Molecular Water Motions of Skim Milk Powder Solutions during Acidification Studied by [17]O and [1]H Nuclear Magnetic Resonance and Rheology

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ABSTRACT: The molecular motion of water was studied in glucono-δ-lactone-acidified skim milk powder (SMP) solutions with various pH values and dry matter contents. NMR relaxometry measurements revealed that lowering the pH in SMP solutions affected ^{17}O and ^{1}H T$_2$ relaxation rates almost identically. Consequently, the present study indicates that the proteins present in the samples do not affect the ^{1}H relaxation behavior markedly, even at relatively high SMP concentrations (15−25%). Comparison of rheological measurements and NMR measurements suggested that the collapse of κ-casein during acidification could contribute to the initial decrease in ^{17}O and ^{1}H relaxation rate in the pH range between 6.6 and 5.5 for 15% SMP and in the pH range between 6.6 and 5.9 for 25% SMP. However, below pH 5.5 the viscosity and ^{17}O and ^{1}H NMR relaxation rates did not correlate, revealing that the aggregation of casein micelles, which increases viscosity below pH 5.5, does not involve major repartitioning of water.

KEYWORDS: ^{17}O relaxation rate, glucono-δ-lactone, κ-casein, calcium phosphate, ^{1}H relaxation rate, water self-diffusion, phase volume, viscosity

■ INTRODUCTION

Acidification of milk is widely used in the food industry to produce yogurt and cheese. Several acidification methods are used, which include addition of lactic acid, starter culture, and glucono-δ-lactone (GDL). GDL is a naturally occurring substance found in honey, fruit juice, and wine, which produces a slow, controllable acidification in aqueous solution. Acidification with GDL mimics acidification by lactic acid bacteria, with GDL gradually hydrolyzing into gluconic acid, which is a weak acid.[1] Casein micelles in milk behave as hard spheres and are stabilized by surface κ-casein protein hairs. However, acidification destroys the capacity of these hairs to provide steric stabilization, causing them to collapse and resulting in a weak attraction between the micelles. The collapse of the κ-casein hairs together with the solubilization of casein monomers from within the casein micelles, which occurs at temperatures below 25 °C, leads to a decrease in viscosity.[2−4] The solubilization of casein monomers below 25 °C is a result of dissolution of calcium phosphate that binds the casein monomers together.[5] When the attractive interactions between the destabilized micelles are strong, the system will aggregate and eventually form a permanent gel network, resulting in a steep increase in viscosity.[4]

Rheological measurements during acidification of different dairy powder solutions have shown increases in storage modulus (G') with acidification time.[6,7] However, only a few studies of changes in viscosity before gel formation have been reported.[4,8] Gastaldi et al.[8] observed a decrease in viscosity as the pH was lowered with a minimum viscosity observed at pH 5.2−5.3, followed by a steep increase at lower pH values.

Using ^{1}H and ^{17}O nuclear magnetic resonance (NMR) relaxometry, the molecular environment of water in skim milk powder solutions has been shown to change when the pH decreased.[9] The dominant ^{1}H relaxation pathway for water is via the T$_2$ mechanism, which is sensitive to both molecular motions of water and the rate of proton exchange with protein surfaces; proteins and other macromolecules have a faster intrinsic relaxation rate than water, so faster water relaxation can be due to either more frequent proton exchange with the protein surface or reduced molecular motions of water molecules. In contrast, as the oxygen atom of water does not exchange with protein surfaces, ^{17}O T$_2$ relaxation reflects only molecular motion of water molecules.[10] Using both methods, a decrease in molecular motions of water was observed when the pH was lowered from 6.6 to 5.3, whereas a small increase was observed for pH levels below 5.3.[9] The decrease in relaxation rate to pH 5.3 could be related to the extent of solubilized micellar calcium phosphate, and it was suggested that the increased molecular motion of water at pH 6.6 was related to the amount of calcium and calcium phosphate bound to the caseins.[9]

As described above, the acidification of milk and milk proteins has been studied using different techniques such as ^{17}O and ^{1}H NMR relaxometry,[9,11−13] pulsed gradient spin echo (PGSE) NMR

for determination of water self-diffusion,[11,14] and rheological characterization including determination of viscosity and G'.[2,4,7,8,15] However, to the authors' knowledge, a comparison of the rheological properties of acidified skim milk powder (SMP) solutions and water properties as revealed by NMR relaxometry has yet to be provided. Consequently, the objectives of the present study were (i) to investigate the effect of pH and dry matter content on water molecular motions using different NMR-based methods in a dairy model system of SMP solutions acidified with GDL and (ii) to compare rheological parameters and water molecular motion, by investigating the relationship between structural changes detectable with rheological parameters (viscosity, storage modulus, and shear viscosity), the molecular motion of water, detectable with ^{17}O NMR, and ^{1}H exchange between water and protein surfaces that can be deduced from ^{1}H NMR.

■ MATERIALS AND METHODS

Sample Preparation. A total of 18 different SMP samples were included in the study. Samples for rheological and ^{17}O NMR measurements were produced from low-heat SMP obtained from Murray Goulburn Co-operative Co-Ltd. (Melbourne, Australia), whereas samples prepared for [1]H T_2 relaxation measurements and water self-diffusion measurements were produced from low-heat SMP obtained from Arla Foods Ingredients amba (Viby J., Denmark). SMP solutions corresponding to concentrations of 15 and 25% (w/v), respectively, were prepared by mixing with demineralized water with stirring at 40 °C for 30 min, followed by equilibration at 22−23 °C for at least 30 min. The SMP solutions were produced as large batches divided into 36 small containers, 2 for each combination of SMP concentration and pH, before acidification.

The pH was adjusted to nine different levels (pH 4.3, 4.5, 4.7, 5.0, 5.3, 5.5, 5.7, 6.0, and 6.7) by adding different amounts of glucono-δ-lactone (GDL) (Sigma-Aldrich, St. Louis, MO). The GDL was added in the form of 50% (w/v) solution, which was mixed in demineralized water at a temperature of 56 °C for 2 min and adjusted to room temperature for 1 min prior to addition to the SMP solutions. Subsequently, the samples were mixed and stored at 22 °C for at least 19 h before analysis.

The final SMP concentrations, which depended on the added amount of GDL solution, were in the ranges of 14.3−15 and 23.2−25% within the samples labeled 15% SMP and the samples labeled 25% SMP, respectively.

Rheology Measurements. Rheological characterization was carried out on a Haake Mars III rheometer (ThermoFischer Scientific, Germany). All samples were analyzed in duplicate 19−25 h after addition of GDL. For samples that had formed a gel, a vane geometry (vane rotor FL22, diameter 22 mm) was used; this involved slowly inserting the vane into a plastic vial (with a diameter of 40 mm and a height of 50 mm) containing the preset gel to minimize disturbance of the microstructure upon loading (prior to measurement). The room temperature was set at ∼23 °C. G' was obtained by applying an oscillatory frequency sweep from 0.01 to 1 Hz at a constant stress value of 0.500 Pa so that all measurements were performed within the linear viscoelastic regimen. Because the G' was relatively invariant with frequency, as is typical for gels, for all samples G' was compared at 0.1 Hz for further analysis. In addition, using the vane geometry an apparent shear viscosity was measured in rotation for increases in stress from 0.5 to 500 Pa. The measurement time per data point was 60 s.

The liquid samples were analyzed using a cone-and-plate geometry (cone 35/0.5° Ti L), and the viscosity was obtained by applying a rotation step program analyzing the steady state viscosity from stress 0.03 to 30 Pa at 22 °C. All liquid samples were found to be newtonian,

and the viscosity obtained for further analysis was taken as the mean value over shear rates from ∼800 to ∼17000 1/s.

The effective phase volume of casein micelles was determined by assuming that they behave as hard spheres[4] and following the hard sphere model described by Quemada,[16] eq 1

$$\eta/\eta_s = (1 - \phi/\phi_m)^{-2} \tag{1}$$

where η is the viscosity of the solutions, η_s is the solvent viscosity ϕ is the phase volume, and ϕ_m is the phase volume for maximum packaging fraction of hard spheres. In the present study it was assumed that η_s = 1.15 mPa·s (solvent viscosity at 20 °C)[2] and ϕ_m = 0.64.[16]

The effective phase volume, calculated from eq 1, was used to calculate an effective radius of the micelles, as the effective radius is directly proportional to $\phi^{1/3}$. The change (%) in effective radius was calculated between pH levels, and by assuming that the radius of the casein micelles is 100 nm at pH 6.6,[4] an estimate for the actual change in radius was calculated.

NMR Analyses. ^{17}O NMR spectroscopy was carried out at 67.8 MHz on a 500 MHz Bruker spectrometer (Advance 500 MHz, Bruker, Germany) equipped with a high-resolution 5 mm probe. The experiments were carried out at 22 °C using a spectral width of 6000 Hz performing 2048 scans with a recycle time of 0.3 s. The Fourier transform of the free induction decay (FID) revealed a single peak. The width at half-height of the peak was determined, and the transverse relaxation rate R_2 was calculated according to eq 2.[9]

$$\frac{1}{T_2} = R_2 = \pi\Delta\nu_{obs} \tag{2}$$

^{1}H NMR T_2 relaxation measurements were performed on a Maran Benchtop Pulsed NMR Analyzer (Resonance Instruments, Witney, U.K.) operating at 23.2 MHz and equipped with an 18 mm variable-temperature probe, applying a CPMG sequence[17,18] at a temperature of 25 °C, and using a recycle delay of 4 s. All samples were analyzed in duplicate 20−25 h after addition of GDL.

The obtained T_2 relaxation data were analyzed using distributed exponential fitting analysis according to the regularization algorithm by Butler et al.[19] and carried out in MatLab (The Mathworks Inc., Natick, MA) version 7.0 using in-house scripts. Distributed exponential fitting results were presented in a plot of relaxation amplitude versus relaxation time, over a predefined range of characteristic relaxation times. In this study a fitting of 256 logarithmically distributed relaxation times from 0.5 to 3000 ms was established. Areas, relaxation times, and relaxation rates of the relaxation populations found were calculated.

^{1}H NMR water self-diffusion coefficient measurements were carried out using a Maran Benchtop Pulsed NMR Analyzer (Resonance Instruments) operating at 23.2 MHz and equipped with an 18 mm variable-temperature probe and a gradient amplifier at a temperature of 25 °C, applying a pulse gradient spin echo sequence. For each measurement, a total of 22 echoes were acquired as a function of the gradient pulse duration (δ), which was varied from 200 to 2500 μs, using a recycle delay of 2 s. The gradient pulse amplitude (g) was 5000, and the gradient pulse separation (Δ) was set to 200 ms. The diffusion coefficient was obtained according to eq 3

$$\log(\text{echoamp}) \text{ vs } \left((\gamma \times g)^2 \times \delta^2 \times \left(\Delta - \frac{\delta}{3}\right)\right) \tag{3}$$

where γ is 267538030 s^{-1} T^{-1}.

The diffusion coefficient was obtained as the negative slope of the best straight line fitted to this graph.

pH Measurements. The pH of the samples was determined 19−20 h after addition of GDL. In samples used for rheological measurements and ^{17}O NMR, the pH was measured using a pH Cube instrument (TPS, Australia) equipped with an Ionode IJ44 electrode
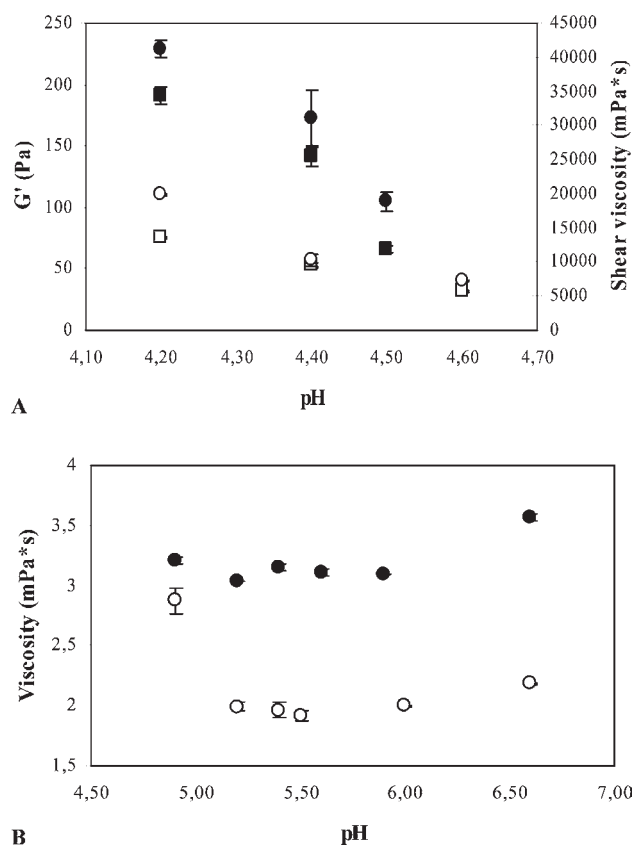
Figure 1. (A) Shear viscosity (●, ○) and G' (■, □) as a function of pH in samples with 25% SMP (●, ■) and 15% SMP (○, □); (B) viscosity as a function of pH in samples with 25% SMP (●) and 15% SMP (○).

Figure 2. ^{17}O transverse relaxation rate as a function of pH in samples with 25% SMP (●) and 15% SMP (○).
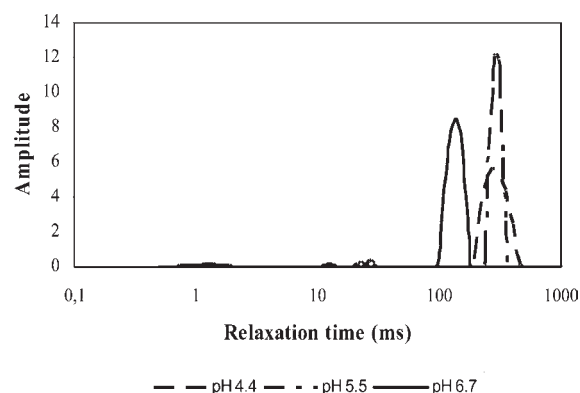
Figure 3. ^{1}H distributed relaxation times for samples with 15% SMP at pH 4.4, 5.5, and 6.7.

(Ionode, Australia), at a temperature of 22−23 °C, whereas the pH of the samples prepared for the ^{1}H NMR T_2 relaxation measurements was determined using a PHM 92 LAB pH-meter (Radiometer, Copenhagen, Denmark) equipped with a Fisherbrand combination electrode (Fisher Scientific, U.K.).

Data Analysis. Analysis of variance and Student's t test were performed using SAS 9.1.3 software (SAS Institute Inc., Cary, NC).

■ RESULTS

In the present study SMP solutions containing two levels of dry mater content (15 or 25% SMP) were acidified with GDL to nine different pH levels. All samples were analyzed in duplicate, and rheological properties, ^{17}O and ^{1}H relaxation rates, and water self-diffusion coefficients were measured for all samples to investigate the relationship between rheological properties and water molecular motion in acidified SMP solutions.

Changes in Viscosity and G'. Samples with a pH of 4.7 or below, which had formed gels, showed an increase in shear viscosity and G' with decreasing pH, which reveals a strengthening of the gel as the pH is lowered (Figure 1A). The difference between the two levels of SMP was expected, as a higher level of dry matter increases the strength of the gels.

Samples with a pH above 4.7 were liquid, and in these samples the viscosity was analyzed (Figure 1B). The higher dry matter concentration in samples with 25% SMP resulted in a significantly higher viscosity compared to samples with 15% SMP. In addition, the results also revealed a decrease in viscosity between pH 6.6−5.9 and 6.6−5.5 for solutions containing 25 and
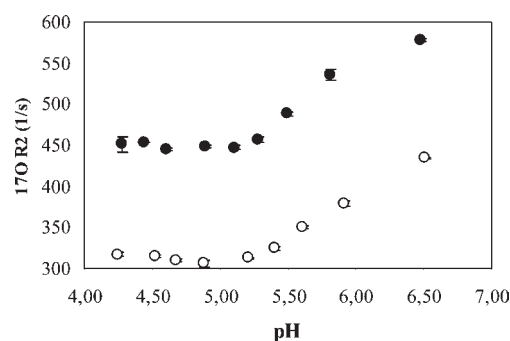
15% SMP, respectively, followed by a steep increase between pH 5.2 and 4.9 in samples with 15% SMP.

NMR Results. All ^{17}O peaks had Lorentzian line shapes (data not shown), and from the width at half-height the relaxation rate was calculated according to eq 2. The ^{17}O relaxation rate decreased with decreasing pH and displayed a minimum relaxation rate at pH 4.9 in samples with 15% SMP, followed by a small increase when the pH was further lowered to 4.3 (Figure 2). Samples with 25% SMP also decreased in relaxation rate to a pH of 5.1, below which a constant relaxation rate of around 450 s^{-1} was observed (Figure 2).

Distributed analysis of the obtained ^{1}H T_2 relaxation data revealed the presence of three relaxation populations in most samples (Figure 3). These three populations, designated T_{21}, T_{22}, and T_{23}, were characterized by relaxation times around 1.2−1.6, 3.0−28.0, and 80−330 ms and represented approximately 1.0−3.0, 0.5−2.0, and 90.0−97.5% of the total amplitude, respectively. In addition, in two samples (25% SMP at pH 4.4 and 4.5) a free water population with a relaxation time around 2 s, which represented approximately 1.5% of the total amount of water, was detected.

The T_{22} and T_{23} relaxation rates were calculated according to eq 2, and in Figure 4 the relaxation rates are displayed as a function of pH. Samples with 25% SMP were characterized by a higher relaxation rate for both T_{22} and T_{23}. The T_{22} and T_{23} relaxation rates decreased as the pH was lowered. The T_{23} relaxation rate displayed a minimum at pH 5.3 for samples with 15% SMP and at pH 5.1 for samples with 25% SMP, and at lower
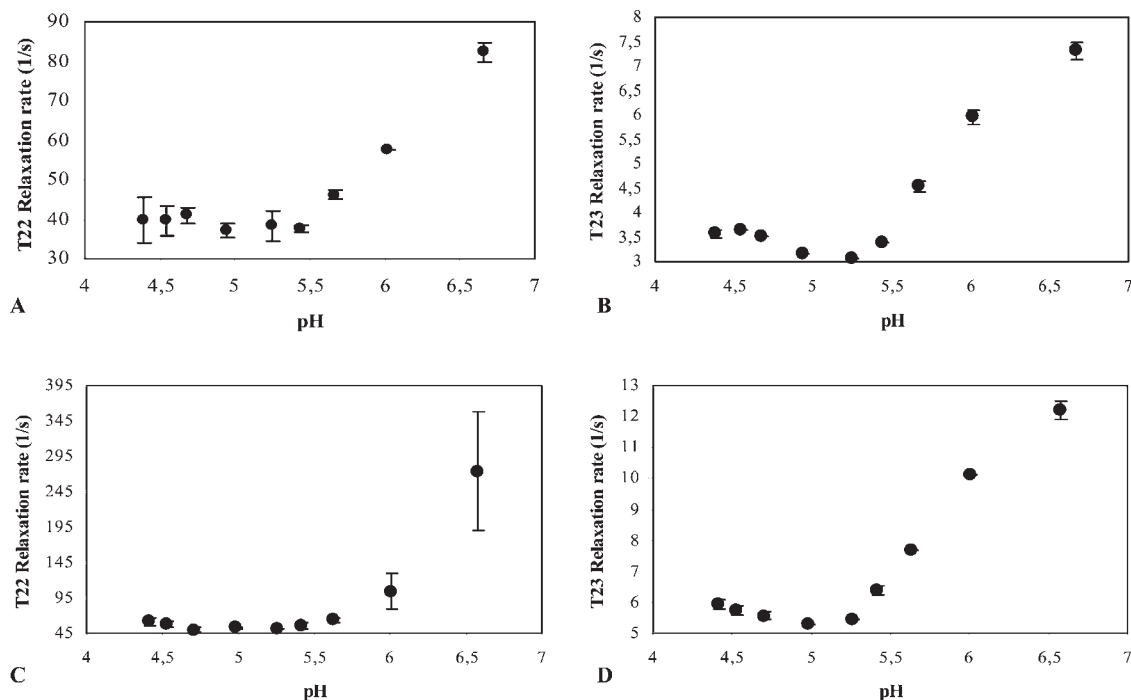
Figure 4. ^1H relaxation rate as a function of pH: (A) 15% SMP population T_{22}; (B) 15% SMP population T_{23}; (C) 25% SMP population T_{22}; (D) 25% SMP population T_{23}.
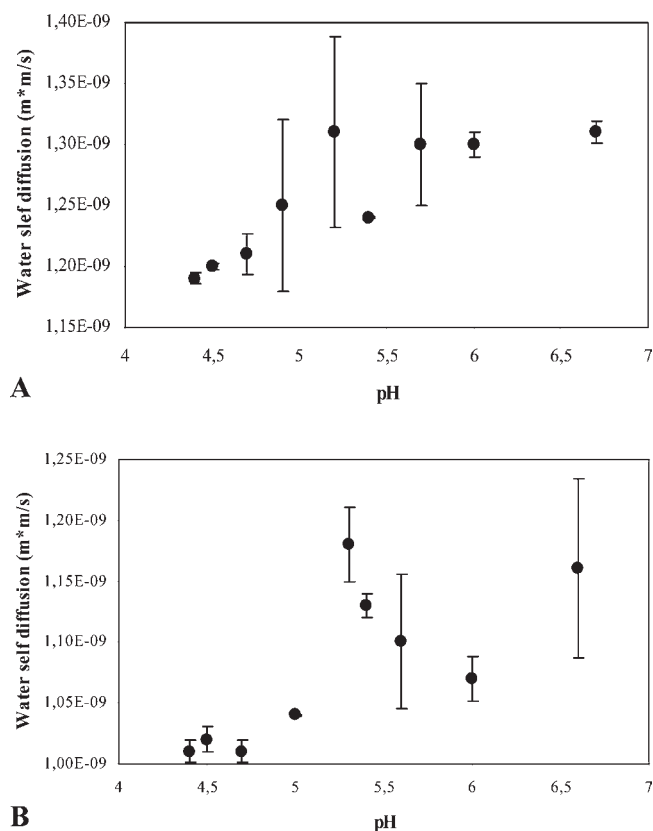

Figure 5. Water self-diffusion coefficient as a function of pH: (A) 15% SMP; (B) 25% SMP.

pH a small increase in the T_{23} relaxation rate was observed. For the T_{22} relaxation rates a similar response to pH as for the T_{23}

relaxation rates was observed with a minimum around pH 5.3—5.5. As the T_{23} relaxation population represented at least 90% of the total ^1H T_2 relaxation population, only the T_{23} relaxation rates are considered in the comparison with ^{17}O relaxation rates and rheological parameters.

Plotting of water self-diffusion coefficients as a function of pH reveals a tendency for a decrease in water self-diffusion coefficient as the pH decreases (Figure 5), although changes are small. Consequently, the lowest diffusion coefficient was observed for samples with pH 4.4, 4.5, and 4.7. Analysis of variance test revealed no significant effect of pH on the diffusion coefficient in samples with 15% SMP, whereas a significant effect of pH ($P <$ 0.01) on the diffusion coefficient was observed for samples with 25%. However, the difference in water self-diffusion, within each SMP concentration, is numerically small, and some samples reveal very large standard deviations.

Comparison between Rheological and NMR Measurements. All analytical methods applied in the present study revealed a difference between the two levels of SMP, where solutions with 25% SMP were characterized by higher ^{17}O and ^1H T_{22} and T_{23} relaxation rates and water self-diffusion coefficients and increased viscosity, shear viscosity, and G' compared with solutions containing 15% SMP. These findings reveal that the analyzed parameters are highly affected by the dry matter content.

The ^{17}O relaxation rate is a measure of the molecular mobility of water,[10,20] and this analysis detects differences only in the behavior of the water, not in the SMP polymeric components. It is therefore of interest to investigate if the changes in viscosity gave rise to concomitant alterations in the ^{17}O relaxation rate. An initial decrease in ^{17}O relaxation rate was consistent with a decrease in viscosity between pH 6.6 and 5.5 for 15% SMP and between pH 6.6 and 5.9 for 25% SMP. However, although the relaxation rate further decreased with decreasing pH, the

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viscosity began to increase around pH 5.6 for 15% SMP, whereas in samples with 25% SMP only a tendency for increase in viscosity was observed at pH below 5.9 (Figures 1B and 2). A small increase in ^{17}O relaxation rate below pH 4.6 in samples with 15% SMP (Figure 2) was comparable with an increase in shear viscosity and G' (Figure 1A); however, this relationship was not observed for samples with 25% SMP.

Comparison of the rheological measurements with the $^{1}H \, T_{23}$ relaxation rate shows a similar relationship between viscosity and $^{1}H \, T_{23}$ relaxation rate as observed between viscosity and ^{17}O relaxation rate (Figures 1B and 4B,D). In addition, a small increase in the $^{1}H \, T_{23}$ relaxation rate below pH 4.9 for both 15 and 25% SMP (Figure 4B,D) was consistent with an increase in shear viscosity and G' (Figure 1A).

■ DISCUSSION

Several biochemical changes occur upon acidification of skimmed milk systems. One of the initial changes is the collapse of κ-casein, altering the effective phase volume of the casein micelles.[2,4] The present study revealed a decrease in viscosity from pH 6.6 to 5.5 in 15% SMP samples and a decrease in viscosity from pH 6.6 to 5.9 for 25% SMP samples, which is in agreement with previous findings.[8] These authors showed a minimum in viscosity at pH 5.3 in samples with 12% (w/vol) SMP, whereas the present study suggested a broad minimum over the range pH 5.5−5.2 in samples with 15% SMP and at pH 5.9−5.2 in samples with 25% SMP (Figure 1B). The viscosity changes above the isoelectric point (pH 4.6) are anticipated to be due to the effective phase volume occupied by the micelles, which behave as hard spheres.[4] If the polymeric molecules undergo even minor conformational changes, the amount of solvent associated with the micelles alters. In the present study the Quemada model[16] was used to calculate the effective phase volume, and from the effective phase volume, the radius of the casein micelles was calculated. The maximum decrease in radius determined from this analysis was found to be 6.4 ± 1.2 nm, close to the length of the glycomacropeptides of κ-casein, which has been reported to be 7 nm.[4] Consequently, the present study shows that the decrease in viscosity as a function of a pH decrease is quantitatively consistent with its being due to collapse of the κ-casein during acidification, as also shown by Banon and Hardy.[2]

The ^{17}O NMR spectra revealed single Lorentzian line-shaped peaks, which is consistent with a model of fast exchange between free water and water strongly associated with the casein proteins. The results were therefore described using the two-state model described in eq 4.[9,10]

$$R_{obs} = f_b R_b + f_f R_f \qquad (4)$$

A decrease in the overall relaxation rate (R_{obs}) was observed when the pH was lowered, and according to eq 4, this decrease could be due to alterations in the relaxation rate of the individual water populations and/or alterations in the fraction of the bound water.

Intriguingly, the $^{1}H \, T_{23}$ relaxation rate showed a similar trend as a function of pH as the ^{17}O relaxation rates, indicating that the behavior of the $^{1}H \, T_{23}$ relaxation rate is not strongly affected by chemical exchange. A similar finding has been reported at lower SMP concentrations by Mariette et al.,[9] who found that the ^{1}H relaxation rate and ^{17}O relaxation rate had a similar behavior as a function of pH, reflected in a decrease to a minimum at pH ∼5.3 followed by a small increase when the pH was further lowered to

∼4.3.[9] In the present study we found that the minimum relaxation rate was seen at a slightly higher pH when analyzing the $^{1}H \, T_{23}$ relaxation rate compared to the ^{17}O relaxation rate in samples with 15% SMP. A study performed by Le Dean et al.[21] revealed two populations of protons present in native phosphocaseinate dispersion and established that the protons present in the population with the long relaxation time were attributed to water and lactose protons. In addition, Le Dean et al.[21] showed that the relaxation rate of the slowest-relaxing T_2 population was primarily influenced by the concentration of native phosphocaseinate, but also to a minor degree by pH, lactose, and whey protein.[21] These findings suggest that it could be the presence of the combination of casein, lactose, and whey protein in the SMP samples that shifts the pH at which the minimum $^{1}H \, T_{23}$ relaxation rate occurs when compared to the ^{17}O relaxation rate. In addition, Mariette et al.[9] showed that increasing the SMP concentration (to 10%) decreased the pH at which the minimum ^{1}H relaxation rate was observed.[9] This is extended in the present study, with a minimum in $^{1}H \, T_{23}$ relaxation rate at a pH of 5.3 and 5.1 for SMP concentrations of 15 and 25%, respectively. Furthermore, the $^{1}H \, T_{23}$ relaxation rate revealed a difference between the two SMP concentrations: 15% SMP irrespective of pH resulted in a lower $^{1}H \, T_{23}$ relaxation rate compared with samples containing 25% SMP. This is consistent with the findings of Mariette et al.[9] and Le Dean et al.,[21,22] both revealing that increasing the level of SMP, native phosphocaseinate, the major protein in whole milk, and phosphocaseinate in dispersion with added lactose and whey proteins increased the $^{1}H \, T_2$ relaxation rate.

Earlier studies at 3−15% SMP concentration reported no differences in water diffusion coefficient between solutions and gels of casein and whey protein systems, but only differences due to dry matter content.[11,14] A strong effect of dry matter content on the water diffusion coefficient was likewise observed in the present study at similar and higher concentrations. A significant effect of pH ($P < 0.01$) on the water self-diffusion of 25% SMP samples was observed, but only a tendency of an effect of pH ($P = 0.07$) was observed for 15% SMP samples. However, the changes in the water self-diffusion are numerically small, and several of the diffusion coefficients were determined with large standard deviations. These numerically small differences in water self-diffusion within each level of SMP indicate that major microstructural changes (e.g., gelation) occurring during acidification have only a small effect on water self-diffusion in these systems.

Mariette et al.[9] reported a minimum in ^{17}O relaxation rate and $^{1}H \, T_{21}$ relaxation rate at pH ∼5.3 in samples with 8−10% SMP, and a study by Gastaldi et al.[8] reported a minimum in viscosity around the same pH in samples with 12% SMP. Furthermore, a study by Donato et al.[23] on nonconcentrated milk revealed a decrease in apparent radius of casein micelles to a minimum at pH around 5.3 followed by an increase. Together these earlier findings indicate a connection between the decrease in viscosity and the decrease in water relaxation properties. Intriguingly, the present study revealed a simultaneous decrease in viscosity, ^{17}O relaxation rate, and $^{1}H \, T_{23}$ relaxation rate between pH 6.6 and 5.5 for solutions with 15% SMP and between pH 6.6 and 5.9 for solutions with 25% SMP (Figures 1B, 2, and 4B,D). Furthermore, the maximum change in radius of casein micelles (6.4 ± 1.2 nm) calculated from the viscosity was found to be consistent with the length of the glycomacropeptides of the κ-casein. Consequently, the observed relationship between the viscosity and water relaxation rate, at pH between 6.6 and 5.5 for 15% SMP

(pH 6.6 and 5.9 for 25% SMP), can be ascribed to the collapse of κ-casein, affecting the amount of water associated with the casein micelles and further causing alterations in the water relaxation. However, at pH <5.5 for samples with 15% SMP and at pH < 5.9 for samples with 25% SMP (Figures 1B, 2, and 4B,D), different trends in viscosity, ^{17}O relaxation rate, and ^1H T_{23} relaxation rate were observed. This finding suggests that the molecular motion of water and viscosity below this pH are perturbed by the formation of extensively aggregated micelle clusters at the higher concentrations used in this study compared with previous studies, such that the viscosity and molecular motion of water are affected by different mechanisms. For example, the small increase in ^{17}O relaxation rate for pH values below 4.9 and in the ^1H T_{23} relaxation rate for pH values below 5.3 for 15% SMP (Figures 2 and 4B) could be a weaker version of a more pronounced increase found for lower SMP concentrations,[9] whereas at 25% SMP there is no significant change below pH 4.9 in ^{17}O relaxation rate and a small increase in the ^1H T_{23} relaxation rate at pH below 5.1. We therefore propose that at 25% SMP, in contrast to lower SMP concentrations, any potential alteration in molecular motion of water below pH 4.9 is prevented by the presence of already large micelle clusters that at pH 4.6 (isoelectric point of casein) lead to network formation with little or no effect on molecular motion of water. The changes in the ^{17}O NMR relaxation rate and the ^1H T_{23} relaxation rate below pH 5.6 and 5.9 for 15 and 25% SMP, respectively, could be ascribed to additional dissociation of calcium phosphate causing dissociation of individual casein proteins,[3,9] whereas the increase in viscosity at the lower pH could be ascribed to the dissociation and subsequent aggregation of casein proteins.[2]

Samples at pH 4.7 and below formed gels, and as the pH was further lowered the complex viscosity and G' increased. This pattern was observed for both levels of SMP; however, the increase was more pronounced for 25% SMP compared with 15% SMP (Figure 2A). This finding could also be expected, as increases in complex viscosity and G' reflect strengthening of the gels, and higher levels of SMP result in stronger gels.[14] With the acid-induced strengthening of the gels, the ^{17}O relaxation rate showed only a small increase in the samples with 15% SMP, whereas the ^1H T_{23} relaxation rate showed a small increase for both 15 and 25% SMP. However, no difference in ^{17}O relaxation rate as a function of the acid-induced strengthening of the gels was observed in the samples with 25% SMP. Therefore, the gelation process is not accompanied by a major change in either the intrinsic ^{17}O relaxation properties of bulk or protein-associated water or the relative amounts of the two or in the ^1H relaxation of the bulk water or the relative amounts of this. Together with the water self-diffusion coefficients, which showed very limited changes with pH (Figure 5), this is consistent with a predominantly hydrophobic gelation mechanism that does not involve major repartitioning of water.

The present study suggests that the initial changes in molecular mobility of water during acidification as detected by ^{17}O relaxometry and ^1H relaxometry cannot be explained solely by solubilization of calcium phosphate. In contrast, the decrease in viscosity caused by the collapse of κ-casein, changing the amount of water associated with the micelles, may also contribute to the observed changes. This is proposed for both levels of SMP; however, the connection is more apparent in the samples with 15% SMP compared to 25% SMP.

In addition, the limited changes in ^{17}O and ^1H T_{23} relaxation rate and water self-diffusion below pH 5.5 reveal no major repartitioning of water during aggregation of casein micelles and gel formation, at 15 or 25% SMP. However, because of the limited number of samples included in the present study, additional work increasing the levels of dry matter contents and the number of pH levels has to be carried out.

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