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Properties of casein micelles in high pressure-treated bovine milk

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Abstract

High pressure (HP) treatment of milk alters many of its constituents and properties; in this study, HP-induced changes in casein micelles were examined. HP treatment of milk at 250 MPa increased casein micelle size by ~30%, whereas treatment at 400 or 600 MPa reduced it by ~50%. Pre-denaturing whey proteins in milk by heat treatment or preventing interactions between β-lactoglobulin and κ-casein, by addition of sulphydryl-blocking or reducing agents to milk prior to HP treatment, had little influence on HP-induced changes in casein micelle size. Addition of ethanol to milk, prior to HP treatment, resulted in the formation of large casein aggregates. On treatment at 250 MPa, larger increases in casein micelle size were observed in milk reconstituted from larger micelles, suggesting that increases in micelle size at this pressure are probably due to the formation of casein aggregates. HP treatment reduced the L^*-value of milk; these changes were rapidly reversed on storage of milk at 37 °C, but maintained on storage at 5 °C. Similar to untreated milk, micelles in HP-treated milk were susceptible to dissociation by urea and tri-sodium citrate. Altered properties of casein micelles in HP-treated milk may influence the properties of products made from such milk.

Keywords: High pressure; Milk; Casein micelles; Aggregation

1. Introduction

Under high pressure (HP), considerable changes occur to a wide variety of properties and constituents of milk (for review, see Huppertz, Kelly, & Fox, 2002). Casein micelles are influenced considerably by HP treatment. Under HP, the light transmittance of milk increases (Kromkamp, Moreira, Langeveld, & Van Mil, 1996), suggesting disruption of the casein micelles, probably as a result of solubilisation of colloidal calcium phosphate (CPP) and disruption of hydrophobic and electrostatic interactions (Needs, Capellas, Bland, Manoj, MacDougal & Paul, 2000a; Schrader & Buchheim, 1998). Hydrogen bonds, another important structural feature in casein micelles, are thought not to be disrupted by HP treatment (Hendrickx, Ludikhuyze, Van den Broek, & Weemaes, 1998). On release of pressure, the increase in light transmittance of milk is at least partially reversible (Kromkamp et al., 1996), suggesting reassociation of casein particles, probably due to the reformation of hydrophobic bonds (Needs et al., 2000a) and possibly also electrostatic interactions.

At 20 °C, treatment at a pressure up to 200 MPa had little effect on casein micelle size (Desobry-Banon, Richard, & Hardy, 1994; Huppertz, Fox, & Kelly, 2004a; Needs, Stenning, Gill, Ferragut, & Rich, 2000b), treatment at 250 MPa increased average casein micelle size (Huppertz et al., 2004a; Huppertz, Fox, & Kelly, 2004b) and treatment at >300 MPa reduced micelle size by ~50% (Desobry-Banon et al., 1994; Gaucheron, Famelart, Raulot, Mariette & Le Graet, 1997; Huppertz et al., 2004a, 2004b; Needs et al., 2000b). Large casein aggregates were observed in milk treated at 200–400 MPa at 40–45 °C (Garcia-Risco, Olano, Ramos, & Lopez-Fandino, 2000; Gaucheron et al., 1997; Law et al., 1998).

HP-induced increases in micelle size may be due to interactions between casein micelles and denatured whey proteins (Huppertz et al., 2004a; Schrader & Buchheim, 1998) or to the formation of casein aggregates (Huppertz

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et al., 2004a). When milk is treated at >100 MPa, β-lactoglobulin (β-lg) is denatured and considerable amounts of denatured β-lg interact with the casein micelles (Huppertz et al., 2004a; Needs et al., 2000b; Scollard, Beresford, Needs, Murphy, & Kelly, 2000). However, the nature of associations between casein micelles and β-lg has not been established.

The effects of various pre-modifications of milk, such as pre-heating, addition of ethanol or sulphydryl-blocking or -oxidising agents, and fractionation of micelles into different size classes, on HP-induced changes in casein micelles are reported in this communication. The susceptibility of casein micelles, in HP-treated milk, to dissociation by various agents was also investigated.

2. Materials and methods

2.1. Milk supply

Raw whole bovine milk, obtained from a local dairy (CMP Dairies, Cork), was defatted by centrifugation at 2000*g* for 20 min at 20 °C, followed by filtration through glass wool to remove fat particles. Sodium azide (0.05%, w/v) was added to raw skimmed milk to prevent microbial growth.

Pre-heated skimmed milk was prepared by heating raw skimmed milk at 90 °C for 10 min and cooling rapidly to 20 °C in ice-water.

2.2. Addition of sulphydryl-modifying agents

To study the possible influence of interactions with whey proteins on the effects of HP on casein micelle size, a sulphydryl-blocking agent, *N*-ethylmaleimide (NEM), was added to milk prior to HP treatment. NEM was dissolved (20 mg ml^{-1}) in *N*,*N*-dimethylformamide (DMF); this stock solution was added to milk at 16.7 ml l^{-1}, an estimated 2-fold molar excess (Needs et al., 2000b). To control samples, an equivalent volume of DMF, without NEM, was added; DMF was also added to milk at levels of 10–100 ml l^{-1}, pre- and post-HP treatment. In separate experiments, a sulphydryl oxidising agent, KIO_3 (0.1 mmol l^{-1}), was added to raw skimmed milk prior to HP treatment.

2.3. Addition of ethanol to milk

Ethanol (100%) was mixed with raw skimmed milk, prior to HP treatment, to a final ethanol concentration of 10 or 20% (v/v). Samples were mixed rapidly in hermetically sealed containers to prevent evaporation of ethanol, transferred to polyethylene bags and vacuum-packaged, as described in Section 2.5.

2.4. Preparation of milks with a modified casein micelle size

Casein micelles were fractionated by sequential centrifugation of raw skimmed milk at 50,000*g*, 60,000*g* and 70,000*g* for 15 min and at 90,000*g* for 45 min at 20 °C; the pellets from each successive stage contained casein micelles of decreasing size, which will be referred to as Large, Intermediate-1, Intermediate-2 and Small. The pelleted micelles were separately dispersed to give a protein level similar to that in the original skimmed milk in the supernatant from the final centrifugation step, which was essentially milk serum containing ~0.3% non-sedimentable casein.

2.5. High pressure treatment

Aliquots of milk, packaged as described by Huppertz et al. (2004a), were HP-treated, using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK) for 30 min 100–600 MPa at 20 °C, as described by Huppertz et al. (2004a).

2.6. Determination of average casein micelle size and casein micelle distribution

The size distribution and average size of casein micelles in milk were determined as a function of micelle number by photon correlation spectroscopy, using a Malvern Zetamaster (Malvern Instruments Ltd., Malvern, Worcestershire, UK), as described by Huppertz et al. (2004a). Average casein micelle size, expressed as a percentage of the value for untreated raw skimmed milk, and casein micelle distribution were determined in triplicate for each sample.

2.7. Colorimetry

To examine the effect of HP on the appearance of milk, samples were incubated in sterile containers after HP treatment for up to 14 days at 37 °C or 28 days at 5 °C. The CIE L^*-value of milk was measured using a Minolta CR300 colorimeter (Minolta Camera Company, Osaka, Japan), following calibration as described in the Minolta CR300 operating manual. The L^*-value (reflectance) of milk is related to the state of the casein micelles (dissociated or non-dissociated; O'Sullivan, Kelly, & Fox, 2002), a lower L^*-value indicating dissociation of the micelles. Analysis was performed in triplicate at the storage temperature of the milk (5 or 37 °C).

2.8. Studies of dissociation of casein micelles

Dissociation of casein micelles in untreated or HP-treated milk by urea (final concentration, 0.0–8.0 mol l^{-1})

or tri-sodium citrate (final concentration, 0.00–0.10 mol l^{-1}) was studied; the extent of dissociation was estimated by determination of the L^*-value of the mixture.

3. Results and discussion

3.1. Influence of pre-heating on the effects of high pressure on casein micelles

In agreement with previous reports (Huppertz et al., 2004a, 2004b), HP treatment of raw skimmed bovine milk at 250 MPa increased the average size of casein micelles, whereas treatment at 400 or 600 MPa reduced it by ~50% (Fig. 1), as previously observed by Needs et al. (2000b) and Huppertz et al. (2004a, 2004b).

Pre-heating milk at 90 °C for 10 min denatures >95% of β-lg (Jelen & Rattray, 1995), most of which interacts with κ-casein on the surface of the micelles (Anema & Li, 2003); in this study, heating milk under such conditions had no effect on average casein micelle size (Fig. 1), indicating that heat-induced interactions with β-lg did not influence casein micelle size. After treatment at 250 MPa, the average size of casein micelles was slightly (~10%) higher in pre-heated than in unheated milk, whereas treatment at 400 or 600 MPa caused similar decreases in casein micelle size in unheated and pre-heated milk (Fig. 1). These results suggest that denaturation of whey proteins, by heat prior to HP treatment, had only a small effect on HP-induced increases in casein micelle size.

3.2. Influence of sulphydryl-modifying agents on the effects of high pressure on casein micelles

On HP treatment at ⩾250 MPa, a considerable amount of β-lg interacts with the casein micelles (Huppertz et al., 2004a); however, the mechanism for these interactions has not been established. Needs et al. (2000b) suggested that the interactions could be prevented by the sulphydryl-blocking agent, NEM. To further study the effect of denatured β-lg-casein interactions on the size of casein micelles, the influence of NEM thereon was studied.

Addition of DMF alone, or NEM in DMF, had no effect on casein micelle size in untreated milk (Fig. 2). However, while HP treatment at 250 MPa increased casein micelle size in control milk by ~25%, this treatment increased the average micelle size in milk containing DMF or DMF plus NEM by ~100% (Fig. 2). DMF, with or without NEM, had little influence on the effect of treatment at 400 or 600 MPa on casein micelle size. The sulphydryl-oxidising agent, KIO_3, had no effect on casein micelle size in untreated milk and did not influence the effects of HP treatment at 250, 400 or 600 MPa on casein micelle size (data not shown).

The small influence of the sulphydryl-modifying agents, NEM or KIO_3, on HP-induced changes in micelle size suggests that the interaction of denatured β-lg with casein micelles has little influence on micelle size, which is at variance with the results of Huppertz et al. (2004a), who reported that casein micelles were larger in control milk than in serum protein-free milk after treatment at 250, 400 or 600 MPa. If interactions between β-lg and

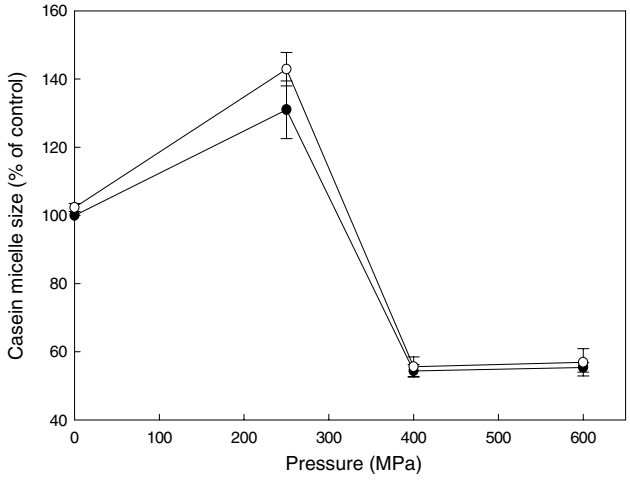

Fig. 1. Effect of high pressure treatment at 0–600 MPa for 30 min at 20 °C on average casein micelle size in raw skimmed milk (–●–) or skimmed milk pre-heated for 10 min at 90 °C (–○–), expressed as a percentage of the value for untreated raw skimmed milk. Values are means of data from triplicate experiments on individual milk samples, with the SD indicated by vertical bars.

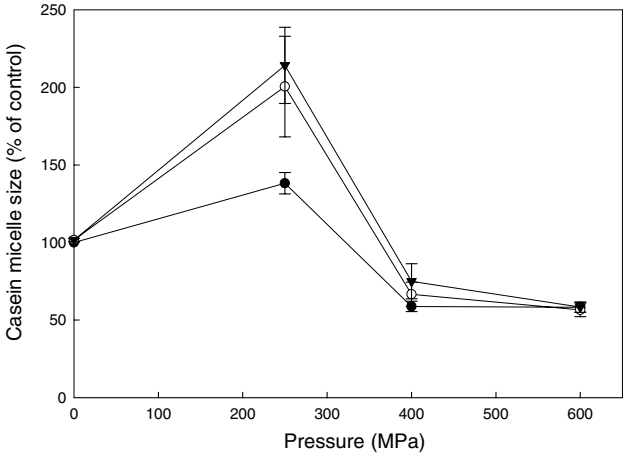

Fig. 2. Effect of high pressure treatment at 0–600 MPa for 30 min at 20 °C on average casein micelle size in raw skimmed milk (–●–), raw skimmed milk containing 16.7 ml l^{-1} DMF (–○–) or raw skimmed milk containing 16.7 ml l^{-1} DMF containing 20 mg ml^{-1} NEM in (–▼–), expressed as a percentage of the value for untreated raw skimmed milk. Values are means of data from triplicate experiments on individual milk samples, with the SD indicated by vertical bars.

casein micelles are responsible for HP-induced increases in micelle size, as discussed previously (Huppertz et al., 2004a; Schrader & Buchheim, 1998), such interactions may be due to a mechanism other than disulphide bonding. However, increases in casein micelle size on treatment at 250 MPa may also be due to formation of large casein aggregates.

3.3. Influence of dimethylformamide on the effects of high pressure on casein micelles

On treatment at 250 MPa, a considerably larger increase in micelle size occurred in milk containing 1.6% DMF than in milk without DMF (Fig. 2); the large increase in micelle size in milk containing DMF suggested the presence of large casein aggregates therein. Further study revealed that, after treatment at 100, 150 or 300 MPa for 30 min, little difference in casein micelle was observed between milk with or without 1.6% DMF (Table 1); however, treatment at 200 and, especially at 250 MPa, in the presence of DMF, resulted in a considerable increase in micelle size.

The influence of DMF on the effects of treatment at 250 MPa for 30 min on casein micelle size was dependent on its concentration and the point of addition (Table 2). DMF at 1–10% had little effect on casein micelle size. HP treatment at 250 MPa increased micelle size in milk containing 0%, 1%, 2.5% or 5% DMF, the extent of the increase being larger for milk with a higher level of DMF; however, treatment of milk containing 10% DMF at 250 MPa had little effect on casein micelle size (Table 2). Addition of 1–10% DMF to milk, after HP treatment at 250 MPa for 30 min, had little influence on casein micelle size (Table 2).

Addition of a high level of DMF (~20%) to milk dissociates κ-casein from the micelles (MacKinlay & Wake, 1965); however, considerably lower concentrations of DMF (1–10%) were used in this study and it is not known whether these low concentrations cause dissociation of the micelles. HP treatment at a pressure ⩾200 MPa also promotes the release of κ-casein from the micelle (Arias, Lopez-Fandino, & Olano, 2000; Lopez-Fandino, De la Fuente, Ramos, & Olano, 1998). The absence of κ-casein on the surface of the micelles prevents the formation of κ-casein/β-lg interactions on the micellar surface; it has been suggested that such interactions hinder reformation of casein micelles on release of pressure (Huppertz et al., 2004b; Johnston, Murphy, Rutherford, & McCreedy, 2002). Thus, DMF-mediated HP-induced dissociation of κ-casein from the surface of the casein micelles may promote the formation of hydrophobic interactions between casein particles on the release of pressure, thus leading to the formation of large casein aggregates. However, further study may be required to establish the exact nature of this phenomenon.

Table 1
Effect of high pressure treatment at 0–300 MPa for 30 min at 20 °C on average casein micelle size in control milk or milk containing 16.7 ml l^{-1} DMF, expressed as a percentage of the value for untreated control milk

Pressure (MPa)	Average casein micelle size (% of control)	
	Control milk	Milk + DMF
Untreated	100.0 ± 0.0	99.8 ± 0.3
100	98.8 ± 2.2	100.9 ± 1.5
150	94.7 ± 0.6	94.6 ± 1.6
200	94.9 ± 0.5	129.4 ± 5.5
250	126.4 ± 2.8	175.7 ± 2.9
300	88.0 ± 5.0	87.2 ± 3.5

Values are means of data from triplicate experiments on individual milk samples ± SD.

Table 2
Influence of DMF added to raw skimmed milk before or after HP treatment on the effects of treatment at 250 MPa for 30 min at 20 °C on average casein micelle size, expressed as a percentage of the mean for untreated raw skimmed milk without added DMF

DMF (%, v/v)	Average casein micelle size (% of control)		
	Untreated	30 min 250 MPa	
		DMF added before HP treatment	DMF added after HP treatment
0	100.0 ± 0.0	128.5 ± 1.8	129.8 ± 2.4
1	100.1 ± 1.4	153.7 ± 5.8	127.9 ± 1.7
2.5	99.3 ± 0.8	181.5 ± 2.3	130.9 ± 1.5
5	98.5 ± 1.7	181.6 ± 2.6	126.3 ± 2.3
10	100.3 ± 2.1	98.5 ± 3.3	131.2 ± 2.7

Values are means from triplicate data on individual milk samples ± SD.

3.4. Influence of ethanol on the effects of high pressure on casein micelles

Addition of ethanol to milk, to a final concentration of 10 or 20% (v/v), had little effect on the average casein micelle size in unpressurised samples (Fig. 3); however, treatment of milk containing 0%, 10% or 20% ethanol at 250 MPa increased micelle size by ~30%, 140% or 90%, respectively. In milk containing 0% or 10% ethanol, treatment at 400–600 MPa reduced micelle size by ~40% or ~20%, respectively, but in milk containing 20% ethanol, HP treatment at 400 or 600 MPa increased average casein micelle size by ~50% (Fig. 3).

The large increases in casein micelle size observed on HP treatment of milk containing ethanol suggest HP-induced aggregation of casein micelles. O'Connell et al. (2003) reported that casein micelles dissociated on heating a 1:1 mixture of milk and 70% ethanol to 70 °C, but reassociated into large aggregates on subsequent cooling. Under HP, dissociation of casein micelles also occurs (Kromkamp et al., 1996) and the presence of ethanol may enhance the formation of casein aggregates on pressure release (Fig. 3).

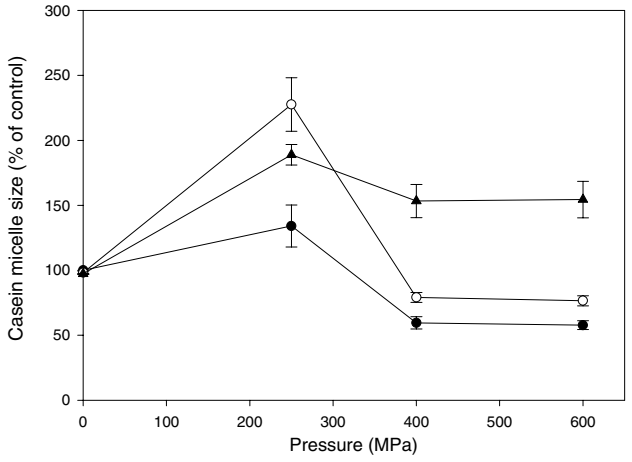

Fig. 3. Effect of high pressure treatment at 0–600 MPa for 30 min at 20 °C on average casein micelle size in raw skimmed milk containing 0 (–●–), 10 (–○–) or 20 (–▲–) % (v/v) ethanol, expressed as a percentage of the value for untreated raw skimmed milk. Values are means of data from triplicate experiments on individual milk samples, with the SD indicated by vertical bars.

3.5. Effects of high pressure on casein micelles in milk with modified casein micelle size distributions

The average casein micelle sizes in milk reconstituted from the Large, Intermediate-1, Intermediate-2 or Small micelle fractions were 220.3 ± 9.0, 176.8 ± 10.0, 149.5 ± 4.1 or 118.2 ± 5.1 nm, respectively; in comparison, the milk from which the micelle fractions were separated, i.e., control milk, had an average casein micelle size of 183.2 ± 7.1 nm (Fig. 4(a)). HP treatment at 250 MPa considerably increased micelle size and broadened the micelle size distribution in all samples, compared to the respective untreated samples, especially in milk reconstituted from the Large and Intermediate-1micelle fractions (Fig. 4(b)). The presence of a larger number of micelles of $\geqslant$400 nm in milks, from various size fractions treated at 250 MPa (Fig. 4(b)), than in corresponding untreated samples (Fig. 4(a)), again suggests the presence of casein aggregates in the HP-treated milk. These aggregates, observed also in milk treated at 200–400 MPa at 40–45 °C (Garcia-Risco et al., 2000; Gaucheron et al., 1997; Law et al., 1998), and possibly formed through hydrophobic bonding on decompression (Needs et al., 2000a), may be responsible for the increases in casein micelle size observed after treatment at 250 MPa (Figs. 1–4), as discussed by Huppertz et al. (2004a).

Treatment at 400 MPa reduced casein micelle size in all samples, and little difference was observed between micelle size distributions in milks reconstituted from Intermediate-1, Intermediate-2 and Small micelle fractions and control milk, with all micelles being $\leqslant$200 nm (Fig. 4(c)); however, after treatment at 400 MPa, milk containing the Large micelle fraction had a broader size distribution than the other milk samples treated at this pressure, with micelles of size up to 300 nm being present. Treatment at 600 MPa resulted in similar micelle size distribution for all milks, with all micelles being $\leqslant$200 nm (Fig. 4(d)). These results suggest that the reduction in micelle size on treatment at 400 or 600 MPa (Figs. 4(c) and (d)), through solubilisation of CCP and disruption of hydrophobic and electrostatic interactions (Needs et al., 2000a; Schrader & Buchheim, 1998), is virtually independent of the initial micelle size; this is in contrast to effects observed at 250 MPa, where a larger initial micelle size resulted in a larger increase in micelle size after treatment at this pressure (Fig. 4(b)).

3.6. Effects of HP on the appearance of milk

HP treatment at 600 MPa for 30 min reduced the L^*-value of milk from ~78 to ~42 (Table 3). HP-induced reductions in the L^*-value of milk (Table 3) are presumably due to disruption of casein micelles and were consistent with results from previous studies (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Huppertz et al., 2004b; Johnston, Austin, & Murphy, 1992). During storage at 5 °C, the L^*-value of untreated milk, or milk treated at 100 MPa, decreased slightly; the L^*-value of milk treated at 200 or 250 MPa did not change significantly, whereas L^*-value of milk treated at 300–600 MPa increased slightly, most of these changes occurring during the first 24 h of storage (Table 3). The L^*-value of all HP-treated samples remained lower than those in untreated milk on storage at 5 °C (Table 3).

Storage of untreated milk at 37 °C for 1 day had little effect on the L^*-value, but the L^*-value of HP-treated milk increased during storage for 1 day at 37 °C (Table 3); the extent of this increase was greater for samples treated at higher pressures and was >20 units for samples treated $\geqslant$400 MPa, thereby reversing most of the HP-induced reductions in this parameter. On further storage at 37 °C, the L^*-value of milk increased gradually, to values between ~70 for milk treated at a pressure $\geqslant$300 MPa or ~80, for untreated milk or milk treated at 100 or 200 MPa (Table 3).

The reversal of the HP-induced reductions in the L^*-value of milk during storage at 37 °C (Table 3) is probably a result of reformation of micellar particles through hydrophobic interactions, as suggested by Needs et al. (2000a). The limited reversal of HP-induced reductions in the L^*-value of milk on storage at 5 °C (Table 3) suggests that reformation of micellar particles does not occur at this temperature, probably due to the low strength of hydrophobic interactions at this temperatures (Walstra, 2003); this is in agreement with observations by Johnston et al. (1992), who reported that HP-induced increases in exposure of hydrophobic groups in milk were unaffected by subsequent storage of the milk for up to 8 days at 5 °C.

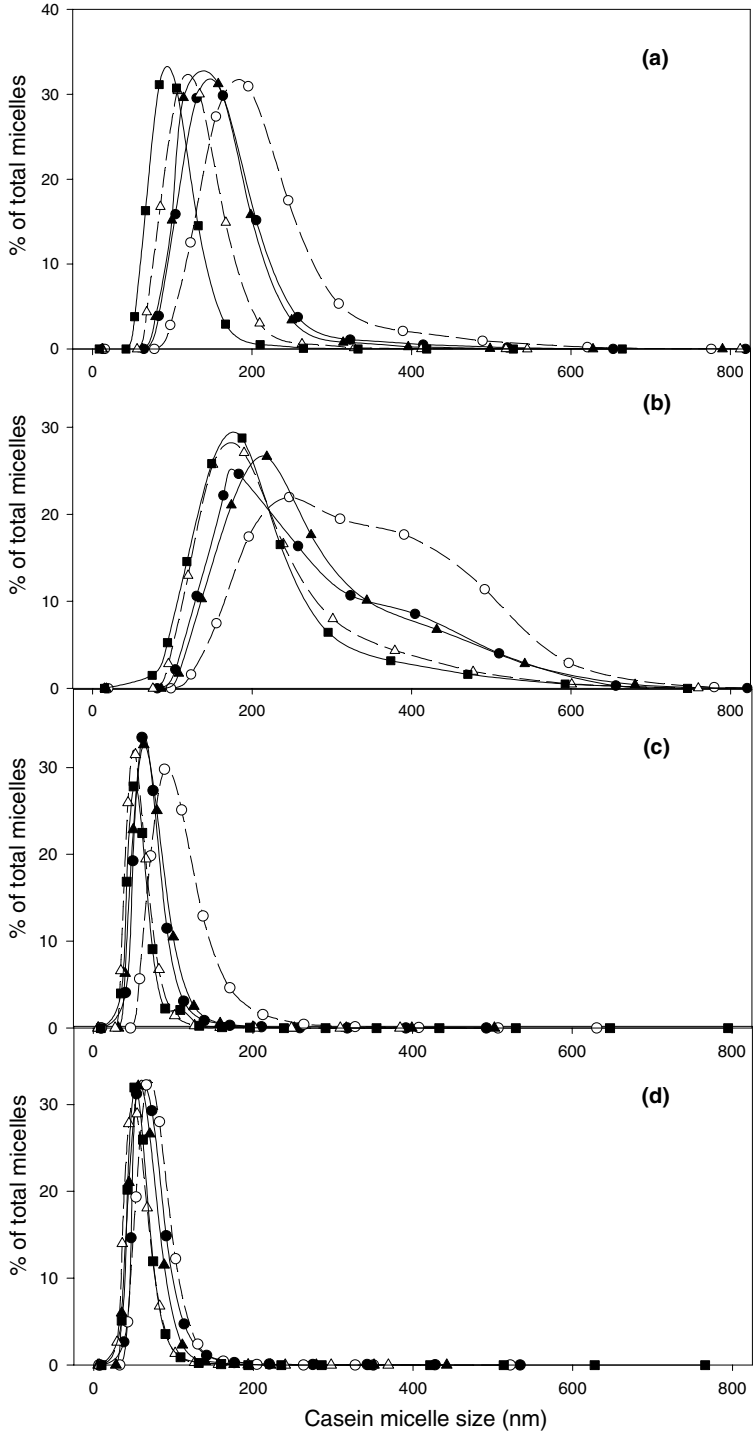

Fig. 4. Casein micelle size distribution in control milk (–●–) or milk reconstituted from the casein micelle fractions Large (–○–), Intermediate-1 (–▲–), Intermediate-2 (–△–) or Small (–■–) before (a) or after high pressure treatment at 250 (b), 400 (c) or 600 (d) MPa for 30 min at 20 °C. Values are means of data from triplicate experiments on individual milk samples.

3.7. Effects of HP on dissociation of casein micelles by urea or citrate

Addition of urea to milk dissociates the casein micelles by disrupting hydrophobic and hydrogen bonds in the micelles, without rupturing casein–calcium phosphate linkages (Holt, 1992; McGann & Fox, 1974). The decrease in the L^*-value for both untreated and HP-treated milk on addition of urea (Fig. 5) indicates disruption of casein micelles in these milks. All untreated and HP-treated milks reached the plateau L^*-value at urea concentrations $\geqslant 6$ M, suggesting that the strength

Table 3
Effect of high pressure treatment at 0–600 MPa for 30 min at 20 °C and subsequent storage for up to 28 or 14 days at 5 or 37 °C, respectively, on the L^*-value of raw skimmed bovine milk

Pressure (MPa)	L^*-value						
		5 °C			37 °C		
	Day 0	Day 1	Day 7	Day 28	Day 1	Day 7	Day 14
Untreated	77.8 ± 0.2	73.8 ± 0.5	72.2 ± 0.1	70.1 ± 0.2	77.7 ± 0.5	77.3 ± 0.9	78.6 ± 1.3
100	73.9 ± 0.3	71.1 ± 0.3	69.7 ± 0.3	68.9 ± 0.1	76.6 ± 0.6	77.5 ± 1.0	78.2 ± 0.7
200	69.0 ± 0.7	68.0 ± 0.4	67.9 ± 0.7	67.6 ± 0.7	75.6 ± 0.4	77.3 ± 0.6	79.6 ± 0.5
250	55.8 ± 1.4	56.9 ± 1.0	57.5 ± 1.2	57.5 ± 1.0	68.5 ± 0.8	73.7 ± 0.5	75.0 ± 0.7
300	45.9 ± 0.8	49.8 ± 0.8	51.2 ± 0.6	51.7 ± 0.8	66.1 ± 0.8	68.4 ± 0.4	69.3 ± 0.1
400	41.6 ± 0.5	47.7 ± 0.6	50.1 ± 0.1	51.0 ± 0.6	65.9 ± 0.7	68.1 ± 0.8	69.1 ± 0.8
600	41.5 ± 1.0	49.0 ± 0.4	53.1 ± 0.3	54.6 ± 0.5	68.5 ± 0.6	70.5 ± 0.7	72.9 ± 0.5

Results are means of data from triplicate experiments on individual milk samples.

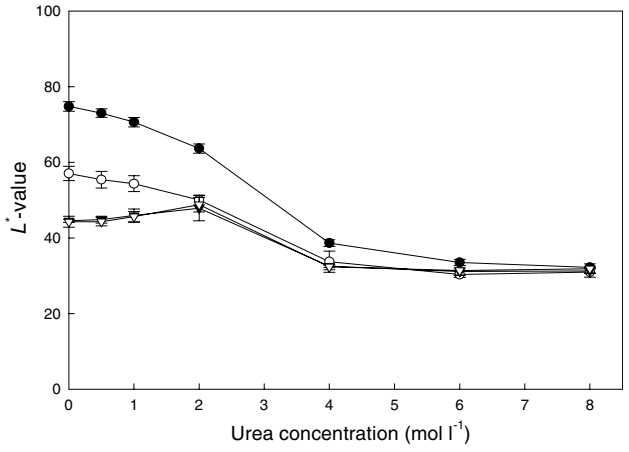

Fig. 5. Effect of adding urea to a final concentration of 0.0–8.0 mol l^{-1} on the L^*-value of untreated raw skimmed milk (–●–) or raw skimmed milk treated at 250 (–○–), 400 (–▼–) or 600 (–▽–) MPa for 30 min at 20 °C. Values are means of data from triplicate experiments on individual milk samples with the SD indicated by vertical bars.

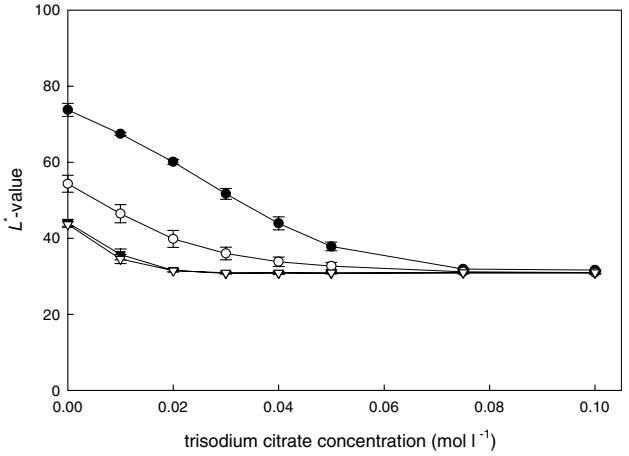

Fig. 6. Effect of adding trisodium citrate to a final concentration of 0.00–0.10 mol l^{-1} on the L^*-value of untreated raw skimmed milk (–●–) or raw skimmed milk treated at 250 (–○–), 400 (–▼–) or 600 (–▽–) MPa for 30 min at 20 °C. Values are means of data from triplicate experiments on individual milk samples with the SD indicated by vertical bars.

of hydrophobic and hydrogen bonds in these milks were similar, and thus not significantly affected by HP treatment. This is consistent with reports by Hendrickx et al. (1998) who suggested that hydrogen bonds are not disrupted by HP; hydrophobic interactions are disrupted under HP (Mozhaev, Heremans, Frank, Masson, & Balny, 1996), but are reformed rapidly at $\geqslant$20 °C after release of pressure (Needs et al., 2000a).

The L^*-value of untreated or HP-treated milk was reduced to ~30 by addition of tri-sodium citrate (Fig. 6). This value was reached at tri-sodium citrate concentrations $\geqslant$0.75, $\geqslant$0.50, $\geqslant$0.02 or $\geqslant$0.02 mol l^{-1} for untreated samples or samples treated at 250, 400 or 600 MPa, respectively. Addition of a calcium-binding agent, such as citrate or EDTA, to milk disrupts casein micelles due to sequestration of CCP (O'Sullivan et al., 2002; Walstra, 1990). Addition of tri-sodium citrate to milk reduced the L^*-value of both untreated and HP-treated milk (Fig. 6); the reduced concentration of tri-sodium citrate needed to reach the plateau L^*-value for milk HP-treated at 400 or 600 MPa confirms previous reports of HP-induced solubilisation of CCP (Buchheim, Schrader, Morr, Frede, & Schütt, 1996; De la Fuente, Olano, Casal, & Juarez, 1999; Lopez-Fandino et al., 1998).

4. Conclusions

Two possible mechanisms have been suggested to explain HP-induced increase in casein micelle size: interaction of denatured β-lg with the casein micelles (Huppertz et al., 2004a; Schrader & Buchheim, 1998) or aggregation of casein micelles (Huppertz et al., 2004a). The results from this study support the latter mechanism, i.e., HP-induced formation of casein aggregates. Preventing or enhancing HP-induced sulphydryl/disulphide associations of β-lg with casein micelles had only a slight effect on micelle size. Structural integrity of casein

micelles in HP-treated milk appears to be maintained by forces similar to those in micelles of untreated milk, i.e., CCP and hydrogen and hydrophobic bonds.

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References

Anema, S. G., & Li, Y. (2003). Association of denatured whey proteins with casein micelles in reconstituted skim milk and its effect on casein micelle size. *Journal of Dairy Research, 70*, 73–83.

Arias, M., Lopez-Fandino, R., & Olano, A. (2000). Influence of pH on the effects of high pressure on milk proteins. *Milchwissenschaft, 55*, 191–194.

Buchheim, W., Schrader, K., Morr, C. V., Frede, E., & Schütt, M. (1996). Effects of high pressure on the protein, lipid and mineral phase of milk. In *Heat treatments and alternative methods* (pp. 202–213). Special Issue No. 9602. Brussels: International Dairy Federation.

De la Fuente, M. A., Olano, A., Casal, V., & Juarez, M. (1999). Effects of high pressure and heat treatment on the mineral balance of goats' milk. *Journal of Dairy Research, 66*, 65–72.

Desobry-Banon, S., Richard, F., & Hardy, J. (1994). Study of acid and rennet coagulation of high pressurized milk. *Journal of Dairy Science, 77*, 3267–3274.

Garcia-Risco, M. R., Olano, A., Ramos, M., & Lopez-Fandino, R. (2000). Micellar changes induced by high pressure. Influence in the proteolytic activity and organoleptic properties of milk. *Journal of Dairy Science, 83*, 2184–2189.

Gaucheron, F., Famelart, M. H., Mariette, F., Raulot, K., Michel, F., & Le Graet, Y. (1997). Combined effects of temperature and high-pressure treatments on physicochemical characteristics of skim milk. *Food Chemistry, 59*, 439–447.

Hendrickx, M., Ludikhuyze, L., Van den Broek, I., & Weemaes, C. (1998). Effects of high pressure on enzymes related to food quality. *Trends in Food Science and Technology, 9*, 197–203.

Holt, C. (1992). Structure and stability of bovine casein micelles. *Advances in Protein Chemistry, 43*, 63–151.

Huppertz, T., Kelly, A. L., & Fox, P. F. (2002). Effects of high pressure on constituents and properties of milk. *International Dairy Journal, 12*, 561–572.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). High pressure treatment of bovine milk: Effects on casein micelles and whey proteins. *Journal of Dairy Research*, 71.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). Heat and alcohol stabilities of high pressure-treated bovine milk. *International Dairy Journal*, 14.

Jelen, P., & Rattray, W. (1995). Thermal denaturation of whey proteins. In P. F. Fox (Ed.), *Heat-induced changes in milk* (pp. 66–85). Special Issue 9501. Brussels: International Dairy Federation.

Johnston, D. E., Austin, B. A., & Murphy, R. J. (1992). Effects of high hydrostatic pressure on milk. *Milchwissenschaft, 47*, 760–763.

Johnston, D. E., Murphy, R. J., Rutherford, J. A., & McCreedy, R. W. (2002). Acidification of high pressure treated milk: The role of whey protein denaturation. *Milchwissenschaft, 57*, 605–608.

Kromkamp, J., Moreira, R. M., Langeveld, L. P. M., & Van Mil, P. J. J. M. (1996). Microorganisms in milk and yoghurt: Selective inactivation by high hydrostatic pressure. In *Heat treatments and alternative methods* (pp. 266–271). Special Issue No 9602. Brussels: International Dairy Federation.

Law, A. J. R., Leaver, J., Felipe, X., Ferragut, V., Pla, R., & Guamis, B. (1998). Comparison of the effects of high pressure and thermal treatments on the casein micelles in goat's milk. *Journal of Agricultural and Food Chemistry, 46*, 2523–2530.

Lopez-Fandino, R., De la Fuente, M. A., Ramos, M., & Olano, A. (1998). Distribution of minerals and proteins between the soluble and colloidal phases of pressurized milks from different species. *Journal of Dairy Research, 65*, 69–78.

MacKinlay, A. G., & Wake, R. G. (1965). Fractionation of *S*-carboxymethyl-κ-casein and characterization of components. *Biochimica et biophysica acta, 104*, 167–180.

McGann, T. C. A., & Fox, P. F. (1974). Physico-chemical properties of casein micelles reformed form urea-treated milk. *Journal of Dairy Research, 41*, 45–53.

Mozhaev, V. V., Heremans, K., Frank, J., Masson, P., & Balny, C. (1996). High pressure effects on protein structure and function. *Proteins: Structure, Function and Genetics, 24*, 81–91.

Needs, E. C., Capellas, M., Bland, A. P., Manoj, P., MacDougal, D., & Paul, G. (2000a). Comparison of heat and pressure treatments of skim milk, fortified with whey protein concentrate, for set yoghurt preparation: Effects on milk proteins and gel structure. *Journal of Dairy Research, 67*, 329–348.

Needs, E. C., Stenning, R. A., Gill, A. L., Ferragut, V., & Rich, G. T. (2000b). High-pressure treatment of milk: Effects on casein micelle structure and on enzymic coagulation. *Journal of Dairy Research, 67*, 31–42.

O'Connell, J. E., Steinle, S., Reiter, F., Auty, M. A. E., Kelly, A. L., & Fox, P. F. (2003). Properties of casein micelles reformed from heated mixtures of milk and ethanol. *Colloids and Surfaces A, 213*, 265–273.

O'Sullivan, M. M., Kelly, A. L., & Fox, P. F. (2002). Influence of transglutaminase treatment on some physico-chemical properties of milk. *Journal of Dairy Research, 69*, 332–341.

Schrader, K., & Buchheim, W. (1998). High pressure effects on the colloidal calcium phosphate and the structural integrity of micellar casein in milk II. Kinetics of the casein micelle disintegration and protein interactions in milk. *Kieler Milchwirtschaftliche Forschungsberichte, 50*, 79–88.

Scollard, P. G., Beresford, T. P., Needs, E. C., Murphy, P. M., & Kelly, A. L. (2000). Plasmin activity, β-lactoglobulin denaturation and proteolysis in high pressure treated milk. *International Dairy Journal, 10*, 835–841.

Walstra, P. (1990). On the stability of casein micelles. *Journal of Dairy Science, 73*, 1965–1973.

Walstra, P. (2003). *Physical chemistry of foods*. New York: Marcel Dekker.