50 (○), 100 (▼), or 200 (▽) mmol L^{-1} added NaCl, during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

as indicated by a decrease in maximum values for Tr_n with an increasing level of added sodium chloride (**Figure 4**). The rate of reformation of micellar particles increased with an increasing level of added sodium chloride (**Figure 4**).

Influence of Milk pH on Changes in Casein Micelles during HP Treatment. Milk pH had a considerable influence on HP-induced changes in Tr_n of 8.4% SPF milk (**Figure 5**). As compared to milk at pH 6.6, the rate of disruption of casein micelles, as indicated by increases in Tr_n, was considerably higher in milk at pH 6.2, as was the rate of reversal of HP-induced increases in Tr_n. At pH 7.0, the rate and extent of the increase in Tr_n were considerably lower than in milk at pH 6.6 and little reversal of HP-induced increases in Tr_n was observed throughout the 40 min of treatment (**Figure 5**).

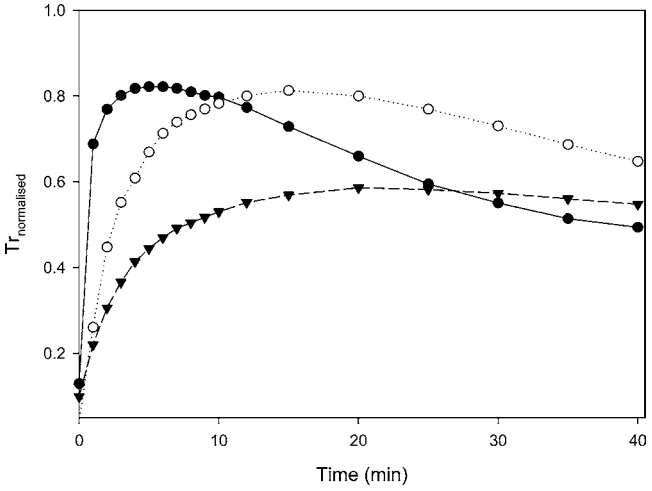

Figure 5. Normalized light transmission of 8.4% (w/v) reconstituted SPF skim milk powder at pH 6.2 (●), 6.6 (○), or 7.0 (▼) during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

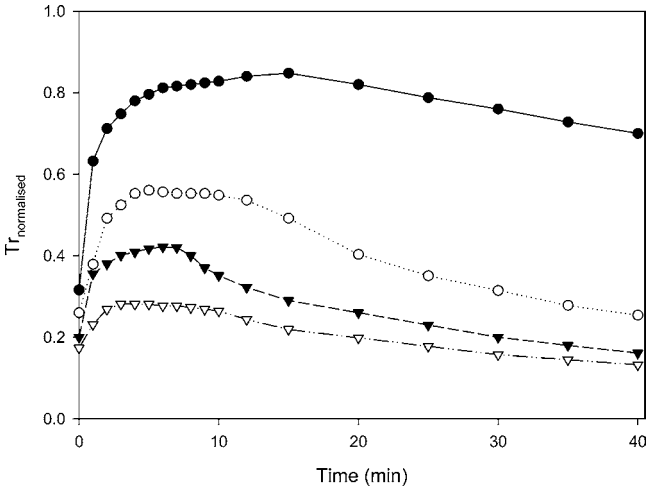

Figure 6. Normalized light transmission of 8.4% (w/v) reconstituted SPF skim milk powder, containing 0 (●), 5 (○), 10 (▼), or 20 (▽) % (v/v) ethanol, during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Values are means of data from experiments on three individual milk samples ($n = 3$); the coefficient of variation was <5% for all data points.

Influence of Ethanol on Changes in Casein Micelles during HP Treatment. The extent of HP-induced disruption of casein micelles was reduced progressively and extensively with increasing concentration of ethanol in SPF milk (**Figure 6**). The rate of reformation of micellar particles during prolonged treatment was comparable in SPF milk containing 0, 10, or 20% ethanol but was higher in milk containing 5% ethanol (**Figure 6**).

Modeling of HP-Induced Changes in Casein Micelles. Using kinetic modeling, we investigated if HP-induced disruption of casein micelles is, as previously suggested (*27*), the result of solubilization of MCP. Assuming that unconcentrated milk contains 30 mmol L^{-1} calcium, of which 20 mmol L^{-1} is micellar and the remainder is in the serum, we estimate that at 250 MPa the calcium concentration increases of the milk serum increases from 10 to 20 mmol L^{-1}, since in 0.5× concentrated milk, the casein micelles dissociate completely at 250 MPa (**Figure 1**), therewith solubilizing 10 mmol L^{-1} of micellar calcium. Furthermore, we assume that the residual calcium concentration in the casein micelle determines the extent of disruption of the casein micelles, so a high level of residual micellar calcium leads to a low extent of micellar disruption and vice versa. On the basis of these assumptions, the following simplified model is proposed, where HP-induced dissolution of calcium is driven by the concentration gradient:

$$\frac{dCa_{serum}}{dt} = K \times [Ca_{max} - Ca_{serum}(t)]$$

where $Ca_{max} = 20$ mmol L^{-} and $Ca_{serum}(t)$ is the serum calcium concentration at time t:

$$Ca_{serum}(t) = Ca_{max} - x0 \exp(-k \times t)$$

where $x0 = [Ca_{serum}(0) - Ca_{max}]$, which follows from the boundary conditions.

We assume that the disruption of the casein micelles is proportional to dissociation of the calcium and that turbidity τ [from Lambert Beer's law; transmission $\propto \exp(-\tau)$] is

$$\tau \propto C \times M$$

where C is the concentration and M is the molar mass of light scattering particles, i.e., the casein micelles (*30*), so turbidity scales quadratically in M, since C is proportional to M. We further observed that after the initial disruption of the micelles, indicated by increases in Tr_n, there was a subsequent, much slower decrease in Tr_n (**Figure 1**), indicating reformation of casein particles. Although it is not entirely clear what type of particles are formed, the reformation process appears to resemble a slow Smoluchowski−Fuchs type of aggregation (*29*). We assume that the various caseins, no longer bound by caseinophosphate bonds to calcium in the nanoclusters, rearrange into large(r) protein aggregates. This process will be inherently slower than the initial disruption, because the polar caseinophosphate groups were initially shielded by hydrophobic groups surrounding the nanoclusters, so in a sense the caseins have to invert themselves inside out prior to association. The reassociation process is modeled according to:

$$\upsilon(t): = \frac{\upsilon 0(t)}{1 + \frac{t}{\mathrm{Ts}(t)} \cdot \mathrm{Ws}} \text{ and } \mathrm{Ts}(t): = \frac{1}{8 \cdot \pi \cdot D \cdot a \cdot [\upsilon 0(t)]}$$

where $\upsilon(t)$ is the number of particles at time t, $\mathrm{Ts}(t)$ is a characteristic association time, and Ws is the Fuchs factor, deriving from a Boltzmann factor of an activation energy, basically slowing down the process.

Because there are so many unknown variables, except for the calcium concentration at the start and at HP, we prepared a best fit on the transmission curve for 1× concentrated milk and subsequently used the model to predict curves for 0.5 and 2× concentrated milk by adjusting only the original calcium concentration. This allowed us to predict transmission as a function of casein micelle concentration, as shown in **Figure 7**, from which it is clear that by only adjusting the calcium concentration of the milk system, the influence of casein micelle concentration on the HP-induced increases in Tr_n transmission can be predicted accurately. The reassociation process of micellar fragments during prolonged HP treatment appeared to follow Smoluchowki−Fuchs kinetics (**Figure 7**) but could not be predicted adequately solely by adjustment of the calcium

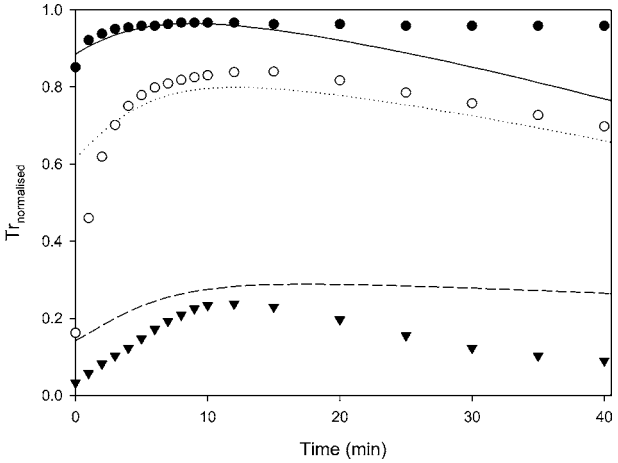

Figure 7. Normalized light transmission data derived from experimental data (symbols) or predictive modeling (lines) of 0.5 (●, —), 1.0 (○, ···), or 2.0 (▼, - - -) × concentrated SPF milk during HP treatment for 40 min at 250 MPa at 20 °C; treatment time $T = 0$ represents the time point at which the pressure reached 250 MPa. Experimental values are means of data from triplicate experiments on individual milk samples.

concentration. This indicates that, to adequately predict micellar reassociation, further adjustable parameters need to be included in the model.

DISCUSSION

The data presented in this communication present an extensive set of new experimental results on the disruption of casein micelles at 250 MPa. The in situ light transmission measurements show an initial steep increase in transmission, subsequently followed by a much slower decrease in transmission; these results clearly indicate that HP-induced disintegration of casein micelles and the subsequent (slower) formation of casein particles from micellar fragments during prolonged HP treatment are influenced by a wide variety of environmental conditions.

Factors Affecting Disruption of Casein Micelles under HP. In general, disruption of casein micelles may be achieved by either one, or a combination of, two mechanisms: (i) disruption of casein−casein interactions, as a result of altered solvent quality, or (ii) disruption of MCP−casein interactions, as a result of solubilization of MCP (*5*). HP-induced disruption of casein micelles as a result of disruption of casein−casein interactions is contradictory to previous results showing that micellization of β-casein is promoted at >150 MPa (*10*), i.e., pressures at which HP-induced disruption of casein micelles is observed (*26*−*28*, **Figures 1**−**6**). As also outlined by Huppertz et al. (*27*), we believe that HP-induced disruption of casein micelles is primarily a result of the increased solubility of calcium phosphate at HP (*24*), thereby leading to MCP depletion of casein micelles. The validity of this hypothesis is strongly supported by the data in **Figure 7**, which show that HP-induced micellar disruption can be accurately predicted when considered as a function of MCP solubilization.

The increasing extent of micellar disruption with reduced concentration of casein micelles (**Figure 1**) can be explained as a function of solubilization of MCP. The solubility of calcium phosphate increases with pressure (*24*), and while the solubility of calcium phosphate at a pressure is independent of casein micelle concentration, the fraction of solubilized MCP decreases with increasing concentration of micelles. Hence, micellar stability against HP-induced disruption increases with increasing casein micelle concentration of casein micelles.

As expected, adding calcium chloride to milk stabilized the casein micelles against HP-induced disruption (**Figure 2**), because added calcium chloride increases the level of MCP (*31*, *32*) and hence is expected to increase micellar stability against HP-induced disruption. A similar increase in stability may have been expected for added sodium phosphate, which also increases the level of MCP (*31*), but adding phosphate decreased, instead of increased, micellar stability against HP-induced disruption (**Figure 3**). However, it should be considered that sodium phosphate has a large negative partial molar volume (*33*, *34*), which indicates that dissociation of mono-, di-, and trihydrogen phosphates is favored under pressure. Thus, milk pH under pressure would decrease with an increasing level of added sodium phosphate, leading to increased HP-induced solubilization of MCP and, hence, micellar disruption.

The reduced extent of HP-induced disruption of casein micelles in milk containing added sodium chloride (**Figure 4**) is perhaps surprising, considering that added sodium chloride induces solubilization of MCP (*35*−*37*) and may thus be expected to reduce micellar stability; however, adding sodium chloride also induces a reduction in intermolecular repulsion between caseins (*37*). As a result, attractive forces may predominate over repulsive forces and micellar integrity may be maintained.

The influence of milk pH on the rate and extent of HP-induced disruption (**Figure 5**) may be, at least partially, explained by the influence of pH on the mineral balance in milk. Acidification of milk increases the level of diffusible (i.e., nonmicellar) calcium and phosphate, whereas alkalanization has the opposite effect (*38*). Hence, the reduced or increased level of MCP in milk adjusted to pH 6.2 or 7.0, respectively, may be responsible for the reduced or increased stability against HP-induced disruption (**Figure 5**).

The reduced extent of HP-induced micellar disruption with increasing ethanol content (**Figure 6**) is probably related to the decreased solubility of calcium phosphate with increasing concentration of ethanol in water−ethanol mixtures (*41*, *42*). Because of the reduced solubility of calcium phosphate in media containing ethanol, the extent of HP-induced solubilization at a given pressure, and hence the extent of HP-induced disruption of casein micelles, decreases with increasing ethanol content.

Factors Affecting Aggregation of Micellar Fragments under HP. Aggregation of micellar fragments to form casein particles of colloidal dimensions during prolonged HP treatment, indicated by partial reversibility of HP-induced increases in Tr_n (**Figures 1**−**6**), occurs because of solvent-mediated association of caseins. However, as outlined in the Introduction, we believe that the limited current knowledge on protein−protein and protein−solvent interactions at HP, particularly in a complex biological system such as milk, does not allow full elucidation of the mechanism at this stage. As reformation of casein particles is more extensive at a higher temperature (*28*), involvement of hydrophobic bonding is likely, but little is thus far known about other contributing forces.

The increased rate of reformation at a lower pH (**Figure 5**) and increased level of added sodium chloride (**Figure 4**) may be related to reductions in the surface charge of caseins on decreasing pH (*39*, *40*) or increasing sodium chloride concentrations (*37*), suggesting a role of electrostatic interactions in the aggregation process. The increased extent of reformation in milk containing 10 mmol L^{-1} added sodium phosphate (**Figure 3**) is probably due to the lower pH in this sample during HP treatment, due to the ionization of hydrogenated phosphates, as discussed in the first section of the Discussion. The increased

rate of reformation of micelles in 1.5× concentrated SPF milk, as compared to in 1× concentrated SPF milk (**Figure 1**), may be due to the presence of a larger number of intact nanoclusters in the former, which was suggested to be required to initiate reformation (*27*); in 2× concentrated SPF milk, the level of micellar disruption may be too low to initiate extensive micellar aggregation.

Huppertz et al. (*27*) previously reported that aggregation of micellar fragments may not occur if disruption is near-complete as a nucleus is required to initiate the aggregation process. However, from **Figures 1**−**3**, it is also apparent that if the extent of HP-induced reduction is limited ($Tr_{n,max} < 0.6$), little or no aggregation occurs; accordingly, little or no reformation was observed at pressures <250 MPa, because these only cause very limited disruption of casein micelles (*26*−*28*). This indicates that an optimal extent of micellar disruption is required to allow casein aggregation during prolonged HP treatment; when too much disruption occurs, no nuclei for the aggregation process are available, whereas too little disruption probably does not reduce intermicellar repulsion sufficiently to induce aggregation. The relatively high rate of micellar reformation on adding ethanol (**Figure 6**), despite the limited extent of HP-induced disruption, may be due to the fact that ethanol facilitates micellar aggregation, primarily due to reduced steric and electrostatic repulsion as a result of altered solvent quality (*43*), and further supports the aforementioned hypothesis that a sufficiently high reduction in the extent of intermicellar steric repulsion is required to facilitate the aggregation process.

In conclusion, the data presented in this communication indicate the strong influence of a wide variety of environmental factors on the disruption of casein micelles under pressure, as well as the formation of casein aggregates of colloidal dimensions during prolonged treatment at 250 MPa. The process of HP-induced disruption of casein micelles appears to be driven primarily by the extent of solubilization of MCP. The unfavorable exposure of hydrophobic surfaces at a pressure > 200 MPa leads to the formation of casein particles of colloidal dimension from fragments of disrupted casein micelles during prolonged HP treatment. The bonding of caseins by van der Waals or hydrophobic interactions is influenced by the charge on the caseins, as well as the extent to which the micelles are disrupted; hence, electrostatic and steric repulsions are diminished during the initial stages of pressure treatment. The results presented in this communication suggest the following simplified mechanism: HP solubilizes MCP; at that point, the hydrophilic caseino-phosphate residues are screened by hydrophobic parts of the caseins. New particle structures are subsequently formed due to a structural rearrangement of hydrophobic and hydrophilic groups.

ABBREVIATIONS USED

HP, high pressure; MCP, micellar calcium phosphate.

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