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ARTICLE

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Size distribution of pressure-decomposed casein micelles studied by dynamic light scattering and AFM

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Abstract Reversible and irreversible states of pressure-dissociated casein micelles were studied by in situ light scattering techniques and ex situ atomic force microscopy. AFM experiments performed at ambient pressure reveal heterogeneities across the micelle, suggesting a sub-structure on a 20 nm scale. At pressures between 50 and 250 MPa, the native micelles disintegrate into small fragments on the scale of the observed sub-structure. At pressures above 300 MPa the micelles fully decompose into their monomeric constituents. After pressure release two discrete populations of casein aggregates are observed, depending on the applied initial pressure: Between 160 and 240 MPa stable micelles with diameters near 100 nm without detectable sub-structures are formed. Casein micelles exposed to pressures above 280 MPa re-associate at ambient pressure yielding mini-micelles with diameters near 25 nm. The implications concerning structural models are discussed.

Keywords Casein micelles · High pressure · Dynamic light scattering · Atomic force microscopy

Introduction

Casein micelles are poly-disperse roughly spherical aggregates in milk with diameters ranging between 100 and 300 nm. Their main physiological task is to dissolve calcium phosphate in neonates. Approximately 1 mM casein in milk binds 10 mM Ca_2PO_4 in aqueous solution, whose solubility is in the range of 10^{-3} mM. In contrast to conventional surfactant systems, casein micelles are heterogeneous, composed of four different phospho-proteins α_{S1}-,α_{S2}-,β-,κ-casein (Walstra 1984). Their structure and stability is still a matter of debate (McMahon and McManus 1998; Dalgleish et al. 2004). Walstra (1999) proposed a well defined sub-structure on a scale of 20 nm, consisting mainly of units, which are linked by small calcium phosphate clusters, while other models deny the existence of casein sub-micelles and consider calcium phosphate clusters as seeds of micelle growth (Holt et al. 2003).

The dual binding model of Horne (Horne 1998, 2003) accounts for distinct hydrophilic and hydrophobic regions of the particular polypeptides: The hydrophilic regions in α_{S1}-, α_{S2}-, and β-casein are rich of phosphoserine, where colloidal calcium phosphate particles are attached. The hydrophobic regions associate, stabilizing the core of the micelle.

The α- and β-caseins mainly make up the interior, while κ-casein is located on the surface of the micelle. The C-terminal end of the κ-caseins supposedly extends into the solution and forms a so-called hairy layer. This hydrophilic layer, common to all models, prevents unlimited growth of the micelle by shielding further hydrophobic contacts. This simple picture has been challenged by new electron micrographs, suggesting a tubular surface structure extending into the interior of the micelle (Dalgleish et al. 2004).

Hydrostatic pressure (HP) generally induces the dissociation of macromolecular assemblies by weakening hydrophobic and electrostatic forces (Mozhaev et al. 1996). Pressure thus serves as a tool to probe the architecture and energetics of protein complexes. A series of review articles on the effects of pressure on bio-molecules can be found in the special issue of Biochim.Biophys.Acta, ''Frontiers in high pressure biochemistry and biophysics'' (2002). The aspect of pressure-unfolding of proteins is reviewed in Doster and Friedrich (2005).

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The application of pressure is very important to food processing, sterilization and the technology of milk products (Cheftel et al. 2002). A variety of studies have shown that casein micelles disintegrate into smaller fragments by pressure treatment as observed by light scattering (Desobry et al. 1994; Anema et al. 1997, 2005) and imaging techniques such as electron microscopy (Needs et al. 2000) or atomic force microscopy (Regnault et al. 2004).

According to these ex situ studies the micelle size is affected only slightly at pressures below 200 MPa (Desobry-Banon et al. 1994; Needs et al. 2000; Huppertz et al. 2004a). High-pressure treatment at 250 MPa is even reported to increase the average micelle size by 30% (Huppertz et al. 2004a, b), while pressures above 300 MPa reduce the micelle size by 50% (Desobry-Banon et al. 1994; Gaucheron et al. 1997; Needs et al. 2000; Huppertz et al. 2002, 2004b). The pressure-induced changes in the size distribution were found to be generally irreversible except for the size increase observed at 250 MPa (Huppertz et al. 2004a, b).

To discriminate between reversible and irreversible induced structural changes in the casein size distribution, we have performed in situ dynamic light scattering experiments using an optical high pressure cell (Gebhardt et al. 2005). In this work we present further in situ light scattering experiments in combination with ex situ atomic force microscopy in a liquid cell.

To avoid the problems with dilution, we analyze native turbid solutions at a concentration of 3%, using a backscattering technique, which records only light scattered from the surface of the sample. This procedure minimizes multiple scattering and yields approximate molecular parameters even of turbid solutions.

Materials and methods

Casein micelles were extracted from commercial-grade skim milk by a combined uniform trans-membrane pressure micro-filtration (mean pore diameter 0.1 μm) and ultra-filtration, concentrated by five washing steps and dried in a spraying tower (Tolkach and Kulozik 2005). Casein powder was dissolved in filtered 0.1 M Mes/Tris-HCl solution, at a concentration of 40 mg/ml. The pH was adjusted to 7.3 with HCl. The solutions were equilibrated by thoroughly stirring for 5 h at 20°C. The samples were exposed to various pressure levels, namely to 0.1, 40, 160, 200, 240, 280, 320 and 400 MPa, using a four-window (sapphire) liquid pressure cell (SITEC-Switzerland). The adjustment of the pressure was controlled by a calibrated pressure gage (SITEC). To reach equilibrium conditions, the pressure level was kept constant for 30 min at 20°C except for the kinetic experiments in Fig. 2 (Gebhardt et al. 2005). Pressure jumps were applied within 100 s, the transient temperature rise was below 1°C depending on the step size.

Static and dynamic light scattering experiments (PCS) were performed with an ALV-NIBS System (ALV-Laser GmbH, Langen). The temperature was stabilized at 20°C. The light of a He–Ne laser ($\lambda = 632.8$ nm, $P = 2$ mW) is focused on the surface of the sample inside the pressure cell to minimize multiple scattering. The back-scattered light is recorded in a 177° back-scattering geometry using a sensitive avalanche diode as detector. The resulting signal is transmitted to a multiple time scale correlator (ALV). The back-scattering set-up with turbid casein solutions (at a concentration of 3%) yielded almost identical size distributions as those derived from more dilute (0.5%) samples. Multiple scattering enhances the initial decay of the intensity correlation function, which leads to a bias in the size distribution toward smaller values.

A major complication of light scattering studies is dust particles in the sample. Therefore careful filtering procedures have to be applied. In the case of casein micelles an efficient filtering is not possible, since dust particles and micelles are of similar size. It is thus essential to work with concentrated solutions of dust-controlled casein preparations (filtered buffers). This guarantees that the scattering signal of casein dominates with respect to the signal of the buffer. However at elevated pressures the scattering intensity decreases due to dissociation of micelles, which enhances the relative contribution of the background. A naive evaluation of the correlation function yields the puzzling result of a constant average particle size in spite of a large reduction in scattering intensity. Only the calculation of the number average (instead of the z-average) reveals that the dominant species has a much smaller size, consistent with the lower average intensity.

Particle size distributions at various pressures were calculated by transforming the intensity -intensity correlation functions with the CONTIN 2DP routine (Provencher 1982), implemented in the ALV data analysis package.

Contact mode AFM measurements were performed using the Nanoscope AFM-3/780 (Digital Instruments Veeco Metrology Group) in a liquid cell. The NSE-436 NanoScope-E scanning probe controller was used. AFM Scanner: AS-12-AT 10 μm×10 μm 2637E. The spring constant of the cantilever was 0.06 N/m. The sample was absorbed on a mica substrate and implemented in the cell. The objects in the AFM maps were classified according to their size and frequency. The resulting size histograms were approximated by Gauss distributions, applying the non-linear-regression package of SIGMA PLOT (SSPS, Chicago, IL).

Results

In situ size distribution of casein micelles from dynamic light scattering

Figure 1 shows the effect of pressure on normalized particle size distributions obtained from a CONTIN analysis of the intensity correlation function. The solid

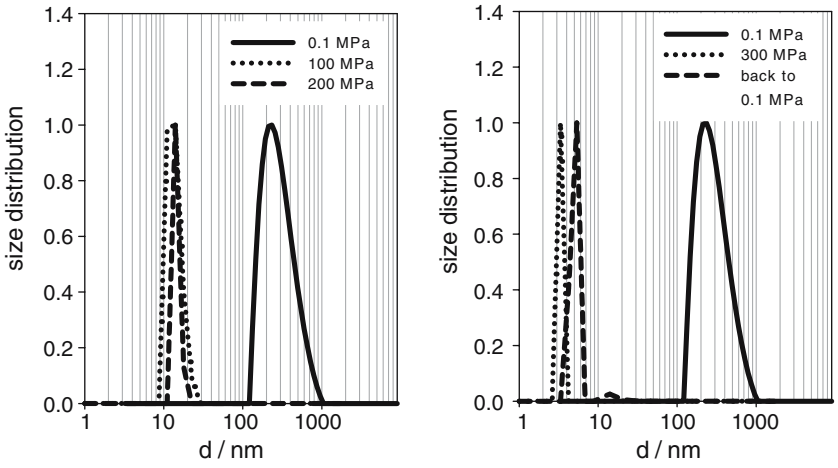

Fig. 1 In situ particle size (diameter) distributions (normalized) versus pressure obtained with dynamic light scattering: **a** intermediate pressure range: (*solid line*) ambient pressure, (*dotted line*) 100 MPa, (*dashed line*) 200 MPa, **b** high pressure range: (*solid line*) ambient pressure, (*dotted line*) 300 MPa, (*dashed line*) ambient pressure after exposure to 300 MPa (30 min). The *solid line* represents the un-weighted or z-averaged distribution, while *dashed* or *dotted lines* refer to number distributions

line in the Fig. 1a or b denotes the z-averaged size distribution obtained by direct ('un-weighted') inversion of the correlation function. The distribution is thus biased by heavy particles $\propto n{\cdot}M^2$, where M denotes the molecular weight and n is the number density. The most probable diameter amounts to 220 nm. However the distribution is about 100 nm wide. Similar values were reported by Anema et al. (2005). Since native micelles at ambient pressure are comparable in size to the wavelength of light (200 vs. 600 nm), intra-molecular interference effects cannot be ignored. This effect can lead to size distributions, which depend on the scattering angle, emphasizing smaller particles at high scattering angles. However at pressures above 100 MPa the turbidity of the casein solution has vanished, indicating the dissociation of micelles into smaller fragments. In contrast, the z-averaged diameter remains constant with pressure. The number average is calculated, assuming that the molecular weight increases with the 3rd power of the diameter, $M \propto d^3$, which is the case for compact spherical particles. In contrast to the z-average, the number average indicates that the most probable particle diameter has decreased to 20 nm as shown in Fig. 1a. The origin of this discrepancy between z- and number average has been explained above by dust particles. The 20 nm species dominates the number average up to 200 MPa, compatible with the existence of stable 'mini-micelles'. At pressures above 250 MPa (Fig. 1b) the mini-micelles dissociate into particles with diameters near 3 nm, the size of casein monomers. Releasing the pressure induces limited re-association to slightly larger particles (5 nm) and mini-micelles (20 nm), but the native micelle is not recovered as shown in Fig. 1b.

In situ pressure association–dissociation kinetics and test of reversibility

Figure 2a shows the time evolution of the light intensity scattered by a turbid casein solution after a pressure step within 100 s of variable size. An increase in pressure leads to a drop in the scattered intensity, indicating a reduction of the average molecular weight. The intensity reaches a plateau within 300 s, depending on the pressure step. The plateau value decreases with pressure suggesting enhance dissociation into fragments. The intensity reaches a minimum value at 300 MPa, indicating the final state of pressure-induced dissociation.

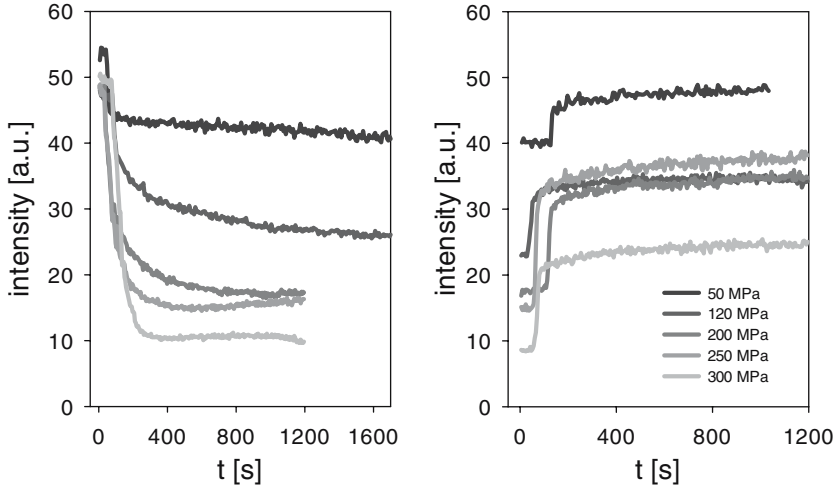

Fig. 2 In situ pressure dissociation–association kinetics of casein-micelles after a pressure jump (< 100 s) monitored by the average (back-scattered) light intensity: **a** dissociation kinetics after a pressure jump from ambient to the indicated pressure levels, **b** association kinetics after a jump from the indicated to ambient pressure (holding time 30 min)

Figure 2b shows the effect of pressure release on the light scattering signal: Up to 50 MPa the signal recovers nearly to the original value at ambient pressure, indicating full reversibility. In the pressure range between 120 and 250 MPa, the intensity curves converge to a common plateau, which is significantly lower than the original value at 0.1 MPa. Above 300 MPa, where the casein micelles dissociate completely into monomer units (Fig. 1), only a small recovery of the intensity is observed.

The results of the pressure cycle experiments are summarized in Fig. 3. The symbols represent the final states of association, which are achieved while the pressure is applied and after pressure release. Increasing pressure (vertical arrows) leads to a continuous decrease in the final degree of association and a regular transition curve. In contrast the pressure release (horizontal arrows) leads to three discrete states: Two well-defined irreversible fragments and the reconstituted micelle.

Ex situ AFM-measurements on casein micelles reveal irreversible pressure effects

To elucidate the surface morphology and size distribution of pressure-treated casein micelles and their irreversible fragments, AFM experiments were performed. The samples were set under pressure for 30 min in discrete steps (0.1–400 MPa) across the dissociation transition (Fig. 3). Instead of a continuous evolution of the structure with pressure we observe three characteristic morphologies, which are displayed in Fig. 4. This result is consistent with the three final states observed with the kinetic experiments in Figs. 2b and 3. The native micelles, observed up to 50 MPa, appear to be composed of elements, suggesting a rather heterogeneous morphology. Moreover, the complex is quite fragile: Repeated scans induce disruption and dissociation due to the mechanical force of the AFM-tip. Thus in spite of obtaining reproducible images, we cannot exclude, that the apparent sub-structure of the micelle is an artifact of the imaging process. In contrast, particles exposed to pressures between 120 and 240 MPa appear to be compact and homogeneous. These particles exhibit little substructure and are less easily perturbed by the AFM tip. Finally the AFM images of samples exposed to pressure levels above280 MPa do not form large micelles. Particles with diameters near 20–30 nm however persist. The result is consistent with the range of casein sub-micelles, observed after pressure release with dynamic light scattering in Fig. 1. AFM images were analyzed in terms of the relative frequency of particles with a particular size. Five images were averaged at each pressure. The resulting histograms are approximately symmetric and can be smoothed using Gauss distributions.

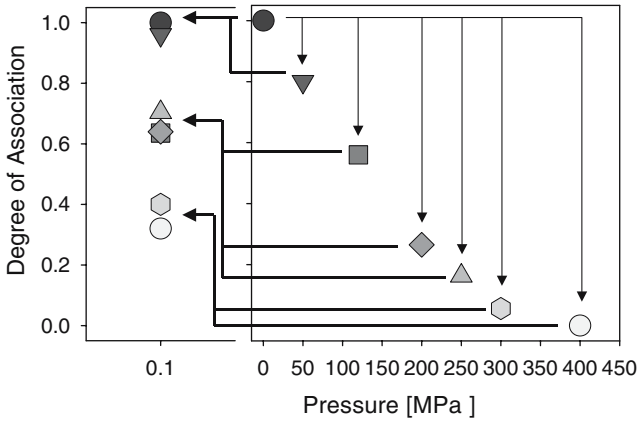

Fig. 3 Dissociation–association cycle of casein micelles: the light scattering signal reflecting pressure dissociation (*vertical arrows*) displays a regular equilibrium transition curve. Re-association (*horizontal arrows*) after pressure release leads to a discrete set of three associated states, which have been studied by ex situ AFM

Figure 5 displays size density plots p(d) evaluated from Gauss fits of the pressure-treated casein samples. Figure 5a demonstrates that the distribution of native micelles diminishes from the large size end. Dissociation and association processes result in a stable species with average size of 110 nm. The decomposition is thus progressively irreversible, starting from moderate pressure-treatment at 40 MPa, where the distribution is bimodal. Figure 5b shows the size distributions resulting from pressure treatment above 240 MPa Their final particle size is close to 25 nm. This result is consistent with the mini-micelles observed in situ with light scattering experiments at low pressure.

Discussion and conclusion

In situ pressure static and dynamic light scattering data, combined with ex situ AFM-measurements provide insight into architecture and stability of casein micelles. In particular the combination of both methods allows discriminating between reversible and irreversible structural changes. From light scattering experiments one derives a most probable size of 220 nm at ambient pressure, which appears to be significantly larger than the AFM result of 170 nm. The discrepancy reflects most likely the bias of the light scattering method toward particles of high molecular weight, the z-average ($\propto M^2$). From the angular dependence of the scattered intensity, which is less biased by the molecular weight ($\propto M$), we derive with dilute samples an average size of 190 nm.

The native 170 nm micelles decompose continuously starting at low pressures (<40 MPa) yielding particles with a discrete size between 15 and 20 nm. The complete decomposition into monomers requires pressures above 280 MPa. The AFM images of untreated samples indicate an internal structure of casein micelles. The respective sub-particles show a rather narrow distribution in size between 10 and 20 nm. Walstra proposed that casein particles are composed of sub-domains, which are linked via colloidal calcium phosphate

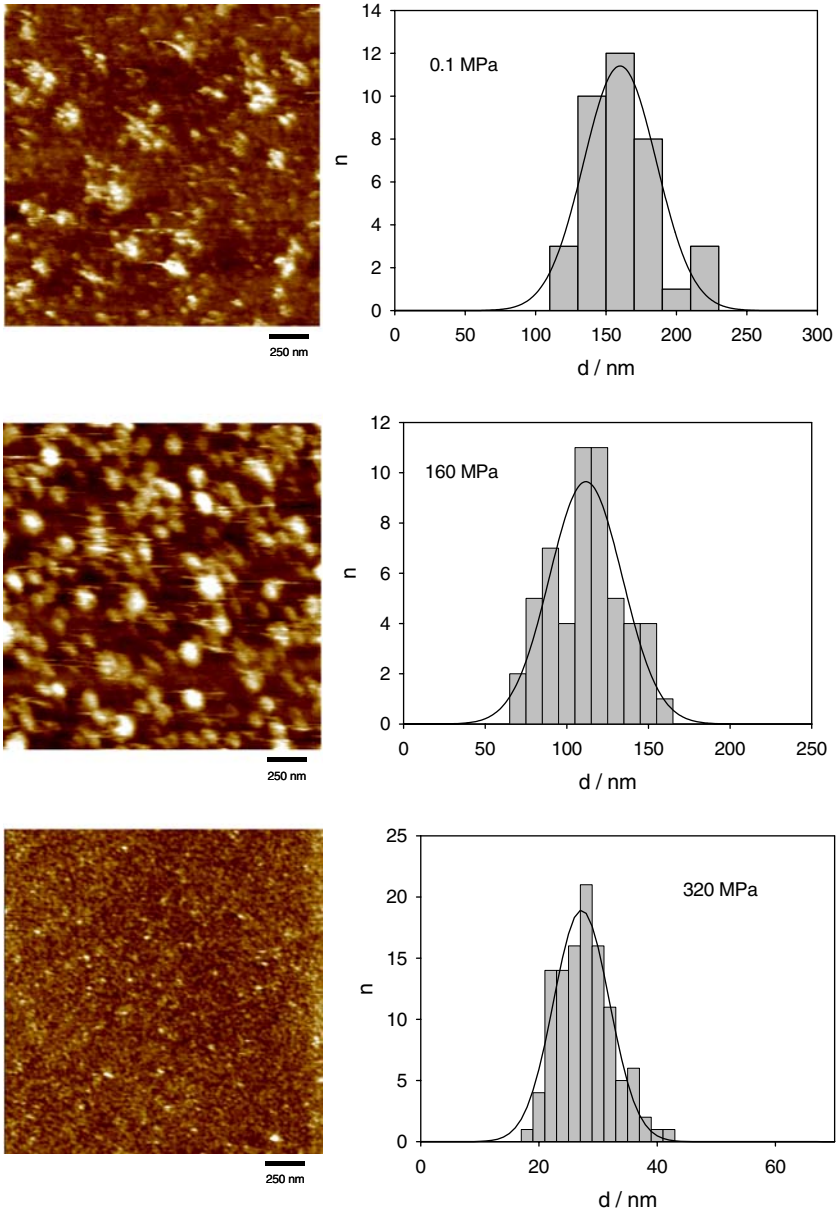

Fig. 4 Three basic structures of pressure-treated casein micelles: representative AFM images together with the associated size-histograms are shown. The *full lines* are fits to Gauss distributions. **a** Intact micelles, $P < 50$ Mpa, **b** compact reconstituted micelles, 120 MPa $< P <$ 240 MPa, **c** mini-micelles, $P > 280$ MPa

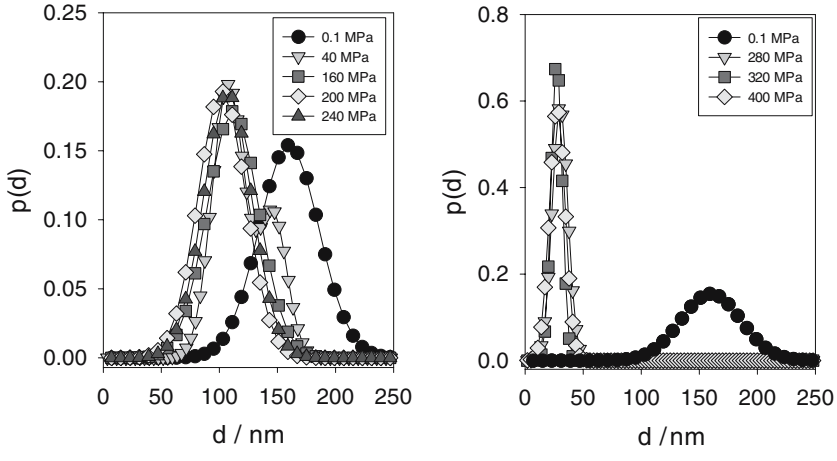

Fig. 5 Size distribution functions p(d) [nm^{-1}] of native and pressure-treated casein micelles derived from the AFM images (area). **a** Intermediate pressure range, **b** high pressure range

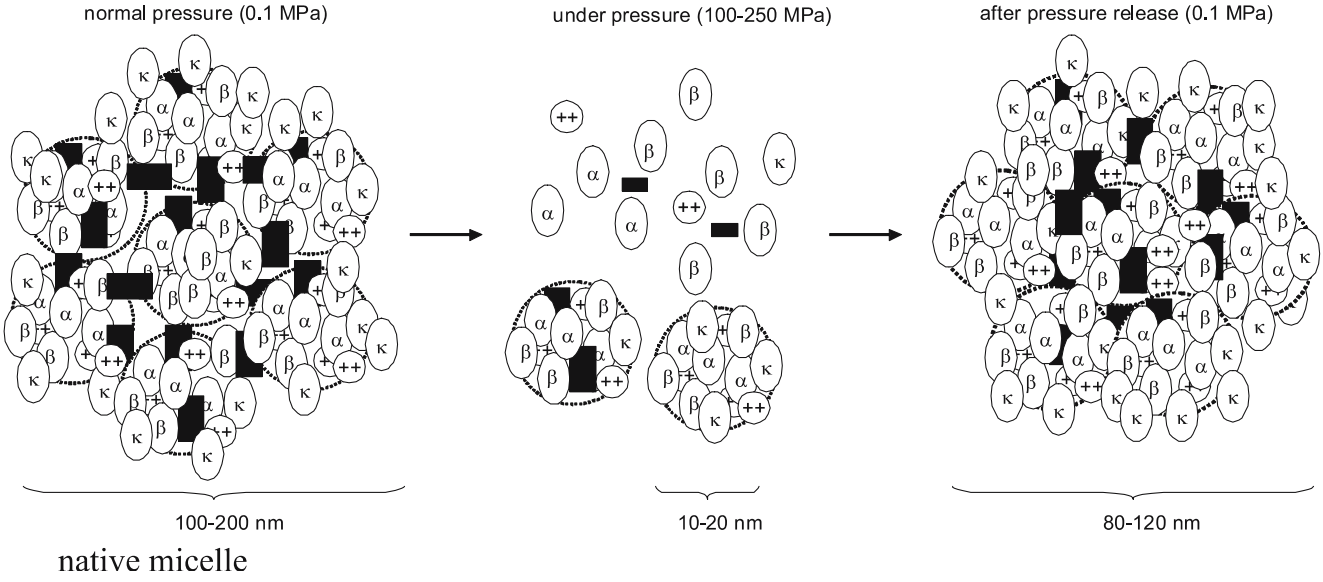

Fig. 6 Schematic view of pressure-induced structural changes at intermediate pressures (below 240 MPa) based on the Walstra model (1999): The native micelles decompose to mini-micelles with diameters in the range of 10–20 nm. After pressure release the mini-micelles re-associate to yield larger compact micelles (80–120 nm) with lower calcium content than the native ones

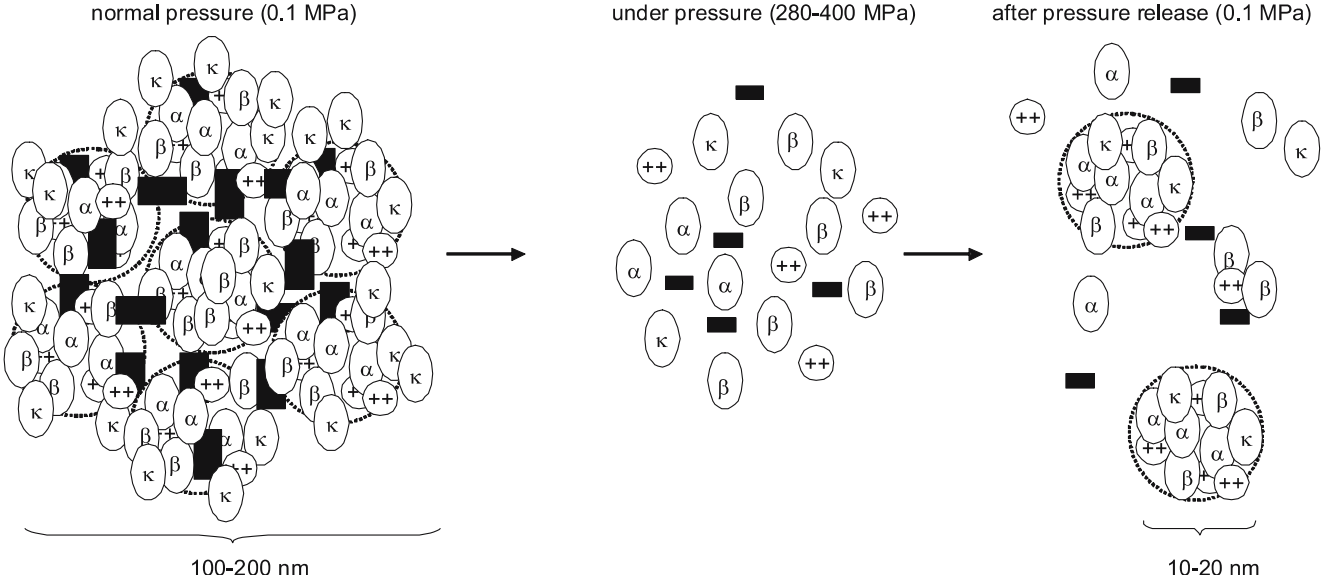

Fig. 7 Schematic view of pressure-induced structural changes at high pressure (above 280 MPa) based on the model of Walstra (1999): the native micelles dissociate completely into monomers. After pressure release, mini-micelles devoid of calcium phosphate are formed with diameters ranging between 10 and 20 nm

(Walstra 1999). Sub-domains are formed by Ca^{++}-induced self-association of the three basic caseins. The inter-domain interactions are mediated by phosphorylated α- and β-caseins. κ-casein is bound to the micelle complex by hydrophobic contacts. Due to its small binding affinity to calcium and due to its tail-train structure, κ-casein associates on the boundary layer, far away from the growing center. In Fig. 6 we use the model of Walstra (1999) to illustrate our results:

Partial recovery of micelles occurs after exposure to pressures between 100 and 250 MPa (Fig. 6). In this range mini-micelles (20–30 nm) are formed. Releasing the pressure leads to further association resulting in a new type of casein micelle, which is somewhat smaller and more compact (80–120 nm) than the native one (170 nm). Moreover, the compact micelles appear to be homogeneous. As a possible mechanism resulting in compact micelles, we assume that a fraction of the mini-micelles formed during pressure treatment below 250 MPa may still contain a high level of colloidal calcium phosphate. After pressure release, these micelles act as aggregation seeds. Fragments with lower calcium

content unite with the growing centers. Therefore particles, which are rich in colloidal calcium phosphate, are found in the interior of the micelle, while hydrophobic particles with lower calcium phosphate content rather gather on the surface. This process leads to a new kind of micelle: Its core is stabilized by colloidal calcium phosphate links, while the shell is dominated by hydrophobic contacts between the caseins. These contacts are enhanced by calcium rebinding to the compact micelle. The resulting matrix is densely packed accounting for the smaller size and the enhanced mechanical stability compared to native micelles. The existence of stable mini-micelles at intermediate pressures with dimensions near 20 nm, detected by in situ by dynamic light scattering experiments, supports the concept of heterogeneous interactions within native micelles even at ambient pressure.

Beyond 280 MPa, the micelles dissociate completely into monomer units due to reduced electrostatic and non-polar interactions. Altogether this leads to an increase in free calcium and phosphate concentration, as observed by Keenan et al. (2003). The pressure-release kinetics show, that casein monomers can re-associate to yield particles with average size of 5–20 nm (Figs. 1b, 5b, 7). The aggregation may be induced by the high level of free calcium resulting from the dissociation of the intact micelles. A full recovery to the native state does not occur because of an irreversible disintegration of the colloidal calcium phosphate particles, which are required as linkers between different casein chains. Pressure-induced dissociation of casein micelles is not fully reversible. Thus in situ pressure studies are necessary to discriminate between reversible and irreversible changes. We used dynamic light scattering in back-scattering geometry to record the structural reorganization of turbid casein solutions under pressure.

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References

Anema SG, Lee SK, Schrader K, Buchheim W (1997) Effect of pH on the turbidity of pressure-treated calcium caseinate suspensions and skim milk. Milchwissenschaft 52:141–146

Anema SG, Lowe EK, Stockmann R (2005) Particle size changes and casein solubilisation in high-pressure-treated skim milk. Food Hydrocolloids 19:257–267

Cheftel JC, Tiehbaud M, Dumay E (2002) High pressure-low temperature processing of foods: a review, p. 327 – 340 In: Winter R (ed) Advances in high pressure bioscience and biotechnology II, Springer Verlag and other articles in this volume

Dalgleish DG, Spagnuolo PA, Douglas Goff H (2004) A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. Int Dairy J 14:1025–1031

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

Doster W, Friedrich J (2005) Pressure-temperature phase diagrams of proteins. In: Buchner J, Kiefhaber Th (eds) Protein folding handbook, Wiley-VCH, Berlin pp 99–126

Gaucheron F, Famelart MH, 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 Chem 59:439–447

Gebhardt R, Doster W, Kulozik U (2005) Pressure-induced dissociation of casein micelles: size distribution and effect of temperature. Braz J Med Biol Res 38:1209–1214

Holt C, de Kruif CG, Tuinier R, Timmins PA (2003) Substructure of bovine casein micelles by small-angle X-ray and neutron scattering. Colloids Surf A 213:275–284

Horne DS (1998) Casein Interactions: casting light on the black boxes, the structure in dairy products. Int Dairy J 8:171–177

Horne DS (2003) Casein micelles as hard sheres: limitations of the model in acidified gel formation. Colloids Surf A: Physicochem Eng Aspects 213:255–263

Huppertz T, Kelly AL, Fox PF (2002) Effects of high pressure on constituents and properties of milk. Int Dairy J 12:561–572

Huppertz T, Fox PF, Kelly AL (2004a) Properties of casein micelles in high pressure-treated bovine milk. Food Chem 87:103–110

Huppertz T, Fox PF, Grosman S, Kelly AL (2004b) Dissociation of caseins in high pressure-treated bovine milk. Int Dairy J 14:125–133

Keenan RD, Hubbard CD, Mayes DM, Tier CM (2003) Role of calcium phosphate in the high-pressure-induced gelation of milk. In: Food colloids-biopolymers and materials. The Royal Society of Chemistry, Cambridge pp 109–118

McMahon DJ, McManus WR (1998) Rethinking casein micelle structure using electron microscopy. J Dairy Sci 81:2985–2993

Mozhaev V, Heremans K, Frank J, Masson P, Balny C (1996) Proteins: Structure Function, and Genetics 24:81–91

Needs EC, Stenning RA, Gill AL, Ferragut V, Rich GT (2000) High pressure treatment of milk: effects on casein micelle structure and on enzyme coagulation. J Dairy Res 67:31–42

Provencher SW (1982) Comput Phys Comm 27:213–229

Regnault S, Thiebaud M, Dumay E, Cheftel JC (2004) Pressurisation of raw skim milk and of a dispersion of phospho-caseinate at 9°C or 20°C: effects on casein micelle size distribution. Int Dairy J 14:55–68

Tolkach A, Kulozik U (2005) Fractionation of whey proteins and caseino-macropeptide by means of enzymatic cross-linking and membrane separation techniques. J Food Eng 67:13–20

Walstra P, Jenness R (1984) Dairy chemistry and physics. Wiley, New York

Walstra P (1999) Casein sub-micelles: do they exist? Int Dairy J 9:189–192