Chapter 6: Acid-induced gelation of heat-treated milk studied by Diffusing Wave Spectroscopy

Abstract

Raw skim milk is a stable colloidal system containing casein micelles and whey proteins. By decreasing the pH the casein micelles become unstable and a gel is formed. During heat treatment at temperatures higher than 70°C the major whey proteins, e.g. α-lactalbumin and β-lactoglobulin denature and start to interact with each other and with casein micelles. This changes the colloidal properties of the casein micelles.

In this article the pH-induced gel formation of heat treated milk and the role of whey proteins was studied. Heat treatment in the range 70 to 90°C induced a shift in gelation pH of skim milk to more alkaline pH values. This shift was directly related to whey protein denaturation. By using WPF milk it was shown that β-lactoglobulin is principally responsible for the shift in gelation pH. α-lactalbumin caused neither alone nor in combination with β-lg an effect on the gelation pH. Heat treatment of milk for 10 min at 90°C resulted in complete denaturation of the β-lg present in skim milk but it is estimated that the casein micelles are coated only up to 40% by whey proteins when compared with pure whey protein aggregates.

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Introduction

The casein micelles in milk are association colloids which consist of different types of casein proteins, i.e. $\alpha_\text{s1}$, $\alpha_\text{s2}$, $\beta$ and $\kappa$. The casein micelles also contain inorganic matter, mainly calcium phosphate, about 8 g per 100 g of casein. The micelles are sterically stabilized by the $\kappa$-casein (17, 7), which is present at the periphery of the micelle. The C-terminal part of the $\kappa$-casein molecule is very hydrophilic and it also has a considerably negative charge. Presumably, this part of the molecule sticks out partly in the surrounding medium as a “flexible” hair. De Kruif and Zhulina (4) consider the presence of the $\kappa$-casein on the surface of the micelles as a poly electrolyte brush. Any property that will effect the stability of the micelle, e.g. ethanol, rennet, acid, will markedly destabilise the poly electrolyte brush and ultimately cause coagulation (6, 8, 11, 17).

On heat treatment at temperatures higher than 70°C the major whey proteins i.e. $\beta$-lactoglobulin ($\beta$-lg) and $\alpha$-lactalbumin ($\alpha$-lac) denature (3), but $\beta$-lg denatures at a much higher reaction rate than $\alpha$-lac. It is well known that $\beta$-lg interacts with casein micelles involving $\kappa$-casein when milk is heated at temperatures above 70°C (9, 15). The whey proteins start to interact with each other and with casein micelles, which changes the colloidal properties of the casein micelles (see Figure 6.1).

In the dairy industry the acidification of milk takes usually place by lactic acid bacteria. However, for model systems it is much

![Figure 6.1: Schematic representation of the heat treatment of milk showing casein micelles (large spheres) and whey proteins (small dots)](image-url)
easier to mimic this process by the addition of glucono δ lacton (GDL); an ester that slowly hydrolyses to produce a weak acid (gluconic acid).

During the acidification of milk the mobility of the particles changes, particularly around the gelation point where the casein micelles start to aggregate. Diffusing Wave Spectroscopy (DWS) is used for continuous monitoring of the coagulation process of the casein micelles during acidification. DWS analyses fluctuations in light back scattered from the sample using correlation techniques to determine a characteristic relaxation time for the mobility of colloidal particles in the system (6, 14). It is therefore a very suitable technique to investigate the influence of process parameters on the gel point. After gelation the particle network still exhibits a thermally driven motion, counteracted by the visco-elasticity of the gel network. As a result the scattered light intensity still fluctuates but now contains information on the visco-elasticity of the gel (4).

In this article we studied the acid-induced gel formation of heat-treated milk by using DWS. Combination of the DWS technique and the use of whey protein free-milk allowed us to determine which whey protein is responsible for the interaction with casein micelles during heat treatment. This is a rather new approach to look at protein interaction mechanisms in milk as until now mostly analytical techniques have been used such as chromatographic and electrophoretic techniques either alone or in combination with ultracentrifugation (1, 2, 12, 16). Furthermore the pH at which gel formation occurs was related to the degree of coating of the micelles. This revealed that casein micelles seem to be coated only partially after a heat treatment even if all the whey proteins are denatured.

**Materials and methods**

**Skimmed milk and Whey Protein Free milk**

Low-heat skim milk powder (Nilac; NIZO food research Ede) was prepared by dissolving 10.45 gram of milk powder in 100 g distilled water while gently stirring. Whey protein free (WPF) milk (microfiltration / UF; NIZO food research) was prepared by dissolving 8.95 g of WPF milk powder in 91.05 gram distilled water (8.95%, w/w). The milk was stirred at 45°C for 1 hour. To prevent bacterial growth 0.02% sodium azide was added and kept overnight at 4°C before use. The initial pH of the milk was 6.67 (± 0.01).
composition of these milks is discussed in the materials and methods section of chapter 2.

**α-lactalbumin and β-lactoglobulin solution**

These powders were obtained by ion exchange chromatography on a Staeck system (Pharmacia). An 8.8% (w/w) β-lg and a 10% (w/w) α-lac solution were prepared by dissolving freeze dried powders in distilled water at 25°C. The solutions were stirred until dissolved and filtered (0.1 µm).

**WPF-milk with addition of α-lac and β-lg**

The WPF-milk was prepared at a slightly higher concentration (8.95% (w/w)) than the finally desired (8.42% (w/w)), which was obtained by mixing 14.1 g WPF milk with 0.9 g of distilled water or the amounts of α-lac and β-lg solutions as required.

**Sample preparation**

The required amount of skim milk or WPF-milk (stored at 4°C), with or without added whey proteins, was stirred for 2 hours at 20°C. Reconstituted skim milk and reconstituted WPF milk enriched with α-lac and β-lg were heat treated for 10 min in aliquots of 5 ml. After heat treatment at temperatures in the range 70-90°C the samples were kept at 32°C for 75 min and then acidified with 1.1% GDL (Sigma Chemical Co.) to pH ~ 4.6 at 32°C. After addition of GDL the milk was stirred gently for 2 min. Gelation was followed by DWS and pH measurements as function of time.

**Diffusing Wave Spectroscopy**

Light from a 5 mW He-Ne laser set at 632.8 nm was passed through a multi mode fiber into the milk. The back scattered light was monitored by a single mode fiber located between 1.8 and 3.5 mm from the input fiber. The scattered light is detected with a Photo Multiplier Tube (ALV SO-SIPD), transforming the light signal into an electronic signal, which is fed to a PC interfaced autocorrelator board (ALV5000/E). The time where the auto correlation curve has decayed to 50% of its maximum plateau level is defined as $\tau_{1/2}$ (see Figure 6.2). The gelation point is defined in the plot of $\tau_{1/2}$ against pH at the
inflexion point. All data were normalised relative to its control blank, which is the sample prior to GDL addition.

**Preparation of β-Ig aggregates**

A solution containing 3.0% β-Ig in 60mM NaCl at pH 7.0 was heated for 12 hours at 68.5°C. After cooling the volume was reduced to a protein concentration of 3.7% by using centriprep YM-3 concentrators (Amicon Bioseparators; nominal molecular weight cut off of 3000 Da). Concentrated simulated milk ultrafiltrate (SMUF(10); 5.5 times concentrated) was added so that a protein concentration of 3.3% was obtained (to mimic the salt and protein concentration in skim milk 2.76% casein + 0.53% whey = 3.29% total protein). After filtration (5 µm) the size of the aggregates was determined by dynamic light scattering and was 225 nm. This solution is acidified with 0.5% (w/w) GDL at 32°C.

**Determination of native protein**

An amount of 0.4 g of milk was mixed with 0.8 g of distilled water (40°C) and 40 µl acetic acid (HAc) (10%) in an eppendorf tube (2
ml). After mixing (vortex) and 10 min waiting, 40 µl of sodium acetate (NaAc; 1M) and 0.72 g of distilled water are added and the solution was mixed again. After 1 hour standing the solution is centrifuged for 5 min at 3000g. The concentration of native β-lg and α-lac in the supernatant is determined by chromatographic analysis.

**Results and discussion**

Figure 6.3 shows the effect of heat treatment on the acid induced gelation of milk measured by DWS and plotted as $\tau_{1/2}$ as a function of pH. The $\tau_{1/2}$ is the time where the intensity autocorrelation function has decayed for 50% and thus represents a “mobility” of the system. The value of $\tau_{1/2}$ correlates in a one to one manner with $G''$ ($\omega$=1s$^{-1}$), where $G''$ is the small deformation loss modulus of the system. The milk at its natural pH was heated, in the temperature range 70-90°C for 10 min. The milk was cooled down to 32°C, the acidification temperature, and after 75 min acidified with 1.1% GDL. Heat treatment of the milk, prior to acidification, induced a shift of the gelation pH to more alkaline pH values. These results are in agreement with the results of Horne and Davidson (6).

Heat treatment of skim milk in the range 70-90°C will result in

![Figure 6.3: Acidification traces, normalised $\tau_{1/2}$ versus pH, of skim milk heat treated for 10 min at 70(●), 75(▼), 80(□), 85(+), 90(○)°C and unheated (▲) with 1.1 % GDL at 32°C](image-url)
whey protein thermal denaturation (3). The results as shown in Figure 6.3 are related to the thermal denaturation of \( \alpha \)-lac and \( \beta \)-lg in the milk prior to acidification. Figure 6.4 shows an intrinsic relationship between the extent in whey protein thermal denaturation and the pH at which gelation occurs.

In order to ascertain the protein responsible for the shift in gelation pH, the effect of adding \( \alpha \)-lac and/or \( \beta \)-lg to WPF-milk was investigated (Figure 6.5). The \( \tau_{\gamma}\)-pH traces of WPF-milk with additions of \( \alpha \)-lac and/or \( \beta \)-lg but without further heat treatment of the milk were all identical to untreated WPF-milk (results not shown). As shown in figure 6.5 heat treatment had no effect on the gelation pH of WPF. This supports the earlier assertion that the whey proteins are responsible for the shift of the gelation pH to more alkaline pH values. Heat treatment had little effect on the gelation pH of WPF with or without \( \alpha \)-lac supplementation. Addition of \( \beta \)-lg caused gelation to occur at a more alkaline pH. This is consistent with literature as it is known that \( \beta \)-lg interacts with \( \kappa \)-casein by intermolecular disulfide bonds. \( \alpha \)-lac has no free thiol group and is therefore not able to interact with \( \kappa \)-casein (13, 17). Supplementation of both \( \alpha \)-lac and \( \beta \)-lg induced no additional shift to \( \beta \)-lg alone, although a large amount of \( \alpha \)-lac is denatured in the presence of \( \beta \)-lg, but apparently this extra denaturation does not influence the gelation
The overall conclusion is that $\beta$-lg is responsible for the shift in gelation pH which was already noticed in milk and that $\alpha$-lac neither alone nor together with $\beta$-lg has any (additional) influence on the gelation pH.

The addition of $\beta$-lg to WPF to an equal concentration as in milk induced a shift of 0.5 pH unit to more alkaline pH. The role of $\beta$-lg was further explored by progressive addition to WPF-milk. The gelation pH values are obtained from the $\tau_{1/2}$ acidification curves and plotted versus the total amount of denatured $\beta$-lg. All the milks were heated for 10 min at 90°C which caused 100% denaturation. Figure 6.6 confirms the role of $\beta$-lg by the observation that with the progressive addition of $\beta$-lg up to 4 g/l there is a concomitant increase in gelation pH; thereafter the gelation pH levels off.

Whey proteins are thought to interact with $\kappa$-casein during heat treatment (9, 15) and thus form a coating on the micelle surface. The properties of the micelles are changed by the coating with whey proteins as the pI of whey proteins is markedly higher than that of the caseins (5.2; 4.6 respectively). This raises the question, in a colloidal context, to what extent do whey protein coated casein micelles start to behave like whey protein particles. To obtain this
information the gelation pH of non-heat treated casein micelles (1.1% GDL, 32°C) and β-lg aggregates ~225nm in SMUF (0.5% GDL, 32°C), assumed as 0 and 100% coating, respectively was determined. These β-lg aggregates were prepared in order to mimic a completely coated micelle with roughly the same size as a micelle (225 nm). The aggregates were acidified in SMUF to simulate a milk milieu. The gelation pH of the β-lactoglobulin aggregates were taken as pH (100% coating) ; pH = 6.00. For non coated casein micelles (WPF) the gelation pH is 5.05. It was assumed (see figure 6.7) that gelation pH is linearly related to the coverage of the micelles. It was then found that the heat treated micelles (10 min at 90°C; gelation pH is 5.40) are covered by 40% by whey proteins. Surprisingly this seems to be a very reasonable estimate, as calculating shows.

The amount of β-lg required to form a monolayer on the surface of the micelles present in milk was calculated. The following parameters were taken: $\phi = 0.11$; $\phi_{\text{max}} = 0.63$; $r_{\text{micel}} = 100$ nm ($\phi$=volume fraction [-]) and a mono layer is formed at 2 mg/m2. By using $\phi = (\text{number} \times \text{volume}) / \text{total volume}$ it was calculated that in case of monodispersity 6 g/l could attach to casein micelle to form a mono layer. In milk there is 3.2 g/l β-lg, which means that not even a mono layer can be formed. The same qualitative result is obtained by the fact that β-lg and κ-casein are present in milk at an equal

Figure 6.6: Effect of β-lg denaturation on the acid-induced gelation point of WPF milk containing 0-5 g/l β-lg, heated (10 min, 90°C) and acidified with 1.1% GDL at 32°C.
molarity of 180 mmol/m3 (17). A small part of the κ-casein is not present at the periphery but in the interior of the micelle (17), which would mean there is hardly enough to get a mono layer. We also have to take into account that during heat treatment β-lg will also form small aggregates by interacting with α-lac and other β-lg molecules. The aggregates will mainly interact with the micelles, but we have indications that a part stays in solution. The amount of available β-lg to coat the micelle by a mono layer is reduced by the formation of these aggregates. Considering the above it is concluded that whey protein coated micelles in milk only behave for 40% like “pure” whey protein aggregates.

Conclusions

Heat treatment in the range 70 to 90°C induced a shift in gelation pH of skim milk to more alkaline pH values. This shift was directly related to whey protein denaturation. By using WPF milk it was shown that β-lactoglobulin is principally responsible for the shift in gelation pH. α-lactalbumin caused neither alone nor in combination with β-lg an effect on the gelation pH. Heat treatment of
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References