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The stabilization mechanism of acidified milk drinks induced by carboxymethylcellulose

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Abstract – In the present work carboxymethylcellulose (CMC) was used as a stabilizer to avoid the floculation of milk proteins in acidified milk drinks. The particle diameter and ζ-potential evolution during acidification of casein micelles in CMC were studied. The experimental results indicate that the adsorption of CMC takes place at and below pH 5.2 and that electroosorption may be the driving force for the adsorption of CMC onto the casein micelles. The stability of acidified milk drinks induced by CMC could be explained presumably by steric stabilization caused by the anchoring of CMC onto the casein micelles’ surface rather than electrostatic repulsion. Above pH 5.2 phase separation of the casein/CMC mixture corresponding to thermodynamic incompatibility was found at high CMC concentration, while below pH 5.2 the adsorption of CMC led to either stabilization or bridging among casein micelles depending on the CMC concentration. In addition, the non-adsorbed CMC (serum CMC) increased the viscosity of the serum and thus contributed to preventing casein micelles from precipitating.

acidified milk drink / carboxymethylcellulose (CMC) / casein micelle / electroosorption / stability

Résumé – Mécanisme de stabilisation de boissons laitières acidifiées induite par la carboxyméthylcellulose. Dans le présent travail, la carboxyméthylcellulose (CMC) a été utilisée comme stabilisant pour éviter la flocculation des protéines laitières dans des boissons laitières acidifiées. Le diamètre des particules et l’évolution potentiel zeta au cours de l’acidification des micelles

摘 要 – 羧甲基纤维素钠稳定酸性乳饮料的机理研究。羧甲基纤维素钠 (CMC) 可作为稳定剂, 阻止酸性乳饮料中乳蛋白发生絮凝。本文研究了酪蛋白/CMC混合体系中, 酪蛋白粒径和ζ-电位随体系 pH 降低的变化。研究表明, 当体系 pH < 5.2 时, CMC 因静电作用在酪蛋白胶粒上发生吸附。酸性乳体系的稳定性可能源于 CMC 在酪蛋白表面上发生吸附产生的空间位阻效应而非静电排斥作用。若体系 pH > 5.2, 当 CMC 浓度较高时, 体系因热力学不相容而发生相分离。而 pH < 5.2 时, 根据 CMC 浓度的不同, 吸附在酪蛋白表面的 CMC 可使体系稳定, 也可使体系因酪蛋白胶粒间的架桥絮凝而发生失稳。此外, 由于体系中非吸附的 CMC 可增加体系的粘度, 从而降低酪蛋白胶束的沉降速度, 这也有利于提高酸性乳饮料的稳定性。

酸性乳饮料 / 羧甲基纤维素钠 (CMC) / 酪蛋白胶束 / 静电吸附 / 稳定性

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Acidified milk drinks are popular dairy products worldwide. This beverage can be described as an acidified protein liquid system with stability and viscosity similar to natural milk. Such drinks are usually composed of an acid dairy phase (fermented base) or a neutral base (milk) with an acidic medium (fruit phases such as pulp, fruit concentration, etc.) which can be flavored. Upon lowering the pH of milk, as is done during the preparation of acidified milk drinks, the native stabilization mechanism of casein micelles fails. This failure is believed to be related to the decrease in steric repulsion after the collapse of the extended conformation of κ-casein which is located on the casein micelle surface [24]. Therefore, acidified milk drinks need the addition of a stabilizer to avoid the flocculation of milk proteins and subsequent macroscopic whey separation. Anionic polysaccharides, such as high-methoxyl pectin, propyleneglycoalginate (PGA), soybean soluble polysaccharides (SSPS) and carboxymethylcellulose (CMC) are often used to achieve this [22]. The stabilizer can prevent aggregation of casein micelles or be the cause of it. These phenomena are believed to be related to the interaction between polysaccharides and proteins; the outcome depends on whether the polysaccharides adsorb onto the casein particles or not.

A lot of work has been reported on the interaction between pectin and milk protein [11, 15, 16]. Pectin has a chain structure of α-(1→4)-linked D-galacturonic acid units interrupted by the insertion of α-(1→2)-linked L-rhamnopyranosyl residues. High-methoxyl pectin has been widely used as a stabilizer in acidified milk drinks to prevent flocculation of milk proteins. Pectin is a non-adsorbing polymer when it is in solution with casein micelles at pH 6.7. The adsorption of pectin onto casein micelles is multilayered and takes place at and below pH 5.0 [25]. The mechanism of stabilization by pectin was postulated to involve the blockwise distribution of charges along the pectin chain. Adsorption of the pectin chain onto the micelle surface would take place only at the charged blocks, while the uncharged stretches in between form entropy-rich loops that extend into the solution. The steric repulsion caused by these loops maintains the pectin-coated casein micelles stable at low pH [10, 23]. However, in a stable system, not all of the pectin addition adsorbs onto the casein micelles. A weak gel or network structure composed of non-adsorbed pectin in the serum and pectin-coated casein micelles promotes the acidified milk drinks’ long-term stability [3].

SSPS, extracted from soybean cotyledons, has recently been used as a stabilizer in acidified milk drinks [2]. SSPS, like pectin, is composed of D-galactose,
L-arabinose, D-galacturonic acid and L-rhamnose. However, the content of neutral monosaccharides in SSPS is much higher than that in pectin [13]. The stabilizing ability and mechanism of SSPS have been investigated in comparison with pectin [12, 14]. SSPS, just like pectin, stabilizes acidified milk drinks by a steric-stabilizing effect due to the adsorbed SSPS layer. However, because of the thick layer of neutral sugar side chains of SSPS on the surface of the protein particles, the stability of acidified milk drinks induced by SSPS is different from that induced by pectin. Less SSPS is more effective than pectin at stabilizing and dispersing protein particles. SSPS could give rise to better stability at pH < 4.2 and the stability was not affected by pH between pH 4.2 and 3.2, while acidified milk drinks homogenized with pectin showed a particle size distribution that depended on pH.

CMC, one of the important cellulose derivatives, is a typically anionic polysaccharide and has been widely used in pharmaceuticals, cosmetics and foods as an emulsifying agent, stabilizer or rheological control, etc. [4, 17]. CMC chains are linear \( \beta-(1\rightarrow4) \)-linked glucopyranose residues. A maximum degree of substitution (DS) of 1.5 is permitted, but more typically DS is in the range 0.6–0.95 for food applications. CMC is generally found in sodium salt form, a water-soluble product for DS > 0.5. One of the important characteristics of the CMC is that it can be dissolved in both hot and cold water and has certain viscosity. CMC aqueous solutions of higher DS exhibit pseudoplastic behavior [4]. On the other hand, CMC is a polar adhesive and as such may allow the formation of complexes with proteins such as caseins at, or around, the isoelectric region of the protein [17]. It has been reported that CMC interacts with \( \beta \)-casein and hinders the thermal or \( \text{Ca}^{2+} \)-induced aggregation of the protein [8].

CMC is commonly used as a stabilizing agent in acidified milk drinks instead of pectin in Asia, especially in China. CMC is chosen because of its large application in the food industry and low cost. The main aim of the present work is to investigate the interaction between CMC and casein micelles as a function of the pH. The mechanism by which CMC stabilizes the acidified milk drinks is addressed.

2. MATERIALS AND METHODS

2.1. Materials

CMC with different molecular weight (700 000; 250 000) and a degree of substitution (DS) of 0.9 were purchased from Acros Organics (New Jersey, USA). Skim milk powders and whole milk powders were given by the Fonterra Co. Ltd., New Zealand. A 500 g·kg\(^{-1}\) citric acid solution was prepared by mixing citric acid monohydrate (from the Shanghai Chemical Reagent Co. Ltd., China) with distilled water.

2.2. Acidified milk drink preparation

The samples were prepared on a pilot scale through the following successive process steps. The 80 g·kg\(^{-1}\) milk solids not fat (MSNF) reconstituted milk was prepared by mixing milk powders and distilled water at 45 °C for 30 min. Meanwhile, 10 g·kg\(^{-1}\) CMC was dissolved in distilled water at 75 °C by stirring for 20 min. Then the CMC solution was added to reconstituted milk and the MSNF was adjusted to 40 g·kg\(^{-1}\). The pH of this solution was adjusted to various values with 500 g·kg\(^{-1}\) citric acid. For some measurements in the present work described in the following sections, additional post-treatment (heating and homogenization) based on actual processing was applied to the samples.
in order to make the final samples similar to commercial products. All measurements were performed after the sample was stored at room temperature overnight.

### 2.3. Dynamic light scattering (DLS) experiments

DLS experiments were carried out with a particle size analyzer (Malvern Zetasizer 3000 HSA, Malvern Instruments, Worcestershire, UK). The system consisted of an optics unit with a 10 W max output He-Ne laser, and a Malvern K7132 correlation used in serial configuration. The Zetasizer 3000 HSA worked at a fixed scattering angle of 90˚ and the wavelength of the laser beam was 633 nm. The PS disposable cuvette (15 mm, 1.5 mL) (Plastibrand, Wertheim, Germany) containing the sample was thermostatted by a Joule-Peltier thermostat at 20 ˚C. The sample was made by dispersing 80 g·kg$^{-1}$ reconstituted skim milk in SMUF (1:100). Then 0.5% CMC was added to the mixture at about neutral pH (6.6–6.7). All solutions in this measurement were prepared with ultra-pure water with 18.2 MΩ·cm$^{-1}$ (Millipore, Bedford, MA, USA), and filtered through 0.22-μm membrane filters prior to use. Before measurement, the samples were filtrated (0.45 μm; Millipore). The apparent diameter of the micelles was monitored while acidifying the diluted skim milk with citric acid. All measurements were performed three times. The biggest variance of the measurement is below 8%.

### 2.4. Measurement of zeta potential

The ζ-potential of casein micelles was determined using a particle electrophoresis instrument (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK) which measures the direction and velocity of droplet movement in an applied electric field at 20 °C. The ζ-potential provides an estimate of the net charge of a particle measured at the ‘shear plane’, which depends on the charge on the actual particle (in this case casein micelle and polysaccharides) plus the charge associated with any ions that move along with the particle in the electric field. An individual ζ-potential was determined from the average of three readings taken on the same sample.

### 2.5. Confocal scanning laser microscopy (CSLM)

The obtained samples were preheated to 65 °C and then homogenized at 200 bar with a two-stage value Rannie TYPE 8.30 H homogenizer (APV Rannie A/S, Denmark). Imaging was performed on the sample using a Leica TCS SP2 confocal scanning laser microscope, configured with a Leica DM IRE2 inverted microscope (Leica Microsystems, Germany). The protein and fat were stained by FITC and Nile Red, respectively. The 488-nm laser beam was used for excitation, inducing a fluorescent emission of FITC, detected between 503 and 543 nm. The 548 nm laser beam was used for excitation of Nile Red, detected between 605 and 673 nm.

### 2.6. Dynamic rheological properties

The samples were preheated to 65 °C and homogenized at 200 bar, then pasteurized at 90 °C for 30 min, and finally cooled to room temperature. Small deformation oscillatory rheology was done at 25 °C using an adapted stirrer vessel geometry on a stress-controlled rheometer (TA Instruments AR 2000, New Castle, USA). To test for the presence of a weak gel structure, oscillation was set at 0.1 Hz and 4.1×10$^{-3}$ Pa after shearing of the sample at 100 s$^{-1}$ for 3 min.
3. RESULTS AND DISCUSSION

3.1. Electrosorption of CMC onto casein micelles

3.1.1. Effect of the concentration of CMC on the diameter of casein micelles during acidification

A similar experiment following Tuinier et al. [25] was done as shown in Figure 1, the variation in particle diameter of casein micelles with different concentrations of CMC added during acidification with citric acid. The sample was made by dispersing 80 g·kg\(^{-1}\) reconstituted skim milk in SMUF (1:100). The filled squares refer to casein micelles without the addition of CMC. Just before citric acid is added, the diameter is close to 230 nm, which is in agreement with earlier DLS results regarding the size of the casein micelle [7, 25]. Upon decreasing the pH from 5.8 to 5.0 the casein micelle diameter decreases due to the shrinking of the casein micelle because of the solution of some fraction of α- and β-caseins [25]. At about pH 5.0, the apparent casein micelle size greatly increases due to the formation of aggregates. The suspension becomes unstable and macroscopic flocculation is observed after a few minutes. This observation is the same as that of Tuinier et al. [25].

The open circles refer to the same sample but with 30 mg·kg\(^{-1}\) CMC added. At neutral pH, we find a small increase in size, as compared with the bare casein micelle size. A similar phenomenon was also
observed in the size evolution during acidification of pectin/casein mixtures [25]. The increase in size has been interpreted based on the Stokes-Einstein relation as follows:

\[ D = \frac{kT}{3\pi \eta_m d_{eff}} \]  

(1)

where \( \eta_m \) is the medium viscosity and \( d_{eff} \) is the effective hydrodynamic diameter. In diluted dispersions, the self-diffusion coefficient, \( D \), is related to the effective hydrodynamic diameter of the sphere, \( d_{eff} \) according to equation (1). The viscosity of SMUF was taken as the medium viscosity, \( \eta_m \). However, upon addition of CMC, the actual medium viscosity will increase. It is thus supposed that the increase in the particle diameter is traceable from the calculation method of DLS [25]. Alternatively, Nakamura et al. [14] also found the initial diameter of skim milk increased with increasing pectin concentration at neutral pH. Taking into account that the DLS measurements were carried out after the samples were extensively diluted in the corresponding ultrafiltration permeate, and assuming that the measurement of particle diameter was not affected by the viscosity of the pectin, they attributed this increase in size to an association of pectin to casein micelles at neutral pH, possibly because of the existence of calcium ions. In our case, the increase in diameter of casein micelles upon addition of CMC at neutral pH needs further clarification. The addition of CMC has no effect on the decrease in the casein micelle size. Upon decreasing the pH to close to 5.2, we find that the diameter of casein micelles in the mixture with CMC increases in comparison with that in the absence of CMC. This is caused by the adsorption of CMC onto casein micelles, which leads to effectively larger casein micelles. The negative charge might be statistically distributed along the CMC chains, presumably yielding a conformation with many loops [9]. For adsorbed CMC, these conceivable loops might extend into the solution and cause a repulsive interaction between the casein micelles at low pH in the same way as \( \kappa \)-casein chains do at neutral pH. The adsorption of CMC onto casein micelles takes place just before casein micelle aggregation would have started in the absence of CMC. The apparent diameter of casein micelles increased with decreasing pH and seemed to level off to ca. 300 nm at a pH range of 4.6 to 4.4 in the presence of 30 mg·kg\(^{-1}\) CMC. At still lower pH values it was observed that the diameter of casein micelles with adsorbed CMC decreased between pH 4.4 and 4.0. With further decreasing pH, the negative charge on CMC chains becomes less and less while the positive charge on casein micelles becomes more and more. More positively charged casein micelles would adsorb more CMC chains. At the same time, CMC chains might absorb in a likely flatter conformation with small loops for the lower charge on CMC when lowering the pH. The collapse of the adsorbed layer might be the reason that the diameters of casein micelles with adsorbed CMC decreased in this pH range. The aggregation of casein micelles exceeding 1 \( \mu \)m was observed at pH 3.9 with 30 mg·kg\(^{-1}\) CMC. Probably, a CMC concentration of 30 mg·kg\(^{-1}\) is not sufficient to provide a thick enough adsorbed layer on casein micelles in the system. The collapse of the CMC adsorbed layer results in a decrease in the steric repulsion between CMC-coated casein micelles, and thus the aggregation occurs at pH 3.9. This phenomenon is similar to the collapse of \( \kappa \)-casein which leads to aggregation of native casein micelles when lowering the pH [24].

The size evolution during acidification of 45 mg·kg\(^{-1}\) CMC in diluted casein micelle suspension in SMUF was monitored as shown, referred to by open diamonds. Upon decreasing the pH, the size evolution is similar to that upon addition of 30 mg·kg\(^{-1}\) CMC. The only difference is that the aggregation takes place at a lower pH of 3.6. The increasing concentration of
Stabilization of acidified milk drinks

3.1.2. Effect of the concentration of CMC on the ζ-potential of casein micelles during acidification

The ζ-potential of casein micelles in the similar samples described in Figure 1 is shown as a function of pH in Figure 2. As the net surface charge of casein micelles is negative, the ζ-potential is also negative. The changes reported refer to the absolute magnitude of the ζ-potential.

The ζ-potentials for native casein micelles, measured in SMUF buffer, ranged from about –16 to –8 mV over the pH range of 6.7 to 4.6. Considering only the protein part of casein micelles, a steady increase in ζ-potential with increasing pH would be expected [20]. However, the observed relationship between the ζ-potential of native casein micelles from milk dispersed in SMUF buffer and pH is considerably more complex. Overall, the ζ-potential pH profile shown in Figure 2 is similar to that reported by Schmidt and Poll [18] and Anema and Klostermeyer [1]. The ζ-potential at pH 4.6 was higher than zero (Fig. 2). This contrasts with the results of Darling and Dickson [5, 6], who reported a ζ-potential of zero at the isoelectric pH of casein (pH 4.6). Due to the complete solubilization of the colloidal calcium phosphate, the calcium and phosphate concentrations, the calcium ion activity and the ionic strength of natural milk serum at pH 4.6 would be considerably higher than in the SMUF buffer used in these experiments, which simulates milk serum at pH 6.6. It is possible that these differences result in a higher ζ-potential of the casein micelles in the SMUF buffer system at low pH.

The addition of CMC to the casein micelles seems to cause some increase in the ζ-potential of casein micelles. A remarkable feature in Figure 2 is the increase in ζ-potential between pH 5.2 and 4.8 upon addition of CMC. The ζ-potential of casein micelles in this pH range became more negative when CMC was added, which confirms that anionic CMC molecules adsorbed onto the surfaces of casein micelles [19, 21]. This result may correspond to the particle size evolution in Figure 1. The fact that negatively charged CMC chains adsorbed onto the surface of casein micelles depending on pH suggests that there are electrostatic attractions between anionic carboxylate groups on the CMC molecules and cationic patches (i.e. –NH₃⁺) on casein micelles. In the pH range 4.8–4.3 the ζ-potential shows a plateau. Nevertheless, the results of the particle size evolution in Figure 1 show that the particle size increased, especially at high concentrations of CMC. As the pH is lowered, in the pH range 4.8 to 4.3, casein micelles will take more positive charge and adsorb more CMC molecules, which leads to the
Figure 2. Zeta potential of casein micelles as a function of pH during acidification with citric acid for 80 g·kg⁻¹ skim milk diluted 100 times in SMUF. The filled squares refer to casein micelles without CMC, the open circles refer to casein micelles and 30 mg·kg⁻¹ CMC, the open diamonds refer to casein micelles and 45 mg·kg⁻¹ CMC, the open triangles to casein micelles and 80 mg·kg⁻¹ CMC, and the crosses to casein micelles and 400 mg·kg⁻¹ CMC. The Mw of CMC is 250 000.

increase in particle size. When the surface charge reached a certain value, there was a strong electrostatic repulsion between the surface and similarly charged CMC in the aqueous phase, which limited further adsorption of the polyelectrolyte [21]. It seems that the adsorption of CMC onto casein micelles was not only induced but also controlled by the electrostatic force. The decrease in the ζ-potential was clearly observed between pH 4.3 and 3. This phenomenon might be explained by the fact that in this pH range the adsorption layer is thick enough to prevent the further adsorption of CMC and the carboxylate groups on the adsorbed CMC layer were protonated at the lower pH. Therefore, the ζ-potential decreases with lowering pH. The reason that the reduction in ζ-potential is higher at lower CMC concentrations (30 mg·kg⁻¹ and 45 mg·kg⁻¹) than at higher CMC concentrations (80 mg·kg⁻¹ and 400 mg·kg⁻¹) is probably attributed to the fact that more CMC adsorbs effectively onto the casein micelle at higher CMC concentrations when lowering the pH.

The ζ-potential of CMC-coated casein micelles in the system with 80 mg·kg⁻¹ and 400 mg·kg⁻¹ CMC is low at pH 3.0 in comparison with that at neutral. Since there are no aggregations if the adsorbed CMC layer of casein micelles is thick enough at high CMC concentrations, as indicated in Figure 1, this result suggests that the steric
stability generated by the CMC layer plays a main role in stabilizing casein micelles at low pH values.

3.2. The stability of acidified milk drinks

The neutral base composed of 4 g·kg⁻¹ CMC/40 g·kg⁻¹ MSNF (skim milk powder) was acidified to different pH values. The stability of the system observed 15 days after preparation is shown in Figure 3. Below pH 5.2, there was no obvious phase separation and the system showed stability. This fact is in good agreement with the abovementioned experimental results, in which the effective interaction between CMC and casein micelles is revealed to take place below pH 5.2. As for the phase separation above pH 5.2, it might be supposed to originate from a depletion flocculation between casein micelles and non-adsorbing polysaccharides [22, 26].

Figure 4 shows the confocal microscopic protein distributions in acidified milk drinks with 4 g·kg⁻¹ CMC and 40 g·kg⁻¹ MSNF (whole milk powder) acidified to pH 6.7, 5.0 and 4.1, respectively. The systems acidified to pH 6.7 and 5.0 have a number of obvious clusters. The degree of aggregation at pH 5.0 was lower than that at pH 6.7. CMC could adsorb onto casein micelles at pH 5.0 and the adsorbed layer would maintain the stability of acidified milk drinks. However, the two samples showed a homogeneous appearance.

When CMC adsorbs onto the casein micelles at below pH 5.2, CMC may give rise to bridging among the casein micelles or make the casein micelles stable, depending on the CMC concentration. Adding a little CMC might consequently result in bridging flocculation, in which a single polymer chain may absorb onto two or more casein micelles, thereby connecting the particles. Above full coverage, the high surface coverage of CMC generally has a stabilizing effect on casein micelles [25]. Our results indicate that the stability of casein micelles in acid medium induced by CMC depends not only on the interaction between them but also on the CMC concentration.

3.3. The role of non-adsorbed CMC (serum CMC) in CMC-stabilized acidified milk drinks

The results of particle size evolution and ζ-potential of casein micelles with different CMC concentrations as a function of pH indicate that the steric stability which was generated by the adsorbed CMC layer maintained the stability of casein micelles at low pH. Here the role of serum CMC is further discussed. Dynamic rheological properties of acidified milk drinks were measured to determine whether there are weak network structures composed of CMC-coated casein micelles/serum CMC.

Figure 6 shows the small deformation rheological properties of acidified milk drinks after shearing the sample at 100 s⁻¹ for 3 min. After the applied shearing is removed, the system shows an obvious liquid-like character; as can be seen, the loss modulus, $G''$ is always higher than the storage modulus, $G'$ with a small loss tangent, tanδ of ca. 2.7. The effect of the serum CMC is to increase the viscosity of the acidified milk drinks. The increase in
Figure 3. Visual appearance of phase behavior of acidified milk drinks at different pH values 15 days after preparation (the concentration of CMC (Mw = 700 000) is 4 g·kg\(^{-1}\)).
Figure 4. Confocal scanning laser micrographs of acidified milk drinks stabilized by 40 g·kg⁻¹ MSNF and 4 g·kg⁻¹ CMC (Mw=700 000) at pH (a) 6.7, (b) 5.0, (c) 4.1. The scale bar is 16 μm.
Figure 5. Confocal scanning laser micrographs of acidified milk drinks stabilized by (a) 1 g·kg$^{-1}$ CMC, (b) 2 g·kg$^{-1}$ CMC, (c) 4 g·kg$^{-1}$ CMC at pH 4.10. The scale bar is 16 μm. The Mw of CMC is 700,000.
viscosity of the system will decrease the sedimentation velocity of casein micelles. Therefore, the serum CMC contributes to the stability by increasing the viscosity of acidified milk drinks.

4. CONCLUSION

The stabilization mechanism of acidified milk drinks induced by CMC was investigated. The present study indicates that steric stability generated by the adsorbed CMC layer may play a key role in the stability of casein micelles at low pH. There is no network composed of the CMC-coated casein micelles and the non-adsorbed CMC. The serum CMC increases the viscosity of the acidified milk drinks, which also contributes to the stability of the system.

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