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Disruption and reassociation of casein micelles under high pressure

Thom Huppertz[1]*, Alan L Kelly[1] and Cornelis G de Kruif[2]

[1] Department of Food and Nutritional Sciences, University College Cork, Ireland
[2] NIZO Food Research, Ede, PO Box 20, 6710 BA, The Netherlands

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High pressure (HP) treatment affects many constituents of milk (for reviews see Huppertz et al. 2002; Needs, 2002); particular in the properties of casein micelles in HP-treated milk differ considerably from their counterparts in untreated milk. In milk treated at 100–200 MPa, average casein micelle size differs little from that of untreated milk (Needs et al. 2000a; Huppertz et al. 2004a; Regnault et al. 2004; Anema et al. 2005), but micelle size in milk treated at 250 MPa for $\geqslant$15 min is considerably higher than in untreated milk, probably due to HP-induced aggregation of casein micelles (Huppertz et al. 2004a, b; Regnault et al. 2004); after treatment at 300–800 MPa, micelle size is $\sim$50% lower than that in untreated milk (Needs et al. 2000a; Huppertz et al. 2004a, b; Anema et al. 2005). HP-induced changes in average casein micelle size are irreversible on subsequent storage, except for the increase in micelle size after treatment at 250 MPa (Huppertz et al. 2004a).

HP-induced changes in casein micelles influence the optical parameters of skim milk, e.g., the turbidity or the *L**-value, considerably. For milk treated at 100–200 MPa, these optical parameters differ little from untreated milk, but they are reduced progressively with pressure in the range 200–400 MPa, with little further effect at higher pressures (Johnston et al. 1992; Schrader & Buchheim, 1998; Needs et al. 2000b; Huppertz et al. 2004b). HP-induced changes in the *L**-value or turbidity of milk are reversible on subsequent storage at 20 (Schrader & Buchheim, 1998) or 37 °C (Huppertz et al. 2004b), but largely irreversible on storage at <10 °C (Huppertz et al. 2004b; Regnault et al. 2004).

Despite an extensive body of knowledge on the differences between casein micelles in untreated and HP-treated milk, only little is known about the changes that occur in casein micelles during HP treatment. Kromkamp et al. (1996) reported that treatment at 200, 250 or 300 MPa progressively increased the light transmission of milk, indicating disruption of casein micelles under HP; at 200 MPa, the increase in light transmission reversed completely and almost instantaneously on release of pressure, but only partial reversal was observed at 250 or 300 MPa, with further reversal on subsequent storage at room temperature (Kromkamp et al. 1996). The aim of the studies presented in this communication was to estimate the extent of disruption of casein micelles under HP, through comparison with the extent of disruption obtained through dissociating agents, i.e., urea and *tri*-sodium citrate. Based on findings of the current and previous studies, a mechanism for changes in casein micelles under high hydrostatic pressure is proposed.

*For correspondence; e-mail: t.huppertz@ucc.ie

Materials and Methods

Milk supply

Serum protein-free (SPF) milk powder, produced from defatted milk by micro-filtration and ultra-filtration at NIZO food research (Ede, The Netherlands), was reconstituted in demineralised H_2O at a level of 84 or 168 g l^{-1}. The SPF milk samples consist of casein micelles suspended in the serum protein-depleted milk serum. Sodium azide (0·5 g l^{-1}) was added to reconstituted SPF milk to prevent microbial growth.

All experiments were repeated on three individual milk samples.

High pressure treatment of serum protein-free milk

A glass cuvette was filled with 4 ml SPF milk (84 g l^{-1}), closed with a movable plunger and placed in the HP unit, which had an internal volume of 25 ml. HP treatment was performed for 60 min at 200–400 MPa at room temperature ($\sim$20 °C), using Baysilon M20 oil (Roland Chemie B.V., Amsterdam, The Netherlands) as the pressure-transmitting medium. Pressure was increased and released manually at a rate of $\sim$100 MPa or $\sim$300 MPa min^{-1}, respectively and readjusted during treatment if the pressure deviated by 0·5 MPa from the desired value. During the 60 min of HP treatment, as well as for 30 min after the release of

pressure, the transmission of laser light (λ=632·8 nm; LGK 7626 50 mW He-Ne laser, Siemens, Munich, Germany) was measured through optical-grade single-crystal sapphire glass windows fitted into the HP vessel in a direct line with the laser beam with a clearance just in front and after the milk sample; sapphire windows are desirable for high pressure studies such as the present one, because of a high light transmission (>95% light transmission) over an extremely wide wavelength range (~150–5000 nm) as well as a high tensile strength and a low occurrence of faults in the material. Transmission values (*Tr*) were normalized using the value of distilled H_2O (*Tr*=1·00) and that detected when the laser beam was off (*Tr*=0·00).

Addition of citrate or urea to milk

SPF milk (5 ml; 168 g l^{-1}) was mixed with 5 ml 0–200 mmol l^{-1} *tri*sodium citrate (Sigma Chemical Co., St. Louis, USA) to yield a final concentration of 0–100 mmol l^{-1} citrate and 8·4% (w/v) milk solids. Urea (Sigma Chemical Co.) and demineralized H_2O were added separately to 168 g l^{-1} SPF-milk to yield a final concentration of 0·0–8·0 mol l^{-1} urea and 84 g l^{-1} milk solids. The light transmission of SPF-milk containing urea or citrate was measured at ambient pressure and normalised as described above.

Results and Discussion

Influence of urea or trisodium citrate *on the light transmission* (Tr) *of milk*

Adding ⩽3·5 mol l^{-1} urea to SPF-milk had little effect on *Tr*, which remained ~0·0, but higher concentrations of urea increased *Tr*, up to ~0·99 on adding 6·0 or 8·0 mol l^{-1} urea (Fig. 1). Milk containing 6 mol l^{-1} urea, corresponding to *Tr*≈1·0 (Fig. 1), contains no centrifugally-sedimentable protein (Holt, 1998), suggesting complete disruption of casein micelles, to small aggregates and/or monomeric caseins. Adding >10 mmol l^{-1} *tri*sodium citrate to SPF-milk increased *Tr* to ~0·98 at ⩾75 mmol citrate l^{-1} (Fig. 1) as a result of disruption of casein micelles due to sequestration of micellar calcium phosphate (MCP) by citrate. McKinnon et al. (1999) reported that adding 50 mmol l^{-1} EDTA, also a calcium-chelating agent, to milk resulted in complete disruption of casein micelles; this corresponds to *Tr*≈1·0 in milk containing ⩾75 mmol l^{-1} *tri*sodium citrate (Fig. 1).

Influence of high pressure on the light transmission of serum protein-free milk

HP-induced increases in *Tr* of SPF milk (Fig. 2), in agreement with the results of Kromkamp et al. (1996), indicate disruption of casein micelles under HP; the extent of disruption increased with pressure up to 200–400 MPa.

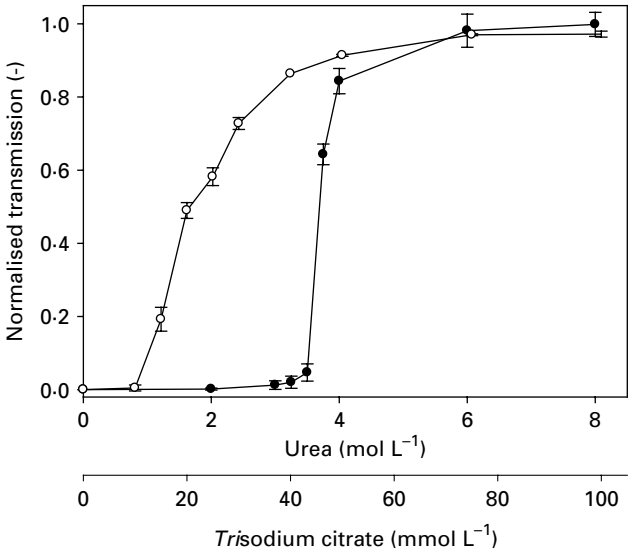

Fig. 1 Normalised transmission of SPF-milk containing 0·0–8·0 mol l^{-1} urea (-●-) or 0–100 mmol l^{-1} *tri*sodium citrate (-○-). Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

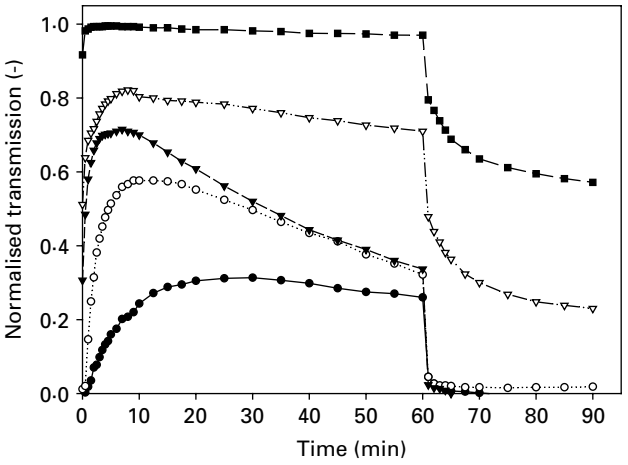

Fig. 2 Normalised transmission of SPF-milk during HP treatment for 60 min at 200 (-●-), 250 (-○-), 300 (-▼-), 350 (-▽-) or 400 (-■-) MPa and during 30 min at atmospheric pressure following the release of pressure. T=0 min represents the time point where the desired pressure was reached. Values are means of data from triplicate experiments on individual milk samples.

Pressurization to 200 or 250 MPa had little effect on *Tr* of SPF-milk ($Tr_{t=0\ min}$<0·01), but after pressurization to 300, 350 or 400 MPa, *Tr* was 0·31, 0·51 or 0·90, respectively (Fig. 2), indicating extensive disruption of casein micelles on pressurization to 300–400 MPa. These effects concur with findings that micelle size in milk treated for 1 sec at 250 or 400 MPa, was similar to, or considerably lower than in untreated milk, respectively (Huppertz et al. 2004a).

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For all pressures studied, *Tr* increased with treatment time to a maximum (Tr_{max}); Tr_{max} increased with pressure, i.e., to 0·31, 0·58, 0·72, 0·82 or 1·00 at 200, 250, 300, 350 or 400 MPa; the time at which Tr_{max} was reached decreased with increasing pressure, i.e., after 30, 15, 7, 5 or 2 min at 200, 250, 300, 350 or 400 MPa, respectively (Fig. 2), suggesting that disruption of casein micelles is more rapid at a higher pressure. Treatment of SPF milk at 400 MPa caused a similar increase in *Tr* (Fig. 2) as addition of $\geqslant 6$ mol l^{-1} urea or $\geqslant 75$ mmol l^{-1} *tri*sodium citrate (Fig. 1), indicating that treatment at this pressure results in complete disruption of casein micelles; lower pressures (200–350 MPa) resulted in less extensive disruption of casein micelles.

After reaching Tr_{max}, a slight reversal in the HP-induced increase in *Tr* of SPF-milk occurred at 200, 350 or 400 MPa, but at 250 or 300 MPa, *Tr* decreased sharply (Fig. 2), which indicates the formation of new, or growth of existing, casein aggregates. These findings agree with the initial reductions in average casein micelle size at 250 MPa with treatment time, followed by increases in micelle size after longer treatment times (Huppertz et al. 2004a). The reversal of the HP-induced increase in *Tr* on prolonged treatment suggests that the casein aggregates, thought to be responsible for the increase in casein micelle size on treatment at 250 MPa (Huppertz et al. 2004a, b; Regnault et al. 2004), are formed during HP treatment.

Immediately following the release of pressure (t = 61 min), *Tr* in milk treated at 200–300 MPa was <0·05, with little further change during the subsequent 30 min at ambient pressure. In milk treated at 350 or 400 MPa, *Tr* decreased by 0·25 or 0·20, respectively, on release of pressure, with a further reduction by 0·25 over the subsequent 30 min at atmospheric pressure (Fig. 2). The reversal of the HP-induced increase in *Tr* on release of pressure (Fig. 2; t = 60 min) indicates the formation of casein aggregates on release of pressure. The only partial reversibility of the increases in *Tr* induced by treatment at 350 or 400 MPa (Fig. 2) concurs with reduced light scattering and increased light transmission of milk treated at such pressures (Johnston et al. 1992; Schrader & Buchheim, 1998; Needs et al. 2000b; Huppertz et al. 2004b).

Changes in casein micelles under high hydrostatic pressure: a possible mechanism

It is apparent that two, counteractive, mechanisms influence casein micelles under HP (Fig. 2): rapid disruption of casein micelles, the extent of which increases with pressure and treatment time, and comparatively slow formation of micellar casein aggregates, which occurs primarily at 250 and 300 MPa. HP-induced disruption of casein micelles was previously suggested to be the result of solubilization of MCP and the disruption of intra-micellar hydrophobic and electrostatic interactions (Schrader & Buchheim, 1998; Needs et al. 2000b; Huppertz et al. 2004a, b), which are probably inter-related, due to the role of calcium in maintaining micellar integrity.

Two forms of micellar calcium can be distinguished, i.e., in amorphous calcium-phosphate clusters and calcium in solution (Holt et al. 1986). The former interacts with the phosphoseryl clusters of the α_{s1}-, α_{s2}- and β-casein, thereby essentially forming the framework micellar nanoclusters of which the micelles are composed, whereas the calcium in solution screens the charges on the caseins and perhaps interacts through ion-bridges between carboxylate groups of the caseins, thus promoting association of caseins. Intra-micellar association of caseins can be seen as a balance between attractive intermolecular hydrophobic and electrostatic interactions and repulsive intermolecular interactions due to electrostatic repulsion and loss of entropy on association (for an extensive review on casein micelle structure see De Kruif & Holt, 2003). Hence, a mechanism to adequately explain changes in casein micelles under HP must consider effects of HP on: (1) the calcium phosphate nanoclusters; (2) charge shielding by calcium in solution; (3) intermolecular electrostatic interactions; and (4) hydrophobic bonding.

The solubility of calcium phosphate increases greatly under HP (Hubbard et al. 2002); this is likely to solubilise the amorphous calcium phosphate nanoclusters, thereby compromising micellar integrity, and decrease intermolecular electrostatic repulsion due to the increased availability of calcium in solution for charge shielding. Intermolecular electrostatic interactions are readily disrupted under HP, due to the electrostrictive effect of separate charges, i.e., the more compact alignment of water dipoles around a charged group than in bulk water (Gross & Jaenicke, 1994).

Effects of HP on hydrophobic bonding are primarily due to pressure-induced changes in the structural arrangement of water molecules (Hvidt, 1975). The exposure of hydrophobic protein surfaces in aqueous solution is favoured at 100–200 MPa, but becomes unfavourable at higher pressures (Kauzman, 1987). This view is in agreement with reports that the light scattering intensity of micelles of β-casein (Payens & Heremans, 1969) or trypsin-treated β-casein (Ohmiya et al. 1989) in solution decreased with increasing pressure up to 150 MPa, but increased with increasing pressure >150 MPa. Ohmiya et al. (1989) related this pressure-dependence to the fact that free bulk water, which has a higher molar volume than water involved in hydrophobic hydration at atmospheric pressure, also has a higher compressibility than this hydrophobic hydration water (Gekko & Noguchi, 1979). Hence, there is a critical pressure above which the molar volume of hydrophobic hydration water becomes larger than the molar volume of free bulk water; under these conditions, one would expect hydrophobic interaction to be favoured over hydrophobic solvation, to minimise the proportion of water involved in thermodynamically unfavourable hydrophobic hydration.

Taking the above into account, it appears reasonable to assume that HP-induced disruption of casein micelles during the initial stages of HP treatment (Fig. 2) is probably a result of solubilization of MCP and disruption of inter-molecular electrostatic interactions. Formation of colloidal casein particles during prolonged treatment at 250 or 300 MPa is probably the result of increased intermolecular hydrophobic interactions at these pressures, possibly aided by additional charge-shielding due to the increased concentration of calcium in solution. The fact that extensive reassociation of casein micelles does not occur on prolonged holding at 350 or 400 MPa indicates that a nucleus may be required for the formation of casein aggregates under pressure; this nucleus may be provided by remaining micellar fragments, which probably are partially-solubilised calcium phosphate nanoclusters. Complete solubilization of the calcium phosphate nanoclusters, leading to complete disruption of the micellar frame-work, may prevent the aggregation of caseins at 350 or 400 MPa.

Reformation of micellar casein particles after release of pressure may be due to hydrophobic bonding (Needs et al. 2000a; Huppertz et al. 2004a, b). The fact that HP-induced increases in *Tr* are only partially reversible after treatment at 350 or 400 MPa (Fig. 1) may be related to increased solubilization of MCP at a higher pressure. A critical level of MCP-removal, through calcium chelation, exists at atmospheric pressure, above which changes in casein micelle structure are not completely reversible (Udabage et al. 2000); this may also apply to HP treatment, i.e., up to a certain level of solubilization of MCP, changes in the micelle are reversible, but at a higher level of solubilization of MCP, resulting in more extensive or complete disruption of the micellar frame-work, changes in the micelle are only partially reversible or irreversible.

It should also be noted that an increase in pressure results in a concomitant increases the temperature of the processing fluid and sample, which dissipates during subsequent holding. It was not possible to determine the temperature of the sample of pressure transmitting medium during HP processing, but consideration of potential HP-induced increases in the temperature of the sample would not appear to contradict the mechanism described above. An increase in temperature reduces the solubility of calcium phosphate and would thus reduce the extent of HP-induced solubilization of MCP and thus stabilise casein micelles against HP-induced disruption; hence, HP-induced disruption during the initial stages of treatment would not appear to be due to the HP-induced increase in temperature. Similarly, hydrophobic bonding, which is thought to be the driving force behind reformation of micelles, is promoted at higher temperatures, but the reformation process only occurs at the latter stages of treatment, where most of the thermal energy generated during treatment has already been lost to the surroundings.

The mechanism discussed above provides considerable understanding on changes in casein micelles during treatment at high pressure and takes into account effects of high pressure on all inter-molecular forces responsible for maintaining micellar integrity; as such, it may provide a frame-work for future studies in this area. It should be noted that the serum protein-free system studied is devoid of whey proteins; association of high levels of denatured whey proteins with casein micelles in HP-treated milk has been reported (Huppertz et al. 2004a) so studies on the influence of whey proteins on changes in casein micelles during HP treatment are desirable for future study.

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