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Alkaline pH does not disrupt re-assembled casein micelles

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ABSTRACT

Characteristics of re-assembled casein micelles were investigated over a broad pH range from 6.35 to 11.4. Turbidity of casein solution decreased as pH increased. The higher the pH, the lower was the turbidity value. This decrease in turbidity was concomitant with the increased diameter of micelles which is attributed to the increased electrostatic repulsion amongst casein molecules and the solvent quality of serum phase. It is hypothesised that looser expanded structure of casein micelles with smaller specific surface area available for light scattering led to a decrease in the observed turbidity of casein solutions with increasing pH. Swelling of re-assembled casein micelles at higher pH values increased the consistency coefficient of casein solutions, indicating an increase in their apparent viscosity.

1. Introduction

Casein micelles, self associated colloids in milk (Horne, 2002), although stable (de Kruif, 1999; Walstra, 1990), but are not fixed structures. Changes in temperature, pH, ionic strength and water activity and imposition of high pressures lead to changes in casein micelles size distribution (de Kruif, 1999; Walstra, 1999) and probably the proportion of sub-structures. This is attributed to their lack of a rigid 3-dimensional tertiary conformation (McMahon & Oommen, 2008). Amongst the various models proposed for the internal structure of casein micelle, sub-micelle model supposes a particulate structure in which the individual caseins form small sub-units via hydrophobic interactions that further cluster together by colloidal calcium phosphate as cement between them (Rollema, 1992; Walstra, 1990, 1999; Horne, 2006). In homogenous internal structure models (Holt, 1992; Horne, 1998; Tuinier & de Kruif, 2002) the micelle is regarded as a self-assembling protein network in which casein chains are cross-linked by colloidal calcium phosphate nanoclusters as building blocks. The interaction sites on casein chains are the phosphoseryl sectors of calcium-sensitive caseins (Horne, 2006). Recently, McMahon and Oommen (2008) proposed an interlocked lattice model in which proteins both surround the calcium phosphate nanoclusters and extend as short chains. This model therefore, views that internal structure of casein micelles contains both globular and linear aggregates of proteins.

Casein micelles can also be re-assembled *in vitro* from caseinate and casein (Liu & Guo, 2008; Semo, Kesselman, Danino, & Livney, 2007; Mounsey, O'kennedy, & Kelly, 2005) even in the absence of calcium (Payens, 1966). This is because of self-assembly tendency of casein monomers (Farrer & Lips, 1999). The overall morphology and size of the re-assembled micelles are neither likely nor expected to be identical to native casein micelles (Huppertz, Vaia, & Smiddy, 2008; Mounsey et al., 2005). They are, however, similar to those of naturally occurring micelles (Semo et al., 2007). For several decades, it has been widely accepted that alkaline pH disrupts casein micelles (Huppertz et al., 2008; Odagiri & Nickerson, 1965). However, recently, Liu and Guo (2008) demonstrated that casein nanoparticles re-assembled from casein are not disrupted by alkaline pH. These conflicting reports persuaded us to carry out a systematic study to explore the influence of pH on casein micelles integrity.

2. Materials and methods

2.1. Materials and sample preparation

Casein and phosphate buffer pH 7.0 (disodium hydrogen phosphate/potassium dihydrogen phosphate) were purchased from Merck (Darmstadt, Germany).

Casein solutions (3%) was prepared by adding 12 g casein powder to 0.5 M phosphate buffer (pH 7.0 prepared by deionised water), stirring at 4600 rpm for 60 min at room temperature and making it up to a final volume of 400 mL; sodium azide (100 mg L^{-1}) was added to prevent microbial growth. The solution was stored at 4 °C for 10 h to allow complete hydration

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(Liu & Guo, 2008). Solution (100 mL) was transferred to a 250 mL Erlenmeyer at its original pH value (6.35 ± 0.05). The pH value of the remainder was then adjusted to 8.0 ± 0.05, 9.7 ± 0.05 and 11.4 ± 0.05 by slow addition of 15 M sodium hydroxide solution to a well-stirred solution. Each time, immediately after the desired pH was reached, 100 mL of solution were transferred to a 250 mL Erlenmeyer. Very strong sodium hydroxide was used for increasing pH value in order to prevent casein solution dilution.

2.2. Turbidity and electric conductivity measurements

Turbidity measurements were carried out using a turbidimeter (WTW, 350 IR., West Chester, PA, USA) and three readings for each sample at 30 °C after 6 h. Banon and Hardy (1992) used a similar method for turbidity measurement of milk samples.

Electrical conductivity of casein solutions was determined after 6 h using a digital conductivity meter (Hanna, model HI 8633, Hanna Instruments Inc., Bedfordshire, UK).

2.3. Particle analysis

Mean particle diameter and size distribution of casein solutions were measured with a laser-diffraction based particle size analyzer (Malvern Master Sizer Hydro 2000 S, Malvern Instruments Ltd., Malvern, UK) operated with program Mastersizer 2000 version 5.22. In this instrument, the angular dependence of the obstruction of a continuous laser beam by particles is used for size measurement (Banavara, Anupama, & Rankin, 2003; Thanasukarn, Pongsawatmanit, & McClements, 2006). Experiments were performed on 5-fold diluted solutions over the range 20 nm–15 μm. Just before measurements, samples were diluted with deionised water having the same pH of sample (adjusted by sodium hydroxide addition) to prevent their foaming during stirring and pumping in the instrument. Water was used as the dispersant with a refractive index was set at 1.330. Particle characteristics of solutions are reported by volume-weighted mean ($D_{4,3}$), specific surface area and span. The latter is an index of particle polydispersity (Tan & Nakajima, 2005) and as expressed by the following equation:

$$\text{Span} = (d_{0.9} - d_{0.1})/d_{0.5} \quad (1)$$

where $d_{0.9}$, $d_{0.1}$ and $d_{0.5}$ are the diameters at 90%, 10% and 50% cumulative volume of particles, respectively.

2.4. Flow behaviour

Viscosity measurements were performed using a rotational viscometer (model DV-II+ pro Brookfield Engineering Lab. Instruments, Middleboro, MA, USA). Samples were sheared from a rate 56.43 s^{-1} to 140.03 s^{-1} and the relevant shear stresses (N m^{-2}) were recorded at 14 time intervals of 6 s. Data were collected by Rheocalc version 3.1-1 Demo application and analysed by Curve Expert version 1.34 (Microsoft Corporation). Consistency (k) and flow (n) indices were calculated by fitting shear rate and shear stress data on power law model.

2.5. Statistical analysis

The experiment was replicated three times in a complete randomised design. The effect of pH value on tested parameters was determined by analysis of variance (ANOVA) using GraphPad Prism 5.0 for windows version 5.00 (GraphPad Software Inc., CA, USA). One-way ANOVA at 5% significant level ($\alpha = 0.05$) was carried out to assess whether different treatments resulted in statistically significant differences in variables evaluated.

3. Results and discussion

Casein solution turbidity decreased rapidly as pH was increased from 6.35 to 8.0 with a marked but more modest linear-like decrease with increasing pH above 8.0. The higher the pH, the lower was the turbidity value (Fig. 1, left axis). Turbidity has been found to be an extremely sensitive, repeatable and noninvasive way of monitoring changes to casein micelles (Martin, Williams, & Dunstan, 2007). Vaia, Smiddy, Kelly, and Huppertz (2006) similarly reported that the extent of decrease in turbidity of reconstituted skim bovine milk at alkaline pH (8.0–11.0) increased with increasing pH. They attributed this to the disruption of casein micelles although they provided no direct evidence for this hypothesis. In another study, increasing milk pH to 10.0 reduced its turbidity to a value close to that of milk serum, indicating complete disintegration of casein micelles. Again they provided no direct evidence for the suggested disintegration phenomenon (Huppertz et al., 2008).

It is reasonable to expect that casein micelles dissociation and disruption would lead to progressively smaller particles. However, particle size measurements in the present study (Fig. 1, right axis) demonstrate a noticeable increase in casein particle diameter as pH was increased from 6.35 to 8.0, and then a milder, linear-like increase with increasing pH above 8.0. Re-assembled micelles diameter at pH 6.35 (282 ± 1 nm) was in good agreement with the results obtained by Mounsey et al. (2005) who formed re-assembled micelles from acid casein with an average size of 250–300 nm at pH 7.0. As expected, the increase in particle diameter is concomitant with a decrease in specific surface area of particles (Fig. 2). The larger the size of particles, the smaller is their specific surface area. Although Zhong, Daubert, and Velev (2007) stated that at a higher pH value casein micelles were larger, no direct evidence for this assumption was provided by them. Recently, Liu and Guo (2008) showed by fluorescence experiments that re-assembled casein micelles were present at high pH, even at pH 12.0. They also observed that the radius of casein micelles increased with increasing pH in the range of 6.0–12.0.

Casein micelle structure is maintained by colloidal calcium phosphate (CCP), a combination of hydrophobic and electrostatic interactions amongst casein molecules (Anema & Li, 2000; Liu & Guo, 2008) and hydrogen bonds (Zadow, 1993). The calcium phosphate ion pairs or CCPs are stabilised, rather than disrupted at high pH values as a consequence of decreased concentration of ionic calcium and phosphate with increasing pH (Vaia et al., 2006). In the present study however, there was logically a minor amount of calcium in the medium and CCP nanoclusters could not play an important role in the formation and structural integrity of micelles. Besides, other authors (Anema, 1998; Anema & Li, 2000; Liu & Guo,

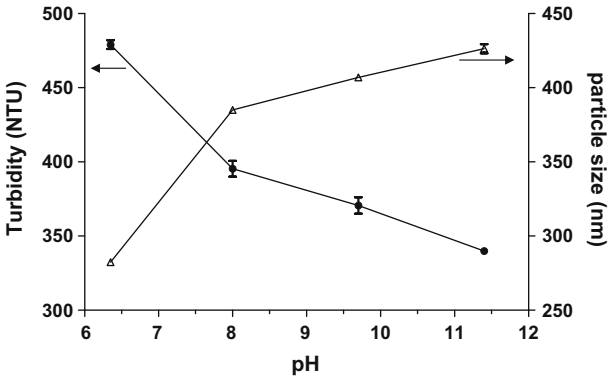

Fig. 1. Turbidity of casein solution (left hand) and particle size of re-assembled casein micelles (right hand) as a result of pH value. All means are statistically different ($p < 0.05$). Error bars indicate standard deviations for each treatment.

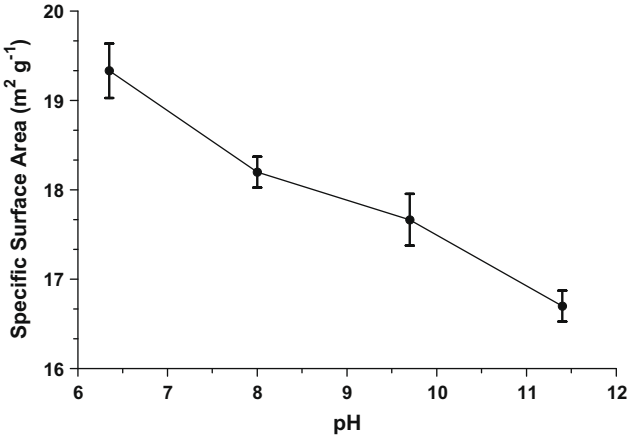

Fig. 2. Specific surface area of re-assembled casein micelles as a result of pH value. All means are statistically different ($p < 0.05$). Error bars indicate standard deviations for each treatment.

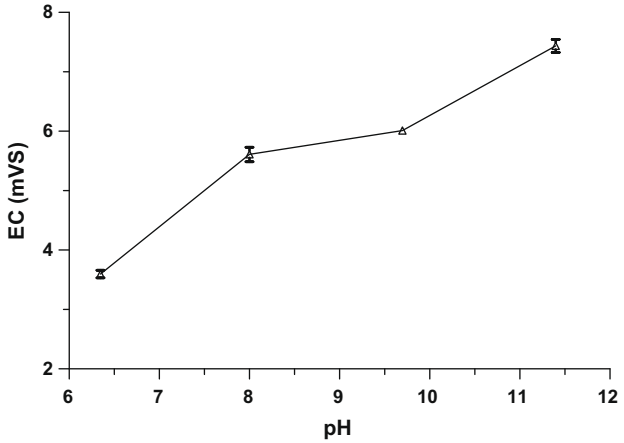

Fig. 3. Electric conductivity (mVS) of casein solutions as a result of pH value. All means are statistically different ($p < 0.05$). Error bars indicate standard deviations for each treatment.

2008) state that CCP probably plays only a minor role in the formation and structural features of casein micelles. In experiments at temperatures above 25 °C, virtually no casein was dissociated from micelles even at pH values where CCP had been extensively solubilised (Dalgleish & Law, 1989). This is due to the concurrent neutralisation of the phosphoserine charge by acid (Horne, 1998). Hydrophobic interactions have little dependence on pH. Therefore, changes to hydrophobic interactions are unlikely to explain the observed effects of pH on micelles (Anema, 1998). This suggests that electrostatic interactions play a critical role in the pH-dependent behaviour of micelles. As pH increases, casein molecules in micelle nanoparticles become more negatively charged. For example, phosphoseryl residues are converted from singly to doubly negatively charged units (Horne, 1998). These high negative charges strengthen electrostatic repulsive forces amongst protein molecules leading to a looser bulky structure of casein micelles (Liu & Guo, 2008). Vaia et al. (2006) proposed a mechanism based on increased casein solubility at alkaline pH, arising from reduced concentrations of ionic calcium and phosphate. They argued that decreased ionic calcium and phosphate increased the solvent quality of milk serum, leading to diminished cohesive interactions between the hydrophobic regions of the caseins. According to their hypothesis, the cohesive interactions between micellar caseins can be described as a solvent mediated association, where interactions are strengthened or weakened as a result of increasing or decreasing solvent quality. In this respect, we considered the role of hydroxyl ions entered to the medium by sodium hydroxide used for increasing the pH titration. These ions increased the electrical conductivity (Fig. 3) and dielectric constant of the medium, likely leading to the increased solvent quality of serum phase. This may increase the number of hydrogen bonds between serum and colloidal phases, most likely causing breakage of some hydrogen and hydrophobic bonds amongst casein chains and eventually weakening the micelle structure. A mechanism based on the reduced dielectric constant of the medium by alcohols has been proposed for alcohol-induced coagulation of milk (Walstra, 1990; Zadow, 1993). The results obtained in the present study suggest that attractive forces amongst casein molecules were still sufficient to maintain the micellar integrity of casein particles even at pH 11.4.

It is well-known that turbidity depends upon three main factors: size, concentration and light scattering properties of particles (Banon & Hardy, 1992). For a given concentration and particle type, solution turbidity has been used as a measure of protein–protein association and aggregation (Banavara et al., 2003). The looser expanded structure of casein micelles with smaller specific surface area available for light scattering and probably more voids in their micellar arrangement which may entrap the light beam, led to a decrease in the observed turbidity of casein solutions with increasing pH in the range of 6.5–7.3. Because higher pH values led to the swelled looser micelles we contrived a treatment with adjusted pH on 12.6 to observe the plausible disruption of casein micelles. However, diameter of micelles was even greater (≈431 nm) than those at pH 11.4 (≈426 nm), indicating no disruption of re-assembled casein micelles even at such a high pH value.

The span index measures the width of particle size distribution. Hence, a small span value indicates a narrow particle size distribution (Tan & Nakajima, 2005). Fig. 4 demonstrates a noticeable increase in span as pH is increased from 6.35 to 8.0, and then a more modest, linear-like increase with increasing pH above 8.0. This means that heterogeneity of particles increases with increasing pH. Flow behaviour of casein solution was influenced by pH value (Fig. 5). It is clear that consistency coefficient (k) increased; whilst flow index (n) decreased with increasing pH. The increased consistency coefficient indicates an increase in apparent viscosity of casein solution with increasing pH due probably to the swelling of casein micelles (Odagiri & Nickerson, 1965). All casein solutions had an n value less than 1.0 (Fig. 5) indicating they were all shear thinning fluids. This index is a dimensionless number that indicates the closeness to Newtonian flow (Bourne, 2002). The higher the pH value, the lower was the flow index (n) and hence the stronger was the shear thinning behaviour. It is attributed to the looser

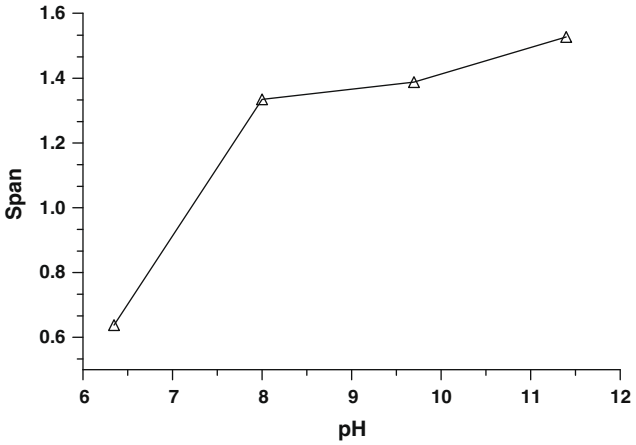

Fig. 4. Polydispersity index (span) of re-assembled casein micelles as a result of pH value. All means are statistically different ($p < 0.05$). Error bars indicate standard deviations for each treatment.

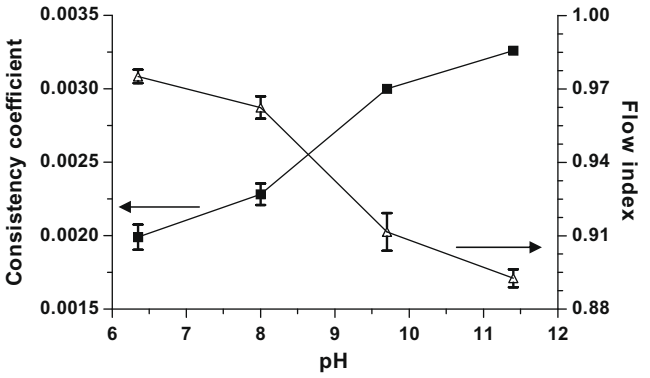

Fig. 5. Consistency coefficient (left hand) and flow index (right hand) of casein solution as a result of pH value. All means are statistically different ($p < 0.05$). Error bars indicate standard deviations for each treatment.

expanded structure of casein micelles at higher pH values which is disrupted more easily with increasing rate of shear.

4. Conclusion

Re-assembled casein micelles swelled as pH increased; however, attractive forces amongst casein molecules are still sufficient to maintain the micellar integrity of casein particles even at pH 12.6. This study showed that a decrease in turbidity value of casein solution and/or milk does not necessarily mean the disruption of casein micelles. The decrease in turbidity has been previously used as the solitary index for assumed disruption of casein micelles at alkaline pH values.

References

Anema, S. G. (1998). Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120 °C. *Journal of Agriculture and Food Chemistry, 46*, 2299–2305.

Anema, S. G., & Li, Y. (2000). Further studies on the heat-induced, pH-dependent dissociation of caseins from the micelles in reconstituted skim milk. *Lebensmittel-Wissenschaft und-Technologie, 33*, 335–343.

Banavara, D. S., Anupama, D., & Rankin, S. A. (2003). Studies on physicochemical and functional properties of commercial sweet whey powders. *Journal of Dairy Science, 86*, 3866–3875.

Banon, S., & Hardy, J. (1992). A colloidal approach of milk acidification by glucono-delta-lactone. *Journal of Dairy Science, 75*, 935–941.

Bourne, M. (2002). *Food texture and viscosity* (2nd ed.). Burlington, MA: Academic Press.

Dalgleish, D. G., & Law, A. J. R. (1989). pH-induced dissociation of bovine casein micelles II. Mineral solubilization and its relation to casein release. *Journal of Dairy Research, 56*, 727–735.

de Kruif, C. G. (1999). Casein micelle interactions. *International Dairy Journal, 9*, 183–188.

Farrer, D., & Lips, A. (1999). On the self-assembly of sodium caseinate. *International Dairy Journal, 9*, 281–286.

Holt, C. (1992). Structure and stability of the bovine casein micelle. In C. B. Anfinsen, J. D. Edsall, F. R. Richards, & D. E. Eisenberg (Eds.). *Advances in protein chemistry* (Vol. 43, pp. 63–151). San Diego, CA: Academic Press.

Horne, D. S. (1998). Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal, 8*, 171–177.

Horne, D. S. (2002). Casein structure, self-assembly and gelation. *Current Opinion in Colloid and Interface Science, 7*, 456–461.

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

Huppertz, T., Vaia, B., & Smiddy, M. A. (2008). Reformation of casein particles from alkaline-disrupted casein micelles. *Journal of Dairy Research, 75*, 44–47.

Liu, Y., & Guo, R. (2008). pH-dependent structures and properties of casein micelles. *Biophysical Chemistry, 136*, 67–73.

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.

Mounsey, J. S., O'kennedy, B. T., & Kelly, P. M. (2005). Comparison of re-micellised casein prepared from acid casein with micellar casein prepared by membrane filtration. *Lait, 85*, 419–430.

Odagiri, S., & Nickerson, T. A. (1965). Micellar changes in skim milk treated with alkali or acid. *Journal of Dairy Science, 48*, 1157–1160.

Payens, T. A. J. (1966). Association of caseins and their possible relation to structure of the casein micelle. *Journal of Dairy Science, 49*, 1317–1324.

Rollema, H. S. (1992). Chemistry of milk proteins. In P. F. Fox (Ed.), *Developments in dairy chemistry-1. Proteins* (pp. 111–140). London, UK: Applied Sciences.

Semo, E., Kesselman, E., Danino, D., & Livney, Y. D. (2007). Casein micelle as a natural nano-capsular vehicle foe nutraceuticals. *Food Hydrocolloids, 21*, 936–942.

Tan, C. P., & Nakajima, M. (2005). β-Carotene nanodispersions: Preparation, characterization and stability evaluation. *Food Chemistry, 92*, 661–671.

Thanasukarn, P., Pongsawatmanit, R., & McClements, D. J. (2006). Utilization of layer-by-layer interfacial deposition technique to improve freeze-thaw stability of oil-in-water emulsions. *Food Research International, 39*, 721–729.

Tuinier, R., & de Kruif, C. G. (2002). Stability of casein micelles in milk. *Journal of Physical Chemistry, 117*, 1290–1295.

Vaia, B., Smiddy, M. A., Kelly, A. L., & Huppertz, T. (2006). Solvent-mediated disruption of bovine casein micelles at alkaline pH. *Journal of Agriculture and Food Chemistry, 54*, 8288–8293.

Walstra, P. (1990). On the stability of casein micelles. *Journal of Dairy Science, 73*, 1965–1979.

Walstra, P. (1999). Casein sub-micelles: Do they exist? *International Dairy Journal, 9*, 189–192.

Zadow, J. G. (1993). Alcohol-mediated temperature-induced reversible dissociation of casein micelle in milk. *The Australian Journal of Dairy Technology, 48*, 78–81.

Zhong, Q., Daubert, C. R., & Velev, O. D. (2007). Physicochemical variables affecting the rheology and microstructure of rennet gels. *Journal of Agriculture and Food Chemistry, 55*, 2688–2697.