Food Chemistry 119 (2010) 202–208

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

High pressure effects on the structure of casein micelles in milk as studied by cryo-transmission electron microscopy

Jes C. Knudsen *, Leif H. Skibsted

Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form 7 May 2009
Accepted 10 June 2009

Keywords:
Caseins
Casein micelles
High pressure
Calcium
Cryo-transmission electron microscopy

ABSTRACT

Milk was processed with high hydrostatic pressure in order to modify the casein micelles. Images, that in details showed the casein micelle structure in untreated and pressure-treated skim milk, were obtained by using cryo-transmission electron microscopy (cryo-TEM). Sizes and shapes adopted by casein micelles in pressurised milk are concluded to be a result of an equilibrium distribution between self-assembling casein molecules in the serum phase and caseins adsorbed to surfaces of casein micelles and are governed by an initial pressure-dependent displacement of caseins into the serum phase. Pressurisation of milk at moderately high pressure, in the range 150–300 MPa, favoured formation of a large number of small micelles that coexisted with a fraction of large micelles, and which appeared perfectly spherical with smooth and well-defined surfaces, features which are suggested to originate from secondary adsorption of caseins. Pressurisation of milk at 400 MPa favoured formation of smaller casein assemblies, with sizes between 30 nm and 100 nm. Measurements of free calcium concentration [Ca^{2+}] showed that calcium was rebound to casein micelles after pressurisation of milk. Furthermore, the electron microscopy images indicated that the substructures were similar for pressure-modified casein micelles and casein micelles in untreated milk.

1. Introduction

Caseins, the major protein fraction in bovine milk, consist of four gene products: α_{S1}-, α_{S2}-, β-, and κ-caseins and are flexible chains of amino acids with an unordered structure (Farrell et al., 2004; Moitzi, Portnaya, Glatter, Ramon, & Danino, 2008). Caseins are surfactants but, unlike small surfactant molecules with a lipid hydrocarbon tail and a polar head group, caseins contain distinct domains dominated by uncharged hydrophobic amino acids, as well as distinct domains dominated by charged hydrophilic amino acids (Farrell et al., 2004; Moitzi et al., 2008). Like many other surfactant molecules, caseins exist as monomers below a critical concentration and associate spontaneously at higher concentrations (Moitzi et al., 2008).

Caseins in milk are associated into large colloidal aggregates, known as the casein micelles (de Kruif & Holt, 2003; Farrell, Malin, Brown, & Qi, 2006; Horne, 2006). The casein micelles are self-assembled caseins and the micelles are formed in the Golgi vesicles through the binding of calcium and phosphate which screen electrostatic charges on the caseins and allow growth of the micelles (de Kruif & Holt, 2003; Farrell et al., 2006; Horne, 2006). Casein micelles in milk are polydisperse, with sizes ranging from 80 nm to 500 nm in diameter and with an average size around 200 nm in diameter (de Kruif, 1998). Caseins contain phosphorylated serine residues that bind calcium phosphate, which form nanometre-sized clusters within the casein micelles (Holt, 2004; Holt, Timmins, Errington, & Leaver, 1998). The calcium phosphate clusters in the micelle interior give rise to inhomogeneous scattering density of casein micelles upon investigations by small-angle X-ray and neutron scattering (Holt, de Kruif, Tuinier, & Timmins, 2003; Marchin, Putaux, Pignon, & Léonil, 2007). Observations of the structure of casein micelles have also been made with transmission electron microscopy and the casein micelles are visible as more or less spherical colloidal particles by this technique (Marchin et al., 2007; Martin, Williams, & Dunstan, 2007; McMahon & Oommen, 2008). Furthermore, a recent study by scanning electron microscopy revealed that caseins were organised into tubular structures within the casein micelle and the surface of the micelles was rather irregular (Dalgleish, Spagnuolo, & Goff, 2004).

Information about effects of processing on the casein micelle structure is of importance, since the casein micelles are the main ingredient in many dairy products. Casein micelles are rather stable in milk and remain as large colloidal particles, even with severe heat treatment, such as boiling of milk. However, processing with high pressure offers the possibility of modifying casein micelles in milk, even at neutral pH (Huppertz, Fox, de Kruif, & Kelly, 2006a). Measurements under pressure (*in situ*), in the range 100–300 MPa,

* Corresponding author. Tel.: +45 35333144; fax: +45 35333190.
E-mail address: jes@life.ku.dk (J.C. Knudsen).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.017

revealed that casein micelles were dissociated into particles of about 5–20 nm (Gebhardt, Doster, Friedrich, & Kulozik, 2006). The mechanism underlying the disruption of casein micelles during pressurisation of milk is not fully understood; however, it has been suggested that dissolution of calcium phosphate within the micelles may play a role (Huppertz et al., 2006a; Huppertz, Kelly, & de Kruif, 2006b) and that water is pressed into the structure as was also proposed for pressure denaturation of globular proteins (Hummer, Garde, García, Paulaitis, & Pratt, 1998). During prolonged pressurisation, in the range 200–300 MPa, the average casein micelle size initially decreases; however, after some time the average size increases and exceeds the average size of casein micelles in untreated milk (Orlien, Knudsen, Colon, & Skibsted, 2006). In line with this, studies of casein micelles in milk after pressurisation (200–300 MPa) show that the size distribution is wider and thus casein micelles with smaller and larger sizes than those in untreated milk are present in the milk. Pressurisation of milk at 400 MPa, and above, renders casein micelles with an average size of about 100 nm, and without any fraction of casein micelles larger than those observed in untreated milk (Anema, Lowe, & Stockmann, 2005; Huppertz, Fox, & Kelly, 2004; Regnault, Thiebaud, Dumay, & Cheftel, 2004).

At present, information about the structure of the casein micelles after treatment at various pressure levels is limited. For example, it is not established whether the large casein micelles, formed at intermediate pressures, are aggregates of smaller micelles. Neither, does any information exist on the shape of the small and large micelles formed as a result of pressurisation. Transmission electron microscopy of casein micelles in pressurised milk has been used mainly for size estimation, although with a resolution that did not provide information about the structure of the casein micelles (García-Risco, Recio, Molina, & López-Fandiño, 2003; Needs, Stenning, Gill, Ferragut, & Rich, 2000; Scollard, Beresford, Needs, Murphy, & Kelly, 2000).

Principally, we have used cryo-transmission electron microscopy (cryo-TEM) to compare the structure of casein micelles in untreated skim milk and after pressurisation of skim milk, with the aim of visualising effects of pressure on sizes and shapes of casein micelles in milk. Additionally, the free ionic calcium concentration [Ca^{2+}] in milk was measured after pressurisation in order to quantify the calcium that is released from casein micelles. The calcium dissolution within the micelles may be linked to the structure of casein micelles, as observed by cryo-TEM.

2. Materials and methods

2.1. Materials

Low heat skim milk powder, Milex® 240, was provided by Arla Foods, Denmark. Imidazole, $CaCl_2$–$2H_2O$, HCl, NaCl, and P_2O_5 were all from Merck, Darmstadt, Germany. Sodium azide and trypsin inhibitor were from Sigma–Aldrich Chemie GmbH, Steinheim, Germany and Boehringer Mannheim GmbH, Germany. All aqueous solutions were made from purified water, Milli-Q Plus®, Millipore Corporation, Bedford, MA, US and all chemicals were of analytical grade.

2.2. Sample preparation

Skim milk was prepared by dissolving 10 g of low heat skim milk powder in 100 g of purified water. Sodium azide (0.1 g kg^{-1}) and trypsin inhibitor (0.01 g kg^{-1}) were added. Fresh skim milk (Arla Foods, Denmark) was treated with sodium azide (0.1 g kg^{-1}) and trypsin inhibitor (0.01 g kg^{-1}) and used only for the pressure experiments shown in Fig. 1. The skim milk was kept at 22 °C and used for pressure experiments on the same day as it was prepared.

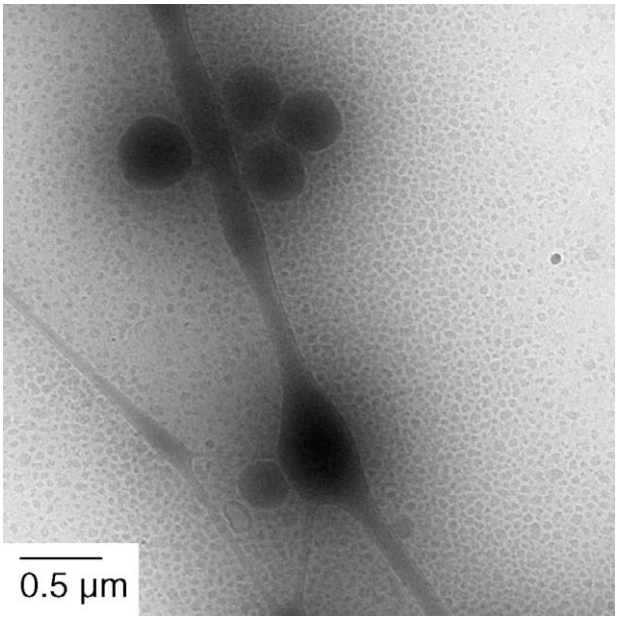

Fig. 1. Cryo-transmission electron micrographs of casein micelles in skim milk subjected to high pressure treatment at 300 MPa. The large continuous black area present in the micrograph is carbon film supporting the thin layer of amorphous ice.

2.3. High hydrostatic pressure treatment

The skim milk was transferred to polyethylene tubes, which were filled without headspace and subsequently vacuum-packed in polyethylene bags. The skim milk samples were subjected to high hydrostatic pressure in a thermostatted hydraulic-pressure chamber from Psika Systems Ltd., Stanford, UK. Isostatic pressures at 20 °C were held for 20 min at 150 MPa, 200 MPa, 300 MPa, or 400 MPa. Rates of pressure increase and release were approximately 100 MPa/min. After pressure release, the skim milk samples were kept at 22 °C overnight prior to further analysis; however, the samples for calcium measurements were analysed immediately after pressurisation.

2.4. Cryo-transmission electron microscopy (cryo-TEM)

The samples for the cryo-TEM analyses were prepared in a chamber with a temperature at 25–27 °C and the relative humidity was close to 100%. Preliminary experiments showed that similar results were found whether microscopic analysis was done 2 h after pressurisation or on the day after pressurisation. Thus, vitrification, on grids was, for the pressurised milk samples, done on the day after pressure treatment and, similarly the untreated milk samples were vitrified the day after preparation. Skim milk sample (5 µl) was put on a lacey carbon film supported by a copper grid, which was gently blotted with filter paper to obtain a thin liquid film, and the grid was then rapidly immersed in liquid ethane (just above its freezing point) and transferred into liquid nitrogen and stored. The vitrified specimens were examined using a transmission electron microscope (Philips CM120 BioTWIN Cryo from Philips Electronics N.V., Amsterdam, The Netherlands) with settings as described earlier (Waninge, Kalda, Paulsson, Nylander, & Bergenståhl, 2004). The cryo-TEM technique is described in detail by Bellare, Davis, Scriven, and Talmon (1988). Representative micrographs, to illustrate effects of high hydrostatic pressure on casein micelles in milk, are selected and shown in the results section.

Repeated experiments showed that the results were absolutely reproducible. The large particles in a polydisperse sample tend to segregate at the edge of the carbon film which, in the present experiments, had a height of about 100 nm (Karlsson, 2001), and smaller particles, tend to segregate along the thickness gradient away from the edge (Bellare et al., 1988; Karlsson, 2001). An example is shown for casein micelles in milk after pressurisation at 300 MPa (Fig. 1). The thickness of the amorphous ice decreases from the edge towards the centre of the hole in the carbon film and a separation of particles by size is seen.

2.5. Measurement of free ionic calcium in milk by a calcium selective electrode

Free ionic calcium (Ca^{2+}) in milk was measured by a calcium selective electrode with a reference calomel electrode, both from Radiometer, Copenhagen, Denmark. For calibration of the electrode, solutions of 1, 2, 3, 4 and 5 mM Ca^{2+} were prepared from a stock solution of 0.100 M $CaCl_2$. The $CaCl_2$–$2H_2O$ was dried in a closed glass container over P_2O_5 prior to use. All calibration solutions were adjusted to an ionic strength of 0.08 with NaCl. From the linear relation between the electrode potential (in mV) in the calibration solutions and the corresponding pCa, measurement of the electrode potential in milk directly gives the Ca^{2+} concentration, assuming that the milk has an ionic strength of 0.08. All measurements were done at 20 °C ± 1 °C, and, for pressurised samples, started 10 min after pressure release. Standard deviations of Ca^{2+} measurements between duplicates of pressure-treated samples were under 5%.

3. Results and discussion

From the cryo-TEM micrograph of untreated milk shown in Fig. 2A, the casein micelles are clearly visible. Micelles with a diameter of about 80–300 nm may be identified, in addition to some smaller micelles. Electron-dense areas, approximately 2–3 nm in diameter are visible inside the casein micelles (Fig. 2B), and this substructure probably originates from the presence of calcium phosphate clusters, as observed by others (Holt et al., 2003; Knoop, Knoop, & Wiechen, 1979; Marchin et al., 2007). The surface structure of the casein micelles in untreated milk is somewhat rough and irregular, and the shape of the micelles does not appear perfectly spherical, which is in accordance with results from previous studies (Marchin et al., 2007; Martin et al., 2007). Pressurisation of milk at 150 MPa (Fig. 3A) and 200 MPa (Fig. 3B) changed the appearance of the casein micelles compared to untreated milk. Notably, a large number of small particles coexisted with larger micelles after treatment at 150 MPa, as well as after treatment at 200 MPa (Fig. 3A and B). Furthermore, some of the large micelles present in milk, after pressurisation at 150 MPa and 200 MPa, had a surface that appeared smoother compared to the other and smaller micelles and also had a smoother surface compared to the casein micelles in untreated milk (Fig. 2A). Fig. 3C and D shows casein micelles in milk after treatment at 150 MPa and 200 MPa, respectively, and small particles with an approximate diameter of 20–50 nm coexist with micelles with diameters up to 200 nm. The effect of pressure on the surface morphology of casein micelles in milk after treatment at 300 MPa, as well as 400 MPa, may be seen in Fig. 4. Pressurisation of milk at 300 MPa further increased the number of small particles; however, a fraction of large casein micelles, which appear perfectly spherical, was also present (Fig. 4A). Fig. 4B shows two micelles with a surface that appears smooth and with a perfectly spherical shape. Furthermore, a number of smaller micelles, with diameters of about 20–50 nm, together with one large micelle, can be identified in Fig. 4C. Pressurisation of milk at 400 MPa limited the growth of casein micelles, and any of the observed protein particles have a diameter of approximately 30 nm to almost 100 nm (Fig. 4D). The size estimates of casein micelles obtained by cryo-TEM lie within the distribution of sizes that have been reported from previous studies by dynamic light scattering of milk subjected to comparable pressure treatment (Anema et al., 2005; de Kruif, 1998; Huppertz et al., 2004; Orlien et al., 2006; Regnault et al., 2004). However, the coexistence of a large fraction of particles from 20 nm to 50 nm, as well as particles with diameters around 300 nm, has not previously been documented in milk subjected to a moderately high

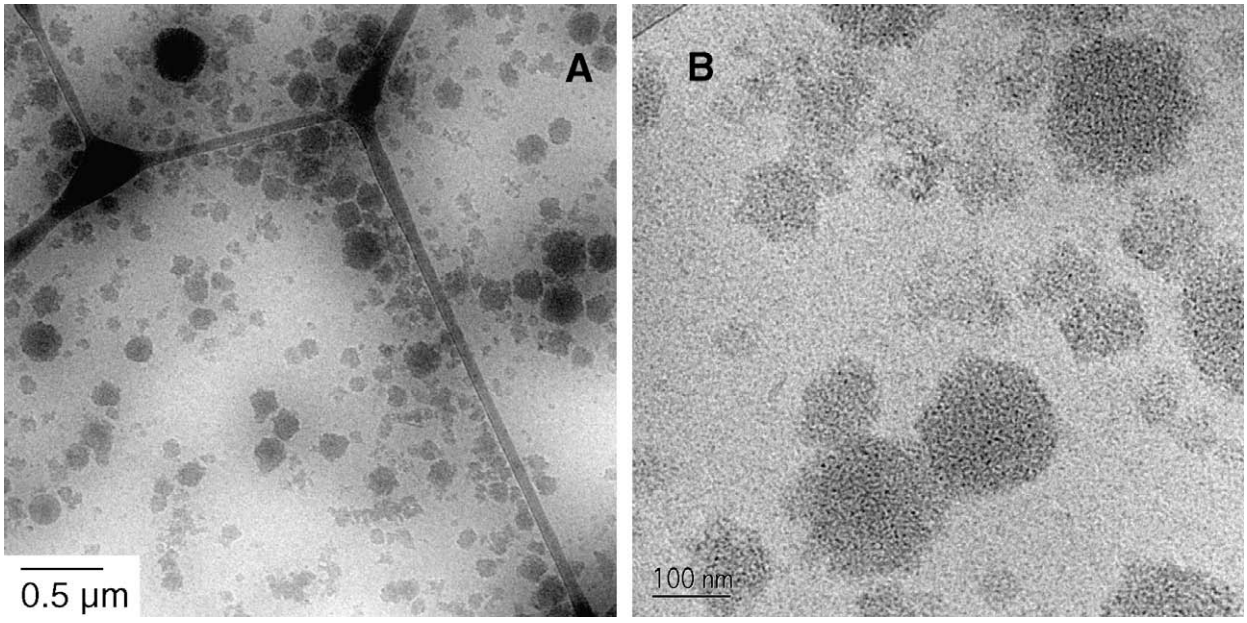

Fig. 2. Cryo-transmission electron micrographs of casein micelles in untreated skim milk shown with different magnification (A and B). The large continuous black area present in micrograph A is the carbon film supporting the thin layer of amorphous ice.

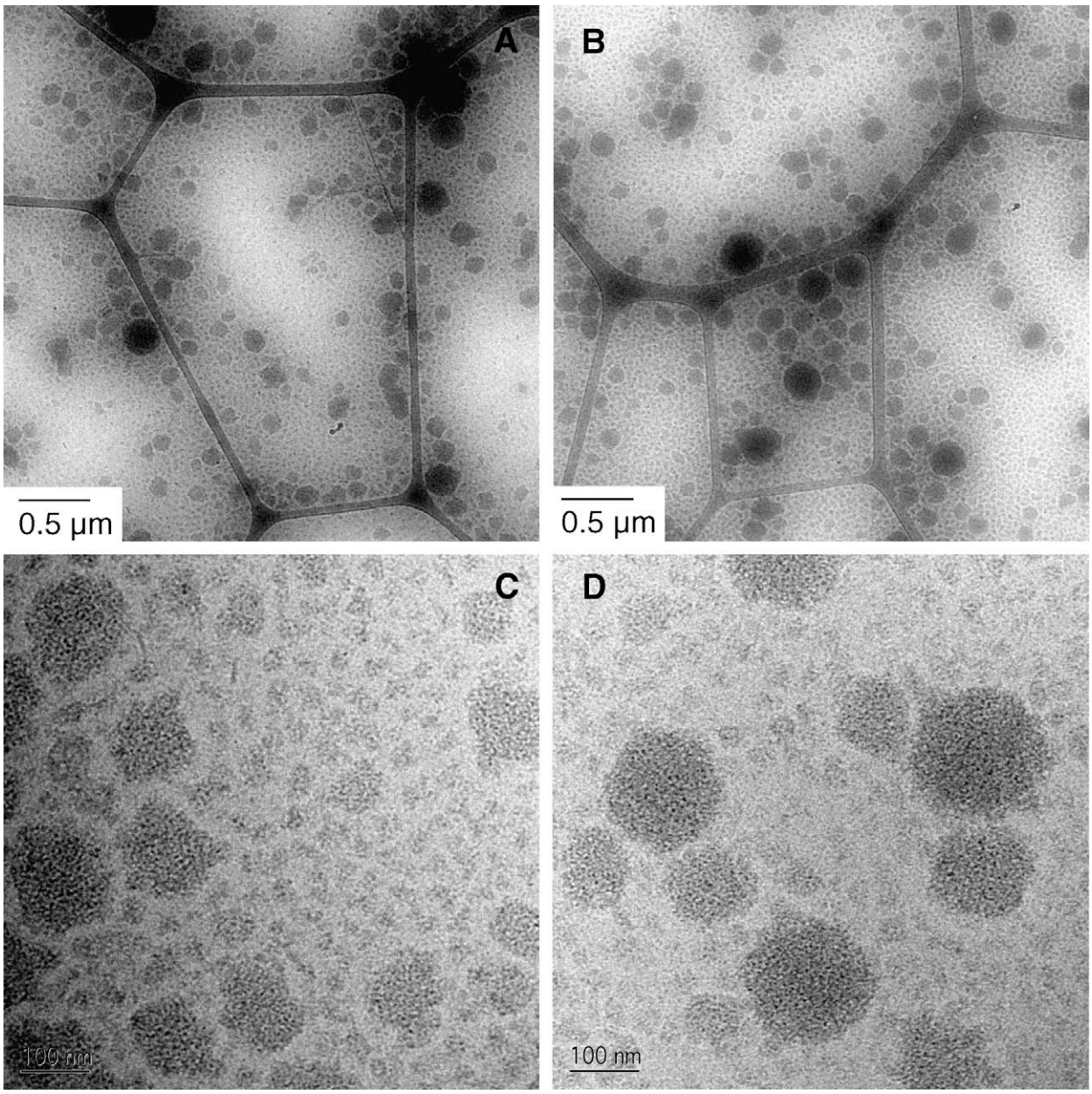

Fig. 3. Cryo-transmission electron micrographs of casein micelles in skim milk subjected to high pressure treatment at 150 MPa (A and C), 200 MPa (B and D). The large continuous black area present in micrograph A and B is the carbon film supporting the thin layer of amorphous ice.

hydrostatic pressure, e.g., 150 MPa or 200 MPa. The scattered light intensity arising from small particles is limited compared to the scattering from large particles and the small particles can hardly be detected by dynamic light scattering of polydisperse samples.

The free calcium concentration in milk, immediately after pressurisation, was increased to about 2–2.4 mM but a gradual decrease in the free calcium concentration toward the level in untreated milk was observed for all samples (Fig. 5). Such measurements have previously only been performed with milk pressurised at 400 MPa and our results agree with those of Keenan, Hubbard, Mayes, and Tier (2003). The pattern, with a gradual decrease in the calcium concentration after pressurisation, was found irrespective of whether the milk was pressurised at 150 MPa, 200 MPa, 300 MPa, or 400 MPa (Fig. 5).

Our results regarding the casein micelle sizes in pressurised milk are consistent with an initially pressure-dependent displacement of casein molecules from the micelles into the serum phase. A release of caseins to the serum phase above a moderately high pressure, e.g., 150 MPa, correlates with the presence of a fraction of particles with an approximate diameter of 20–50 nm, which probably are small assemblies of casein molecules (Figs. 3 and 4). Presence of single casein molecules in the serum phase in milk is energetically unfavourable and these casein molecules may either assemble into micelles or adsorb to surfaces of remaining casein micelles, tending to minimise exposed hydrophobic surfaces. For pressurised milk, it implies a wider distribution of sizes, as observed (Figs. 1, 3 and 4). Furthermore, the surface area relative to the volume of a sphere is decreased when the diameter of the sphere is increased and the sphere in general, represents the minimum ratio of surface area to volume. Accordingly, adoption of a spherical shape is favoured, together with a growth of micelles, as was observed for a fraction of

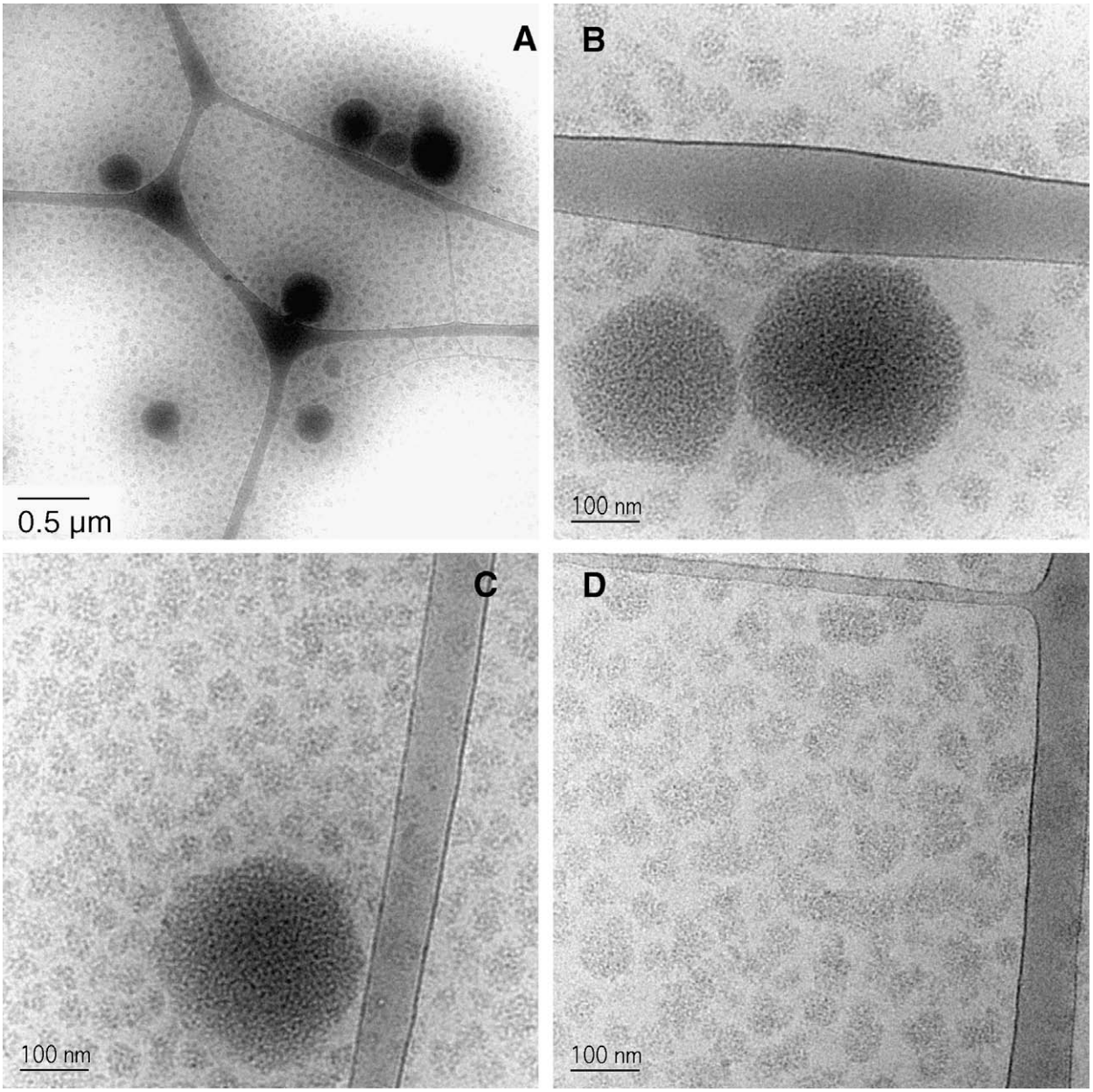

Fig. 4. Cryo-transmission electron micrographs of casein micelles in skim milk subjected to high pressure treatment at 300 MPa (A–C) and 400 MPa (D). The large continuous black area present in the micrographs is the carbon film supporting the thin layer of amorphous ice.

the casein micelles in pressurised milk (Figs. 1 and 4). Growth by secondary adsorption of casein molecules onto surfaces of micelles may explain the clear, distinct boundaries of the large micelles (Figs. 1 and 4). The assembly of casein molecules into large micelles in pressurised milk is driven by hydrophobic interactions. Furthermore, calcium and phosphate association with caseins screen charges and decreases repulsion. The micrographs seem to indicate that both small and large micelles have a substructure with electron-dense areas within the micelles, which may originate from the presence of calcium phosphate clusters (Figs. 3 and 4). Moreover, the free calcium concentration in milk after pressurisation, quite near the concentration in untreated milk, suggests that pressure-modified casein micelles contain calcium (Fig. 5), which has also been shown by others (López-Fandiño, De la Fuente, Ramos, & Olano, 1998). Growth of micelles in pressurised milk is limited and balanced by increased repulsion from adsorbed surface molecules. An increased diameter of a sphere necessarily diminishes the curvature of the sphere and forces the repulsive surface molecules closer together, and further adsorption is energetically unfavourable. Pressurisation of milk at 400 MPa was found to limit growth of micelles and the caseins remained in small assemblies with sizes of 30–100 nm (Fig. 4D). These sizes are slightly larger than the sizes of the fraction of small casein assemblies found in milk after treatment at 150 MPa, 200 MPa, and 300 MPa (Figs. 3 and 4). The presence of exclusively small micelles, and no large micelles, indicates that pressurisation at 400 MPa changes the distribution of casein molecules at the micelle surface in a way that allows an increased total surface area of micelles. Probably, the casein molecules at the surface of the micelles are changed as a result of secondary adsorption/assembly of caseins that occurs either during or immediately after pressurisation.

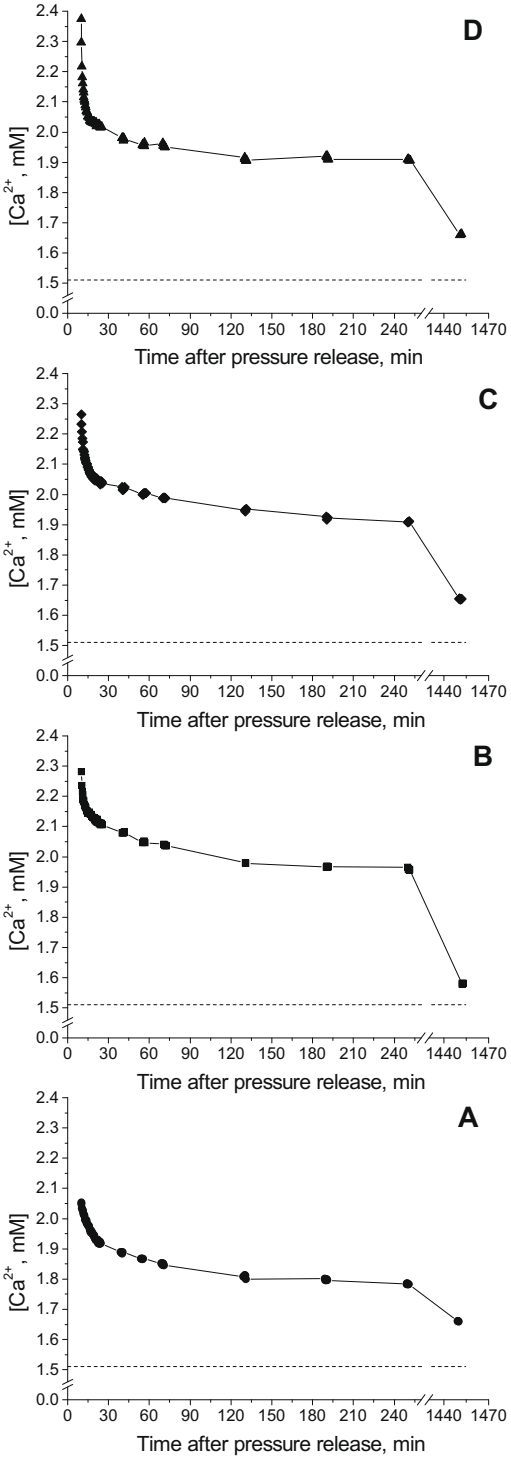

Fig. 5. Ca^{2+} measurements in milk subjected to high pressure treatment for 20 min at 20 °C at 150 MPa (A), 200 MPa (B), 300 MPa (C), and 400 MPa (D). The dashed horizontal line indicates measured level of Ca^{2+} in untreated milk. The Ca^{2+} concentration was measured by a calcium ion selective electrode at 20 °C ± 1 °C from 10 min to 24 h after pressure treatment of milk, which was subsequently kept at 20 °C ± 1 °C.

4. Conclusions

The micelle formation described above represents an extension and simplification of the mechanism for high pressure-induced changes of casein micelles compared to previously proposed mechanisms. Sizes and shapes adopted by casein micelles in pressurised milk are a result of an equilibrium distribution between self-assembly of casein molecules in the serum phase and caseins adsorbed to surfaces of casein micelles. High hydrostatic pressure treatment causes a pressure-dependent displacement of caseins into the serum phase and a secondary assembly and adsorption of caseins to casein micelles. Pressurisation of milk at moderately high pressure, in the range 150–300 MPa, favoured formation of a large number of small micelles that coexisted with a fraction of large micelles, which appeared perfectly spherical with smooth and well-defined surfaces, features which are suggested to originate from secondary adsorption of caseins. Pressure-modified casein micelles and casein micelles in untreated milk were similar with respect to substructure, visualised by cryo-TEM. In agreement, the level of the free calcium concentration in milk, after pressurisation, was quite near the level in untreated milk, which suggests that pressure-modified casein micelles contain calcium.

Acknowledgements

This study was supported by The Danish Dairy Research Foundation and The Danish Food Industry Agency and partly by the project: Calcium in milk. Physiological and technological aspects of protein binding, which was sponsored by The Danish Council for Independent Research, Technology and Production Sciences and The Danish Dairy Research Foundation. The skilful assistance from Gunnel Karlsson, The Biomicroscopy Unit, Chemical Centre, Lund University, Sweden, is gratefully acknowledged. Karsten B. Qvist and Lars H. Øgendal are thanked for their valuable comments. The technical assistance from Anni Nielsen is also gratefully acknowledged.

References

Anema, S. G., Lowe, E. K., & Stockmann, R. (2005). Particle size changes and casein solubilisation in high-pressure-treated skim milk. *Food Hydrocolloids, 19*, 257–267.

Bellare, J. R., Davis, H. T., Scriven, L. E., & Talmon, Y. (1988). Controlled environment vitrification system: An improved sample preparation technique. *Journal of Electron Microscope Techniques, 10*, 87–111.

Dalgleish, D. G., Spagnuolo, P. A., & Goff, D. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal, 14*, 1025–1031.

de Kruif, C. G. (1998). Supra-aggregates of casein micelles as a prelude to coagulation. *Journal of Dairy Science, 81*, 3019–3028.

de Kruif, C. G., & Holt, C. (2003). In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry-1: Proteins. Part A* (3rd ed., pp. 233–276). New York: Kluwer Academic/Plenum Publishers.

Farrell, H. M., Jr., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., et al. (2004). Nomenclature of the proteins of cow's milk – Sixth revision. *Journal of Dairy Science, 87*, 1641–1674.

Farrell, H. M., Jr., Malin, E. L., Brown, E. M., & Qi, P. X. (2006). Casein micelle structure: What can be learned from milk synthesis and structural biology? *Current Opinion in Colloid and Interface Science, 11*(2–3), 135–147.

García-Risco, M. R., Recio, I., Molina, E., & López-Fandiño, R. (2003). Plasmin activity pressurized milk. *Journal of Dairy Science, 86*, 728–734.

Gebhardt, R., Doster, W., Friedrich, J., & Kulozik, U. (2006). Size distribution of pressure-decomposed casein micelles studied by dynamic light scattering and AFM. *European Biophysics Journal, 35*(6), 503–509.

Holt, C. (2004). An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *European Biophysics Journal, 33*, 421–434.

Holt, C., de Kruif, C. G., Tuinier, R., & Timmins, P. A. (2003). Substructure of bovine casein micelles by small-angle X-ray and neutron scattering. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 213*, 275–284.

Holt, C., Timmins, P. A., Errington, N., & Leaver, J. (1998). A core–shell model of calcium phosphate nanoclusters stabilized by β-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. *European Journal of Biochemistry, 252*, 73–78.

Horne, D. S. (2006). Casein micelle structure: Models and muddles. *Current Opinion in Colloid and Interface Science, 11*(2–3), 148–153.

Hummer, G., Garde, S., García, A. E., Paulaitis, M. E., & Pratt, L. R. (1998). The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. *Proceedings of the National Academy of Sciences USA, 95*, 1552–1555.

Huppertz, T., Fox, P. F., de Kruif, K. G., & Kelly, A. L. (2006a). High pressure-induced changes in bovine milk proteins: A review. *Biochimica Biophysica Acta, Proteins Proteomics, 1764*(3), 593–598.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Properties of casein micelles in high pressure-treated bovine milk. *Food Chemistry, 87*, 103–110.

Huppertz, T., Kelly, A. L., & de Kruif, C. G. (2006b). Disruption and reassociation of casein micelles under high pressure. *Journal of Dairy Research, 73*(3), 294–298.

Karlsson, G. (2001). Thickness measurements of lacey carbon films. *Journal of Microscopy, 203*, 326–328.

Keenan, R. D., Hubbard, C. D., Mayes, D. M., & Tier, C. M. (2003). Role of calcium phosphate in the high-pressure-induced gelation of milk. In E. Dickinson & T. van Vliet (Eds.), *Food colloids, biopolymers and materials* (pp. 109–118). Cambridge: The Royal Society of Chemistry.

Knoop, A.-M., Knoop, E., & Wiechen, A. (1979). Sub-structure of synthetic casein micelles. *Journal of Dairy Research, 46*, 347–350.

López-Fandiño, 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.

Marchin, S., Putaux, J.-L., Pignon, F., & Léonil, J. (2007). Effects of the environmental factors on the casein micelle structure studied by cryo transmission electron microscopy and small-angle X-ray scattering/ultrasmall-angle X-ray scattering. *Journal of Chemical Physics, 126*, 045101.

Martin, G. J. O., Williams, R. P. W., & Dunstan, D. E. (2007). Comparison of casein micelles in raw and reconstituted skim milk. *Journal of Dairy Science, 90*, 4543–4551.

McMahon, D. J., & Oommen, B. S. (2008). Supramolecular structure of the casein micelle. *Journal of Dairy Science, 91*, 1709–1721.

Moitzi, C., Portnaya, I., Glatter, O., Ramon, O., & Danino, D. (2008). Effect of temperature on self-assembly of bovine β-casein above and below isoelectric pH. Structural analysis by cryogenic-transmission electron microscopy and small-angle X-ray scattering. *Langmuir, 24*, 3020–3029.

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

Orlien, V., Knudsen, J. C., Colon, M., & Skibsted, L. H. (2006). Dynamics of casein micelles in skim milk during and after high pressure treatment. *Food Chemistry, 98*, 513–521.

Regnault, S., Thiebaud, M., Dumay, E., & Cheftel, J. C. (2004). Pressurisation of raw skim milk and of a dispersion of phosphocaseinate at 9 °C or 20 °C: Effects on casein micelle size distribution. *International Dairy Journal, 14*, 55–68.

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.

Waninge, R., Kalda, E., Paulsson, M., Nylander, I., & Bergenståhl, B. (2004). Cryo-TEM of isolated milk fat globule membrane structures in cream. *Physical Chemistry Chemical Physics, 6*, 1518–1523.