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Dynamics of casein micelles in skim milk during and after high pressure treatment

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Abstract

The effect of high hydrostatic pressure on turbidity of skim milk was measured in situ together with casein micelle size distribution. High pressure (HP) treatment reduced the turbidity of milk with a stronger pressure dependency between 50 and 300 MPa when the temperature was decreased from 20 to 5 °C, while at 30 °C (50–150 MPa) turbidity exceeded that of untreated milk. At 250 and 300 MPa turbidity decreased extremely. During pressurization of milk at 250 and 300 MPa, the turbidity initially decreased, but treatments longer than 10 min increased the turbidity progressively, indicating that re-association followed dissociation of casein micelles. Especially at 40 °C and at 250 and 300 MPa, the turbidity increased beyond untreated milk. Dynamic light scattering was used to investigate casein micelle sizes in milk immediately after long time (up to 4 h) pressurization at 250 and 300 MPa and casein micelle size distributions were bimodal with micelle sizes markedly smaller and markedly larger than those of untreated milk. Pressure modified casein micelles present after treatment of milk at 250 and 300 MPa were concluded to be highly unstable, since the larger micelles induced by pressure showed marked changes toward smaller particle sizes in milk left at ambient pressure.

Keywords: Casein micelle; High pressure; In situ turbidimetry

1. Introduction

High pressure (HP) effects on milk have been studied extensively in the recent two decades with the goal of developing new dairy processes, as reviewed by Huppertz, Kelly, and Fox (2002). The main effect of HP treatment of milk is found to be changes in the mineral balance (López-Fandiño, De la Fuente, Ramos, & Olano, 1998), denaturation of whey proteins (Huppertz, Fox, & Kelly, 2004b), and changes in sizes of casein micelles (Anema, Lowe, & Stockmann, 2005; Huppertz, Fox, & Kelly, 2004c, 2004d; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Regnault, Thiebaud, Dumay, & Cheftel, 2004). HP treatment of milk destabilizes the casein micelles, as shown by a more translucent appearance of milk subjected to HP treatment above 150 MPa compared to the appearance of untreated milk (Gaucheron et al., 1997; Kromkamp, Moreira, Langeveld, & Van Mil, 1996; Regnault et al., 2004). Decreased turbidity and a reduction in average casein micelle diameter, resulting from casein micelle dissociation, have been reported after HP treatment of milk above 150 MPa (Anema et al., 2005; Huppertz, Fox, & Kelly, 2004d; Needs et al., 2000; Regnault et al., 2004). Interestingly, HP treatment of milk at pressures from 250 to 300 MPa seems to induce a broadening of the size distribution of casein micelles, explained by dissociation of casein micelles and re-association and aggregation of a fraction of casein micelles into larger particles than observed in untreated milk (Anema et al., 2005; Huppertz et al., 2004d; Regnault et al., 2004). However, the mechanism

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of dissociation and association of casein micelles during and after high-pressure treatment of milk is unclear. The integrity of casein micelles in milk is stabilized by micellar clusters of calcium phosphate and hydrophobic interactions (De Kruif & Holt, 2003, Chapter 5; Horne, 2002) and HP treatment may affect both (Anema et al., 2005; Huppertz et al., 2004d; Regnault et al., 2004). Re-association and aggregation of casein micelles after dissociation resulting from HP treatment at 200–300 MPa were found to be enhanced by increasing temperature from 5 to 40 °C during the pressure treatment and this endothermic nature of the reaction may indicate that hydrophobic interactions are involved in re-association and aggregation (Anema et al., 2005; Gaucheron et al., 1997; Huppertz et al., 2004c; Regnault et al., 2004).

As the studies cited above indicate, further studies are needed in order to establish the mechanism of the modifications of casein micelles that occur both during pressurization of milk and after release of pressure, and also to explain the reversibility of the modifications. Information about the stability of the pressure-induced changes in milk proteins is also becoming important for an understanding of functional properties of HP modified milk proteins and for practical applications of HP technology in the dairy industry.

In order to contribute to the understanding of HP effects on milk at different temperatures and holding times, turbidity of milk under HP for various temperatures and durations were measured in situ. Furthermore, changes in the casein micelle size distribution in milk as a function of HP holding times at 250 and 300 MPa and time elapsed after pressure treatments were investigated by dynamic light scattering.

2. Materials and methods

2.1. Materials

Low heat skimmed milk powder, Milex® 240, was obtained from Arla Foods, Akafa, Svenstrup, Denmark. Imidazole, $CaCl_2 \cdot 2H_2O$, HCl, and NaCl were of analytical grade and purchased from Merck, Darmstadt, Germany. Sodium azide was from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Trypsin inhibitor was from Boehringer Mannheim GmbH, Germany. All aqueous solutions were made from purified water using a Milli-Q Plus® purification system (Millipore Corporation, Bedford, USA).

2.2. Sample preparation

Skim milk was prepared by dissolving low heat skimmed milk powder in purified water to a final concentration of 100 g/l. Sodium azide (0.1 g/l) and trypsin inhibitor (0.01 g/l) were added. The reconstituted skim milk equilibrated overnight at 20 °C and was kept at this temperature until further experiments, all done within three days.

2.3. Pressure treatment

The reconstituted skim milk was subjected to high pressure in a water thermostated hydraulic-pressure chamber (0.25 l) from Psika Systems Ltd., Stanford, UK. Skim milk was transferred to polyethylene tubes, which were filled without headspace, and the tubes were further vacuum-packed in polyethylene bags. Isostatic pressure at 20 °C was held at 250 and 300 MPa for 1, 2, 3, or 4 h with castor oil as pressure transmitting medium. Rates of pressure increase and release were approximately 100 MPa/min. After pressure release the skim milk samples were kept at 20 °C until further analysis.

2.4. In situ turbidimetry under high pressure

The intensity of light transmitted through the milk sample under HP was measured in situ with a thermostated high pressure optical cell (SITEC Sieber Engineering AG, Switzerland) equipped with a pressure generating system and an UV–vis fiber light source (Hamamatsu Photonics Deutschland GmbH, Germany). In order to allow observation of highly turbid milk samples without dilution, the optical cell was modified to provide a path length of 2 mm. The pressure generating system was filled with skim milk and the intensity spectrum from 350 to 700 nm was recorded. The intensity spectrum was converted to an absorbance spectrum using $A_\lambda = -\log(\text{sample intensity at } \lambda/\text{reference intensity at } \lambda)$ with water as reference (water intensity spectrum recorded prior to milk). The turbidity index (I_{turb}) was calculated as $(\tau - \tau_0)/\tau_0$, where τ_0 is the turbidity of milk at 0.1 MPa and τ is the turbidity at the actual pressure, using the absorbance at 601 nm (after each treatment the system was cleaned). The I_{turb} of milk was calculated at pressures between 0.1 and 500 MPa for every 50 MPa at 5, 10, 20, and 30 °C. In additional experiments, the I_{turb} was calculated as a function of holding time while maintaining the pressure at 150, 200, 250, 300, 350, 400, 450, or 500 MPa at 20 °C, and when maintaining the pressure at 250 and 300 MPa at 5, 20, and 40 °C.

2.5. Dynamic light scattering

The casein micelle sizes in skim milk were determined by dynamic light scattering using a Malvern HPPS particle sizer (Malvern Instruments Ltd., Worcestershire, United Kingdom). The light source was a 3 mW helium neon laser with a wavelength of 633 nm. Light scattering was detected at the 173° angle and all measurements were made at 20.0 ± 0.1 °C. Immediately before recording the

light scattering spectra, the milk samples were diluted 1:1000 in filtered (0.20 μm) 20 mM imidazole buffer containing 5 mM $CaCl_2 \cdot 2H_2O$, adjusted to pH 6.7 and an ionic strength of 0.08 M by adding HCl and NaCl, respectively. For pressurized milk samples the dilution was made 10, 60, and 120 min after pressure release and 10 autocorrelation spectra were recorded for each dilution. Similarly, the untreated milk samples were diluted and measured. Size distributions were derived from the autocorrelation functions using inverse Laplace transformation.

3. Results and discussion

3.1. General

The combination of in situ measurement of turbidity during high-pressure treatment of skim milk and measurement of micelle size distribution of skim milk following release of high-pressure allows a detailed description of pressure effects on the changes in micelles at various temperatures.

3.2. In situ turbidity of milk under HP as a function of temperature

Fig. 1 shows the pressure and temperature dependency of the turbidity index of the reconstituted skim milk. Changes in the turbidity index represent the quantitative variations of the turbidity of milk during the gradual increase in pressure from ambient pressure to 500 MPa, moreover this index indicates changes in the size of the casein micelles. It is seen that dissociation of casein micelles is enhanced upon increased pressure, as indicated by the observed decreased turbidity index value (Fig. 1). Only minor changes in the turbidity index were observed during moderate high-pressure treatment, i.e., at 50, 100, and 150 MPa, where the turbidity of milk was reduced up to 30% at 5 °C. However, the turbidity was markedly reduced at higher pressures and at intermediate pressures marked changes in the turbidity index occurred over a narrow pressure range. Above 300 MPa the turbidity reached a low constant level where the milk appeared almost clear. The observation of decreasing turbidity of milk under increasing pressure is in agreement with similar findings measured after pressure treatment and pressure release (Needs et al., 2000; Regnault et al., 2004; Schrader & Buchheim, 1998).

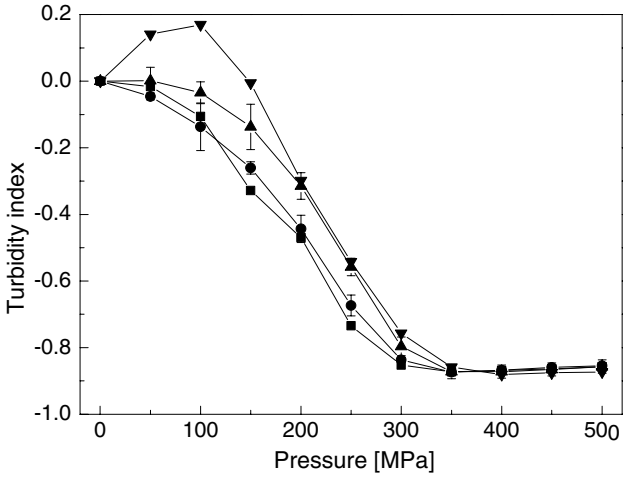

Fig. 1. Turbidity index of reconstituted skim milk under pressure at different temperatures. Milk was subjected to high pressure at 5 °C (■), 10 °C (●), 20 °C (▲), or 30 °C (▼). The pressure was increased manually up to 500 MPa, and for every 50 MPa, a spectrum of the milk was measured. The absorption at 601 nm (measured in situ) was used for calculation of the turbidity index.

The temperature dependence of the changes in turbidity index in skim milk under pressure may be deduced from Fig. 1. Previous investigations have shown that the temperature affects the casein micelle dissociation induced by pressure treatment (Regnault et al., 2004; Schrader & Buchheim, 1998). Regnault et al. (2004) reported that pressure treatment (above 200 MPa) of milk at 9 °C resulted in a lower turbidity index compared to pressurization at 20 °C. Similar, the decrease in turbidity of skim milk was greater after pressure treatment above 200 MPa at 5 and 10 °C compared to 20 and 40 °C (Schrader & Buchheim, 1998). As further seen from Fig. 1 our results confirm this temperature dependency of the turbidity index at pressures between 100 and 300 MPa with a decrease in turbidity index, due to increasing pressure, occurring faster at 5 and 10 °C compared to 20 °C. However, pressurization conducted at 30 °C and between 50 and 150 MPa resulted in an increase in the turbidity index to higher values than observed for untreated milk. This phenomenon has also been observed after pressure treatment of milk after pressurization at 300 MPa and 40 °C (Schrader & Buchheim, 1998).

3.3. In situ turbidity of milk under HP as function of holding time

Turbidity was found to change with time at some pressures and therefore the development in the turbidity index of reconstituted skim milk at different pressure levels was followed as a function of holding times, as shown in Fig. 2, in order to provide a kinetic description. The decreasing turbidity index indicates that the casein micelles are progressively dissociated upon increasing pressure and holding time. The turbidity index measured at 150 MPa remained fairly constant over time as also observed for the turbidity at 200 MPa although after an initial decrease. Pressure treatments at 250 and 300 MPa, however, showed a remarkably different relation with respect to the holding time. During

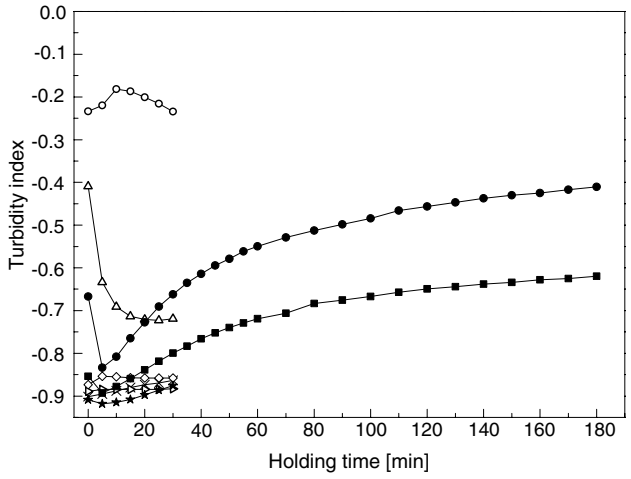

Fig. 2. Turbidity index of reconstituted skim milk as a function of high pressure holding time and working pressure as calculated from in situ absorbance measurements at 20 °C: 150 MPa (○); 200 MPa (△); 250 MPa (●); 300 MPa (■); 350 MPa (★); 400 MPa (+); 450 MPa (▷); 500 MPa (◇).

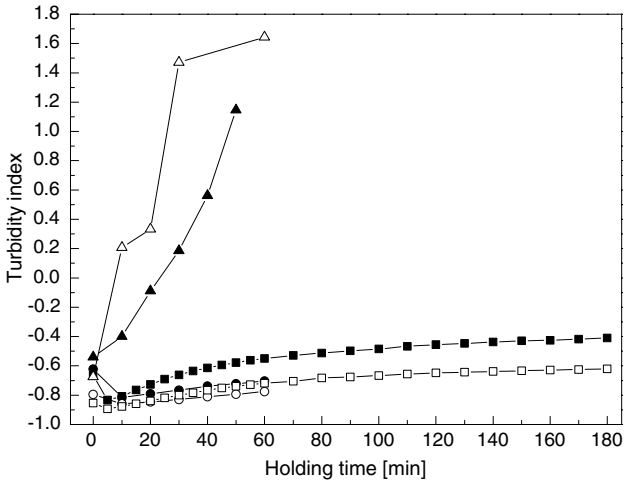

Fig. 3. Turbidity index of reconstituted skim milk as a function of high pressure holding time and temperature as calculated from in situ absorbance measurements at 250 and 300 MPa. Closed symbols represent measurements at 250 MPa conducted at 5 °C (●), 20 °C (■), 40 °C (▲) and open symbols represent measurements at 300 MPa at 5 °C (○), 20 °C (□), 40 °C (△).

pressurization of milk at 250 and 300 MPa for more than about 10 min the turbidity index was found to begin to increase, indicating some type of association or aggregation following initial dissociation. The increase in turbidity is more pronounced for milk subjected to a pressure of 250 MPa than to 300 MPa. At 250 MPa the turbidity index decreased considerably during the first 5 min, followed by a marked increase during further pressurization. Notably, the turbidity of milk pressurized at 250 MPa for 180 min was lower than the turbidity of the original untreated milk. Within the time frame for the experiment, the turbidity index curve did not reach a plateau. This result supports the interpretation that association becomes dominating compared to the initial (within 5 min at 250 MPa) HP-induced casein micelle dissociation, as suggested by Regnault et al. (2004). This association phenomenon is apparently dominating at pressures of 250 and 300 MPa at the measuring temperature of 20 °C. At higher pressures the dissociation of casein micelles dominates and the casein micelles remain dissociated without major changes at least throughout the time of the actual experiment, as indicated by the low and constant turbidity index.

The development in the turbidity index of reconstituted skim milk during pressurization at 250 and 300 MPa was also investigated at different temperatures, and the turbidity index under the conditions of varying temperatures is shown in Fig. 3. Immediately upon pressurization of milk at 250 and 300 MPa at 40 °C the turbidity decreased, however, during pressurization the turbidity increased to a level higher than that of untreated milk. The turbidity index, measured at 40 °C, increased faster for milk pressurized at 300 MPa compared to milk pressurized at 250 MPa. This was in contrast to the development in the turbidity of milk under pressure at 20 °C, where the treatment at 250 MPa reached a higher turbidity index than at 300 MPa (Fig. 3). The development in the turbidity index of milk during pressurization at 250 and 300 MPa at 5 °C was more or less similar to the turbidity development during pressurization at 300 MPa 20 °C.

3.4. Casein micelle size distribution in milk after HP

Since pressure treatment of milk at 250 and 300 MPa induced dynamic changes during pressurization (Figs. 2 and 3) the casein micelle sizes in milk after pressurization at 250 and 300 MPa were further investigated. Figs. 4 and 5 show the casein micelle size distributions measured at ambient pressure following high-pressure treatment of skim milk at 250 or 300 MPa, respectively, and for different HP holding times for both pressures. A major change in the casein micelle size distribution is observed after all pressure treatments. A separation of the rather narrow casein micelle size distribution in untreated milk (bottom row in Figs. 4 and 5) into a bimodal size class distribution was observed. Thus, two distinct groupings of particle size classes were observed after pressure treatment, one grouping with decreased sizes and one grouping with increased particle sizes compared to those in untreated milk. A general feature for the casein micelle size distributions after HP treatments of milk at 250 and 300 MPa was that prolonged pressure holding times increased the normalized scattering intensity amplitude from the fraction of particles that were smaller than those in untreated milk. Concomitantly, the normalized scattering intensity amplitude, corresponding to the fraction of particles that were larger than those in untreated

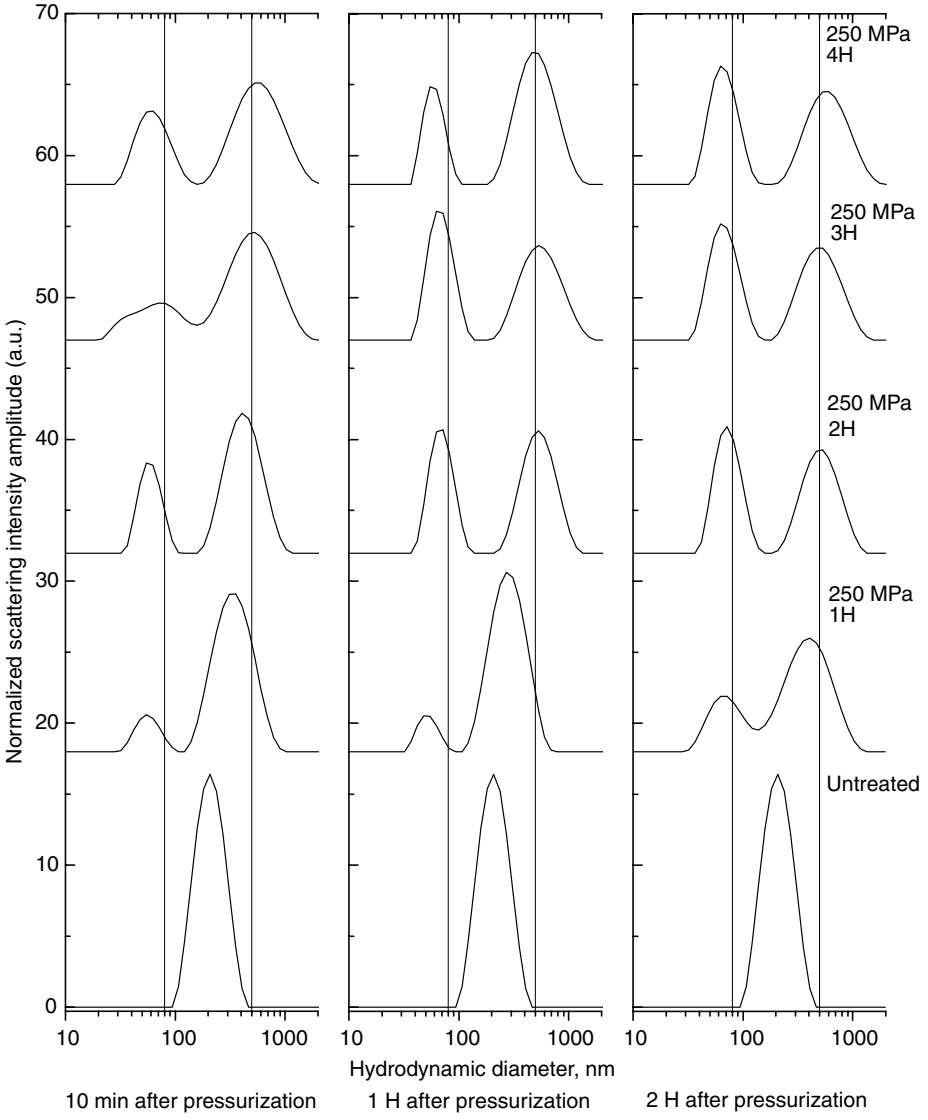

Fig. 4. Casein micelle size distribution derived from dynamic light scattering measurements on reconstituted skim milk. Measurements were conducted on untreated milk (0.1 MPa) and on milk after high pressure treatment at 250 MPa for 1, 2, 3, and 4 h and for each pressure treatment after 10 min, 1, and 2 h storage at ambient pressure. The vertical lines indicate the width of the casein micelle size distribution of untreated skim milk.

milk, decreased. However, only minor changes in the width of the size distribution in these two fractions occurred upon prolonged pressurization.

The stability of the casein micelles in pressure modified milk was studied for up to 2 h after pressure treatment. The pressure modified casein micelles in milk were not stable during these 2 h as indicated from the decreased normalized scattering intensity amplitudes arising from the fraction of particles that were larger than those in untreated milk. In parallel, an increase in the normalized scattering intensity amplitudes, arising from the fraction of particles that were smaller than those in untreated milk, was observed. Hence, the pressure modified casein micelles, present after treatment of milk at 250 and 300 MPa, were highly unstable as also indicated by the decrease in *z*-average hydrodynamic diameters derived from measurements on milk left at ambient pressure for up to 2 h after treatment (Table 1). The main difference between casein micelle sizes in milk after HP treatment at 250 and 300 MPa is that the fraction of smaller micelles increased when pressure treatment was conducted at 300 MPa compared to 250 MPa (Figs. 4 and 5). In agreement, *z*-average hydrodynamic diameters of the casein micelles in milk after HP treatment at 300 MPa were smaller compared to average diameters after HP treatment at 250 MPa (Table 1).

3.5. Influence of HP on casein micelles

The effects of high hydrostatic pressure on casein micelles in reconstituted skim milk during and after HP treatment are summarized in Fig. 6 showing that

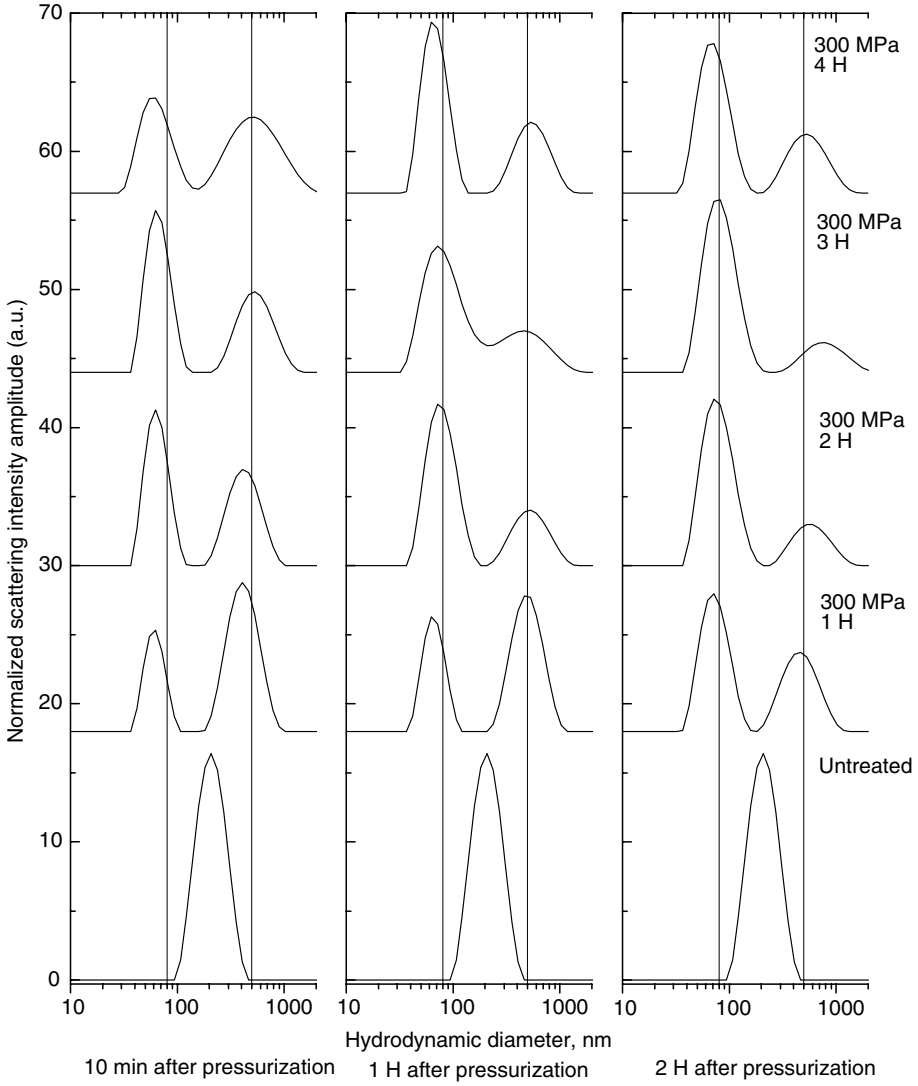

Fig. 5. Casein micelle size distribution derived from dynamic light scattering measurements on reconstituted skim milk. Measurements were conducted on untreated milk (0.1 MPa) and on milk after high pressure treatment at 300 MPa for 1, 2, 3, and 4 h and for each pressure treatment after 10 min, 1, and 2 h storage at ambient pressure. The vertical lines indicate the width of the casein micelle size distribution of untreated skim milk.

Table 1
z-Average hydrodynamic diameter of casein micelles measured at various times after HP treatment of milk at 20 °C and ambient pressure

HP treatment (MPa)	*z*-Average hydrodynamic diameter, 10 min (nm)	*z*-Average hydrodynamic diameter, 1 h (nm)	*z*-Average hydrodynamic diameter, 2 h (nm)
0.1 (control)	207.6 ± 1.3	–	–
250 (1H)	213 ± 11	185.0 ± 6.0	180.3 ± 0.7
250 (2H)	211 ± 33	165.4 ± 1.5	147 ± 19
250 (3H)	183 ± 12	131.4 ± 1.6	116.2 ± 8.8
250 (4H)	245 ± 12	157.0 ± 0.5	161 ± 22
300 (1H)	136 ± 31	126 ± 39	105 ± 13
300 (2H)	113.9 ± 7.1	113 ± 22	94.5 ± 1.0
300 (3H)	108.3 ± 7.3	96.4 ± 3.4	90.7 ± 0.0
300 (4H)	129 ± 10	110 ± 18	94.7 ± 0.3

Average of five measurements is tabulated.

the initial (10 min) dissociation is followed by either unchanged micelle distribution or re-association dependent on pressure and temperature. At HP treatments below 250 MPa and above 300 MPa casein micelles dissociated by the HP treatment remained stable (Figs. 2, 3 and 6). Proteins may be dissociated due to inclusion of water

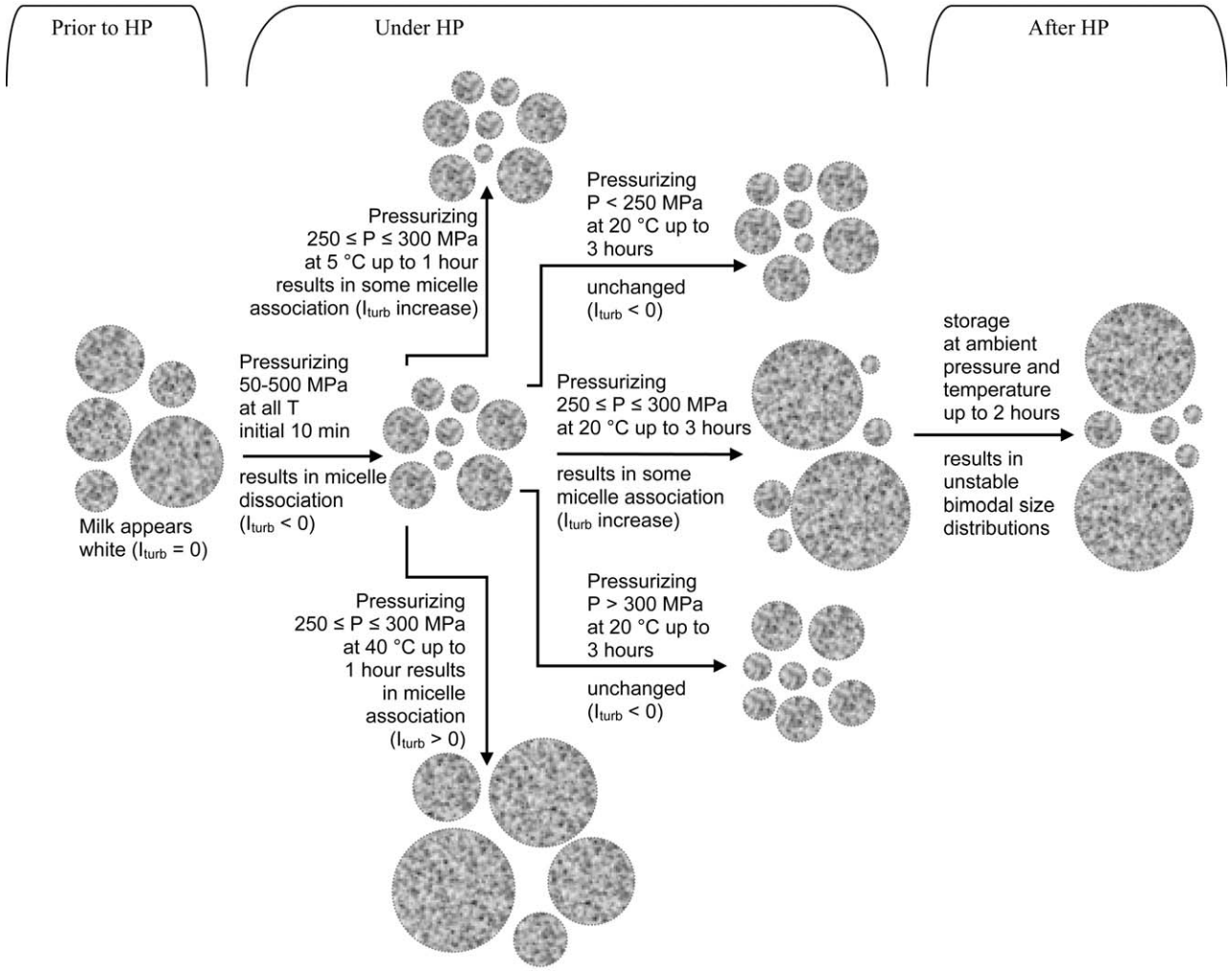

Fig. 6. Changes in casein micelles during and after high pressure (P) treatment of skim milk at various temperatures (T). The sizes of the casein micelles in the figure are based on the results from in situ measurements of the turbidity index (I_{turb}) of pressurized milk and from dynamic light scattering measurements following pressure release.

into the structure (Boonyaratanakornkit, Park, & Clark, 2002; Hummer, Garde, García, Paulaitis, & Pratt, 1998), and such pressure-induced inclusion of water may explain the dissociation of casein micelles. HP treatment of milk, in the range of 100–400 MPa, solubilizes micellar calcium and releases calcium from the casein micelles to the milk serum (López-Fandiño et al., 1998) and additionally caseins are liberated from the casein micelles to the serum upon HP treatment of milk (Anema et al., 2005; Huppertz, Fox, & Kelly, 2004a; López-Fandiño et al., 1998). It has been shown that casein micelles are disrupted into smaller sizes when calcium phosphate is removed from the casein micelles in milk (Holt, 1998). Thus, HP-induced solubilization of micellar calcium may contribute to the disruption of casein micelles into smaller sizes upon pressurization of milk in addition to a possible disruptive effect on the casein micelles caused by HP-induced inclusion of water into the casein micelle structure.

Notably, the present study of skim milk have shown that HP at 250 and 300 MPa represented treatments, where casein micelle sizes are markedly changed, both during and after pressure treatment, and this pressure range seem partly to result in dissociation of casein micelles leaving the micelles highly susceptible to re-association and formation of casein micelle sizes larger than those observed in untreated milk. Casein micelles in untreated milk are remarkably stable and the size of casein micelles is determined by a balance between κ-casein molecules, which terminate growth at the casein micelle surface and availability of calcium phosphate clusters, being able to bind caseins to each other (De Kruif & Holt, 2003; Horne, 1998). Apparently, pressure treatment that initially dissociates casein micelles is a prerequisite for initiation of a subsequent association to form larger micelles (Fig. 6). The highly perturbed and unstable casein micelles, both during (Figs. 2 and 3) and after pressure treatment (Figs. 4 and 5), indicates

a disruptive effect in the balance between stabilizing and repulsive interactions in casein micelles at intermediate pressures. The casein micelles consist of caseins with distinct hydrophobic and charged domains (Farrell Jr. et al., 2004; Swaisgood, 2003). Thus, partly dissociated casein micelles and the dissociated caseins seem particularly prone to re-associate, resulting in formation of larger micelles. This could explain the broad size distribution of the casein micelles as observed in the present study at 250 and 300 MPa (Figs. 4 and 5), and also observed by others (Anema et al., 2005; Huppertz et al., 2004d; Regnault et al., 2004). An increase in temperature markedly favoured association at intermediate pressures (Figs. 3 and 6), as also observed by others (Anema et al., 2005; Regnault et al., 2004; Schrader & Buchheim, 1998). These findings support the assumption that the re-association could be driven by hydrophobic interactions (Anema et al., 2005; Regnault et al., 2004; Schrader & Buchheim, 1998). Stabilization by weak interactions, such as hydrophobic interactions, may render the pressure-induced casein micelle aggregates highly unstable and explain the particle dissociation observed in milk during storage for 2 h after pressure treatment. Interactions between denatured β-lactoglobulin and casein micelles via thiol/disulphide interchange reactions have also been suggested as a mechanism to explain the increase in the size of the casein micelles in milk at a pressure between 200 and 300 MPa (Huppertz et al., 2004c, 2004d). However, the association phenomena were also observed in milk with additions of various thiol blocking agents (Huppertz et al., 2004d), and in microfiltrated milk where only traces of β-lactoglobulin were present (Anema et al., 2005). These authors concluded that other factors than β-lactoglobulin interactions may induce the association of casein micelles at intermediate pressures (Anema et al., 2005; Huppertz et al., 2004d).

4. Conclusion

We have described the dynamic changes in casein micelles during HP treatment of skim milk. HP treatment induces dissociation leaving casein micelles highly susceptible to re-association. Association of casein micelles was found by high temperature and the endothermic nature of the process suggests a major role of hydrophobic interactions during pressure-induced association of casein micelles at intermediate pressures. Stabilization by weak interactions, such as hydrophobic interactions, supports the suggestion that the casein micelle aggregates induced by HP are highly unstable as particle dissociation occurred in milk pressurized at 250 and 300 MPa following pressure release. HP in combination with heating seems to be promising and deserves further attention. Such combinations of treatments can be developed as novel ways to induce aggregation and gelation of casein micelles in milk at neutral pH.

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