

Article

Influence of Ethanol on the Acid-Induced Flocculation of Casein Micelles

Thom Huppertz ^{1,2} ¹ FrieslandCampina, 3818 LE Amersfoort, The Netherlands; thom.huppertz@wur.nl² Food Quality & Design Group, Wageningen University & Research, 6708 WG Wageningen, The Netherlands

Abstract: The influence of ethanol (0–10%, *v/v*) on the acid-induced flocculation of casein micelles was examined using diffusing wave spectroscopy. For this purpose, samples containing 10% (*w/w*) reconstituted skim milk powder and 0–10% (*v/v*) ethanol were acidified with glucono-delta-lactone and acid-induced coagulation was monitored by diffusing wave spectroscopy. The pH at which acid-induced flocculation of the casein micelles commenced (pH_f) increased near-linearly with increasing ethanol content, whereas the rate of flocculation was not affected by ethanol. The results are discussed in terms of the steric stabilisation of casein micelles by a polyelectrolyte brush in a medium of high ionic strength. Ethanol-induced increases in pH_f are probably primarily due to an ethanol-induced reduction in solvent quality; an ethanol-induced reduction in dissociation of carboxylic acid groups in the brush is likely to contribute.

Keywords: milk; casein micelle; flocculation; acid; ethanol; milk coagulation



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1. Introduction

The colloidal instability of casein micelles forms the basis of the gelation of milk, which is readily utilised in the conversion of milk into dairy products such as cheese and yoghurt. Casein micelles in their native state are stabilised sterically by a polyelectrolyte brush [1–4]. Because of the relatively high ionic strength of milk, this polyelectrolyte brush is considered as a salted brush, meaning that electrostatic interactions are short-range and highly screened [5]. This salted brush consists primarily of the hydrophilic C-terminal end of κ -casein, although β -casein also contributes, particularly at low temperature [6].

Since milk can be boiled, dried, or frozen, or considerable levels of salt can be added without impairing the colloidal stability of the casein micelles, the aforementioned salted brush provides the micelles with remarkable colloidal stability. Colloidal instability of the micelles can be induced by either removal or collapse of the brush [7]. Removal of the brush is utilised in cheesemaking, where the proteolytic enzyme chymosin cleaves κ -casein at the Phe₁₀₅-Met₁₀₆ bond; this reduces the steric stabilisation of the micellar surface, therewith facilitating aggregation and gelation [3]. The addition of ethanol to milk induces the flocculation of casein micelles, which can be understood in terms of the influence of the solvent quality of the brush [5,8]. Acid-induced coagulation of milk, which forms the basis of the manufacture of yoghurt, is also the result of the transition of the brush from a stretched to a collapsed state [1,9]. The C-terminal end of κ -casein contains a large number of carboxylic acid groups, primarily from Glu residues, whose dissociation is diminished on acidification. As a result, κ -casein molecules lose their solvency, leading to a collapse of the brush [1,9].

As described above, various processes inducing the destabilisation of the salted brush on the casein micelle surface have been well-characterised, but detailed studies on inter-relationships between various factors affecting brush stability are far less abundant. Partial enzymatic removal of the κ -casein brush, by chymosin, reduces the susceptibility to acid-induced [1] or ethanol-induced [5] coagulation, whereas the addition of ethanol promotes

the aggregation of renneted casein micelles and reduces the amount of enzymatic brush removal required to induce coagulation of the casein micelles [10]. It is also well-known that a reduction in pH decreases the amount of ethanol required to induce the coagulation of milk [8].

However, the influence of ethanol on the acid-induced coagulation of milk has not been studied quantitatively to date and was the subject of the investigation of the studies reported in this article. For this purpose, reconstituted skim milk samples containing various levels of ethanol were prepared. Acid-induced gelation was monitored by diffusing wave spectroscopy (DWS). These studies provide further insight into the matter of the steric stabilisation of the casein micelles and thus aids in our understanding of the colloidal stability of the micelles and provide valuable insights for dairy products containing ethanol (e.g., cream liquors).

2. Materials and Methods

2.1. Sample Preparation

NILAC low-heat skim milk powder (NIZO food research, Ede, The Netherlands) was reconstituted in demineralised water at a level of 125 g L^{-1} ; sodium azide (0.5 g L^{-1}) was added to prevent microbial growth. Milk samples were diluted with appropriate amounts of ethanol and demineralised water to yield a final concentration of 100 g L^{-1} of milk solids and 0, 2, 4, 6, 8, or 10% (*v/v*) of ethanol.

2.2. Monitoring of Acid-Induced Coagulation of Milk

Milk samples were equilibrated at $32 \text{ }^{\circ}\text{C}$ prior to the determination of acid-induced coagulation. Glucono- δ -lactone (GDL; Sigma-Aldrich, St. Louis, MO, USA; 25 g L^{-1}) was added to the thermally equilibrated milk samples and rapidly mixed. Samples were subsequently incubated at $32 \text{ }^{\circ}\text{C}$ and milk pH and acid-induced coagulation were monitored as a function of incubation time. Acid-induced coagulation of milk was monitored by DWS, as outlined previously [11]. The time at which the auto-correlation curve had decayed to 50% of its maximum plateau level was defined as the relaxation time, $\tau_{1/2}$, which reflects the restriction of mobility of the particles in solution. In DWS, a relaxation time is, as in classical dynamic light scattering, directly related to particle diffusivity and therefore to particle size and interaction via the generalised Stokes–Einstein relation. During the initial stages of flocculation, the relaxation time is therefore a direct measure of the particle size and interaction. All data were normalised relative to its control, which is the $\tau_{1/2}$ value of the sample equilibrated at $32 \text{ }^{\circ}\text{C}$, prior to the addition of GDL.

3. Results and Discussion

3.1. Influence of Ethanol on the Rate of Acidification of Milk

During incubation with 25 g L^{-1} GDL at $32 \text{ }^{\circ}\text{C}$, the pH of the milk decreased gradually with the incubation time (Figure 1); this is due to the fact that GDL is the 1,5-intramolecular ester of D-gluconic acid, which, in addition to an aqueous medium, is hydrolysed to an equilibrium mixture of the weak acid D-gluconic acid and the δ - and γ -lactones. The rate of acidification decreased slightly, but progressively, with an increasing ethanol content of milk, although differences between the pH of milk containing 0 or 10% ethanol was never more than 0.1 pH unit (Figure 1). Three factors may be responsible for the reduced rate of acidification in the presence of ethanol: (1) a reduced rate of hydrolysis of GDL; (2) a reduced dissociation of gluconic acid; or (3) an increased buffering capacity of milk. The influence of ethanol on the hydrolysis of GDL has, to the author's knowledge, not been studied to date and thus cannot be ruled out as an influential factor. Although the influence of ethanol on the dissociation of gluconic acid has not been studied to date, it has been shown that a similar solvent, methanol, decreases the dissociation constant of other weak acids [12,13], so an ethanol-induced reduction in the dissociation of gluconic acid is likely. Finally, the buffering capacity of milk is mainly governed by minerals in the milk serum [14]; since the solubility of most salts including calcium phosphate [15] is

actually reduced with the addition of ethanol, the reduced rate of the acidification of milk in the presence of ethanol is unlikely to be a result of increased buffering capacity. Hence, it appears that the ethanol-induced reduction in the rate of acidification could be the result of the reduced dissociation of gluconic acid, potentially aided by the reduced hydrolysis of GDL.

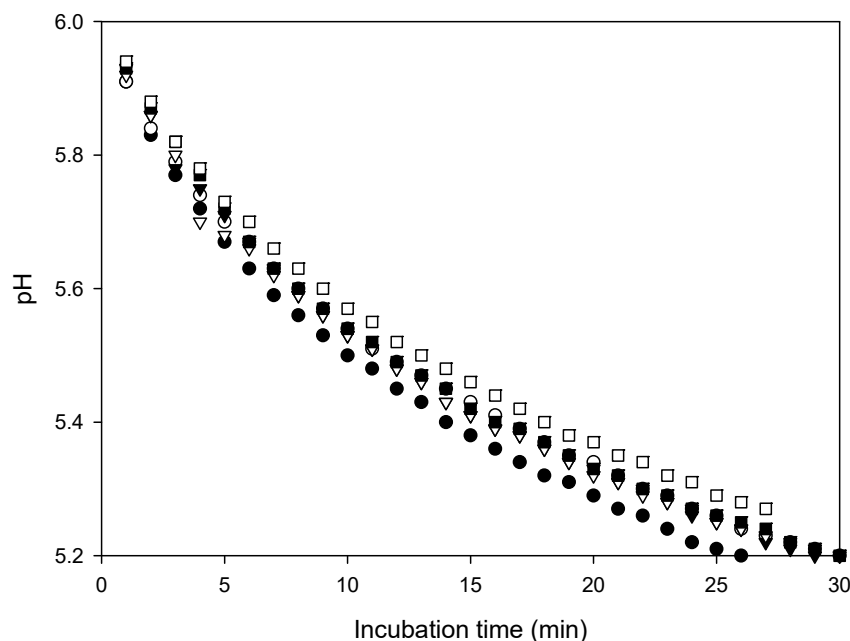


Figure 1. The influence of incubation time at 32 °C with 25 g L⁻¹ GDL on the pH of reconstituted skim milk (100 g L⁻¹ milk solids) containing 0 (●), 2 (○), 4 (▼), 6 (▽), 8 (■), or 10 (□) % ethanol. Values are the means of experiments on three individual milk samples. The coefficient of variation was less than 5% for all data points.

3.2. Influence of Ethanol on the Acid-Induced Coagulation of Milk

On acidification of the control milk (without added ethanol), the DWS trace, which is a direct measure of particle size, decreased slightly with decreasing pH (Figure 2). This decrease was previously observed [11,16] and is presumably due to the decrease in the micelle size on acidification as a result of the collapse of the stabilising brush; De Kruif [9] observed a similar effect by dynamic light scattering. The acid-induced transition of the brush from an extended to a collapsed state is caused by the reduced solubility of side-groups, primarily carboxylic acid-groups of the C-terminal end of κ -casein, on acidification [9]. The collapse of the brush reduces inter-micellar repulsion and eventually induces flocculation of the micelles. The onset of flocculation is highlighted by an upturn in the DWS profile, after which the DWS traces increase rapidly with a further decrease in pH (Figure 2). The pH at which the onset of flocculation occurs is referred to as the flocculation pH (pH_f) and is taken as the pH at which the upturn in the DWS trace occurs. The rate at which $\tau_{1/2}$ increases with decreasing pH for $pH < pH_f$ is a measure of the rate of the acid-induced flocculation of casein micelles.

The addition of low levels (0–10%) of ethanol to milk had a large effect on the pH_f (i.e., the pH at which acid-induced flocculation commenced). The pH_f increased progressively with an increasing ethanol content (Figure 2) in a near-linear fashion ($p < 0.02$; Figure 3). The rate of the acid-induced flocculation of casein micelles was not influenced by ethanol since only extremely minor differences existed between the slopes in plots of $\tau_{1/2}$ as a function of pH for $pH < pH_f$ (Figure 2). In fact, when the data were not plotted as a function of pH, but as a function of $pH - pH_f$, all curves virtually overlapped (Figure 4), suggesting that the point of coagulation is influenced by ethanol, but the coagulation process itself is not. The data in this study hence strongly suggest that the presence of ethanol makes casein

micelles more susceptible to acid-induced flocculation, but does not influence the kinetics of the flocculation process. It is interesting to note that De Kruif and Zhulina [1], who studied the acid-induced flocculation of casein micelles from which the brush was partially removed by enzymatic hydrolysis, observed a profile nearly-identical to that shown in Figures 2 and 4 (i.e., a strong effect on pH_f but little effect on the rate of the aggregation of micelles).

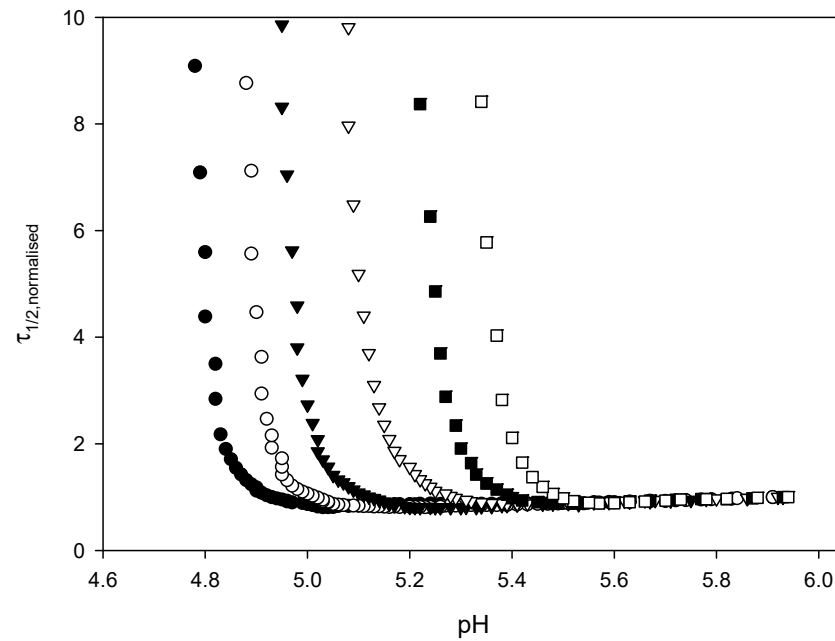


Figure 2. The DWS traces of reconstituted skim milk (100 g L^{-1} milk solids) containing 0 (●), 2 (○), 4 (▼), 6 (▽), 8 (■), or 10 (□) % ethanol as a function of pH during incubation with 25 g L^{-1} GDL at $32 \text{ }^\circ\text{C}$. Values are the means of experiments on three individual milk samples. The coefficient of variation was less than 5% for all data points.

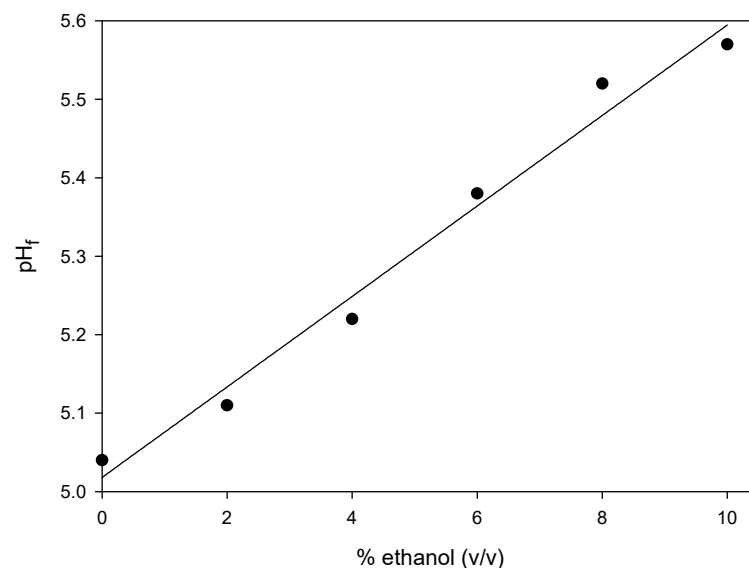


Figure 3. The influence of ethanol concentration on the pH at which acid-induced flocculation of the casein micelles (100 g L^{-1} milk solids) commenced during incubation with 25 g L^{-1} GDL at $32 \text{ }^\circ\text{C}$. The symbols (●) represent data points whereas the solid line (—) represents a best fit linear regression. Values are the means of experiments on three individual milk samples. The coefficient of variation was less than 5% for all data points.

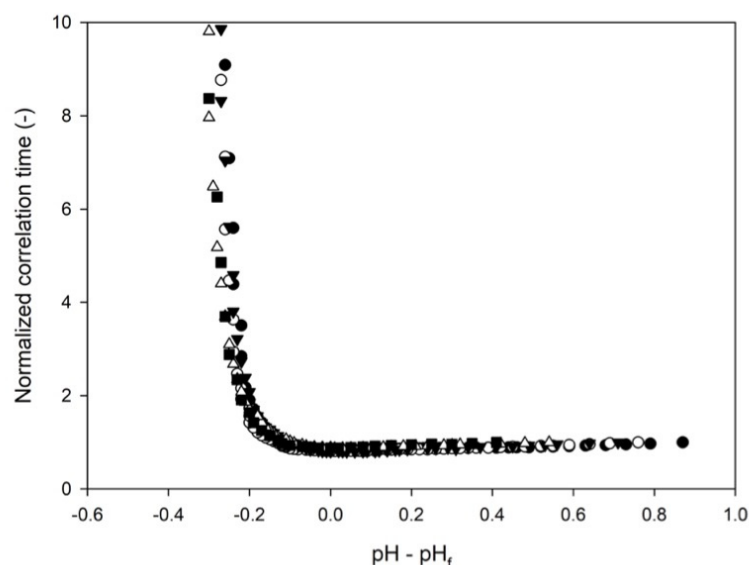


Figure 4. DWS traces of reconstituted skim milk (100 g L^{-1} milk solids) containing 0 (●), 2 (○), 4 (▼), 6 (▽), 8 (■) or 10 (□) % ethanol as a function of $\text{pH} - \text{pH}_f$ during incubation with 25 g L^{-1} GDL at $32 \text{ }^\circ\text{C}$. Values are means of experiments on three individual milk samples. The coefficient of variation was less than 5% for all data points.

De Kruif and Zhulina [1] reported that the acid-induced flocculation of casein micelles can be described by the following scaling equation for a stretched–collapsed transition of a polyelectrolyte brush:

$$|v|^* = (\alpha^2 \sigma)^x \cdot I^y \cdot N^z \quad (1)$$

where $|v|^*$ is the transition point of the brush from an extended to a collapsed state; α is the degree of ionisation of the brush ($\alpha = 1/m$, where m is the distance between neighbouring charges on the chain); σ is the grafting density (i.e., the number of chains per area); I is the ionic strength; and N is the chain length of the brush. The values of the exponents x , y , z are determined by the range of the ionic strength, I , and the interrelationship between N , α , and σ . Considering the influence of ethanol on the components of milk, it seems reasonable to assume that of the variables in Equation (1), N and σ will remain unaffected, since ethanol does not induce hydrolysis or the dissociation of caseins; however, α and I are likely to be influenced by the presence of ethanol in the milk system during acidification.

Dissociation of the carboxylic acid groups of amino acids is reduced in the presence of ethanol [12], which means that the degree of brush ionisation, α , at a given pH, decreases with increasing ethanol content; according to Equation (1), a decrease in α facilitates acid-induced flocculation and may thus be, partially, responsible for the ethanol-induced increases in pH_f . However, the increase in the pK of the carboxylic acid side group of glutamic acid with the addition of ethanol (~ 0.019 per % (v/v) of ethanol; [12]) is too small to be the driving force behind the increase in pH_f by 0.6 pH units with the addition of 10% ethanol (Figure 3). Furthermore, the solubility and ionisation of many minerals including calcium phosphate [15,17] are reduced in the presence of ethanol, which leads to a reduction in ionic strength, I , with increasing ethanol content. Since a decrease in ionic strength is actually likely to lower the sensitivity to acid-induced flocculation of milk (Equation (1)), ethanol-induced reductions in mineral solubility and ionisation would be expected to decrease the pH_f , rather than increase it, as observed in Figures 2 and 3.

Hence, it appears that changes in α and I alone are insufficient to account for the ethanol-induced changes in the acid-induced flocculation of milk and that a further factor must be considered (i.e., the solvent quality). The size of the casein micelles decreased in the presence of ethanol as a result of the collapse of the salted brush [18–21] as well as a contraction of the micellar core [21]). The ethanol-induced collapse of the κ -casein brush is a result of reduced solubility because of the reduced solvent quality [5,8]. At room

temperature, ethanol is a poor solvent for the polyelectrolyte brush and thus at ~25% (*v/v*) ethanol, the brush collapses. Lowering the brush density [5] and/or pH [8] induces the collapse of the brush at a lower alcohol content, which was the basis of the alcohol test in the 1960s. Consistent with polymer solution theory, the solvent quality of the ethanolic media improves with increasing temperature. Dispersing CM in a 35% (*v/v*) ethanol solution and heating it to >65 °C led to an almost transparent solution of casein-stabilised nanoclusters and small casein particles. On lowering the temperature, the caseins self-aggregated again [22,23]. Therefore, the influence of the solvent quality of brush stability is likely to be the primary cause of ethanol-induced increases in pH_f . For successful application of the scaling Equation (1) to the acid-induced flocculation of casein micelles in the presence of ethanol, an additional variable to account for solvent quality needs to be included; this parameter may be approximated by the dielectric constant, ϵ , or polarizability of the medium. The dielectric constant of water–ethanol mixtures decreases in linear fashion with increasing ethanol content, which may thus account for the near-linear increase in pH_f with increasing ethanol content (Figure 3).

4. Conclusions

The addition of ethanol (0–10%, *v/v*) to milk strongly increases the pH at which acid-induced flocculation commences, but does not influence the kinetics of the flocculation, suggesting a synergistic destabilising effect of ethanol and acidification on the polyelectrolyte brush on the micelle surface. This destabilisation is probably primarily due to reduced solvent quality, although ethanol-induced reductions in ionisation of the brush are likely to contribute since a lower charge density reduces solvency of the chains. The results of this study highlight the strongly additive effects of ethanol and acid on the coagulation of casein micelles and provide a further understanding of the steric stabilisation of casein micelles and the factors governing its stability.

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