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ORIGINAL PAPER

Calcium induced skim-milk gelation during heating as affected by pH

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Abstract Milk gels (acid or rennet) are used by dairy industry to produce dairy products such as yogurt and cheese. Enrichment of milk with calcium salts and heat treatment are known to produce "calcium-milk coagulum" as a new type of milk gels, due to reduction of milk protein charges through calcium binding. The combination of heat treatment and calcium addition to milk results in gel structures, but the effect of calcium addition and pH adjustment during heating of milk is still unclear. The role of added calcium and decreasing pH were investigated by addition of calcium chloride (30 mM) to reconstituted skim milk followed by pH adjustment by hydrochloric acid and sodium hydroxide ($4.6<pH<6.6$ investigated), followed by heating at 90 °C for 10 min and overnight storage at 22 °C. In parallel, samples with no addition of calcium chloride were produced under the same conditions. The time and temperature to reach the gelation point, as detected by dynamic measurements of storage modulus (G′), were decreasing as pH decreased without addition of calcium, while calcium addition made gelation time and temperature independent of pH except for pH 4.6. Heat treatment combined with calcium addition was found, using confocal laser microscopy, to provide a fine and dense gel structure for skim milk with higher pH, while at pH lower than 5.6, the gel structure was similar to the structure of acid-induced gels. The last observation helps to establish a pH limit for production of calcium gels.

Keywords Calcium-milk coagulum · Milk gelation · Acid-calcium synergism · Mineral distribution

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1 Introduction

The delicate mineral equilibria between serum and micellar phase in milk are shifted during most of the processes used by the dairy industry such as cooling, acidification, addition of NaCl or other salts, and under heat treatment (Gaucheron 2005). The content and the interactions of calcium with proteins during milk processing are crucial for the final properties of dairy products, and a good understanding is important for future product development. The mineral fraction in milk is composed of calcium, magnesium, sodium, potassium, phosphorus, citrate, and chloride. Calcium and phosphorus play an important role in the milk system because they are part of the colloidal calcium phosphate (CCP) structure which is bound to certain regions of casein micelles and being important for their stability (Gaucheron 2005). Calcium in milk has received considerable attention due to the major contribution of calcium to the stabilization of the casein system during milk processing and during the formation of milk protein gels. During milk acidification, casein micelle integrity becomes affected and casein clusters are formed as building blocks of acid gels (Peng et al. 2009; Lucey 2002). In addition, CCP is solubilized from the casein micelles during acidification (Dalgleish and Law 1989) and calcium, liberated from the casein micelles, can participate in structure formation and influence the final structure and properties of protein gels in milk products. After CCP solubilization, there is a considerable amount of calcium attached to the carboxyl groups of glutamate and aspartate in the micelles which may participate in the structure of acid milk gels by forming bridges between two negative sizes of casein molecules (Dalgleish and Law 1989; van Hooydonk et al. 1986). During heat treatment of milk at elevated temperatures (higher than 40 °C), calcium and phosphorus are transferred from the serum phase to the micellar milk phase and form heat-induced CCP (Holt 1995). The heat-induced CCP connects with the existing CCP in the casein micelles and increases micelle size (Holt 1995). Holt (1995) showed that heat treatment at less than 95 °C for a few minutes will modify the mineral equilibria and that this modification will be reversible at cooling.

Calcium chloride may be added to milk during processing in order to modulate gel formation. Heat treatment of calcium-added milk is, however, challenging from a technological point of view due to the increase in micellar calcium with a concomitant decrease of pH inducing milk gelation (Ramasubramanian et al. 2008; Omoarukhe et al. 2010). At temperature between 4 and 40 °C, for conditions of constant pH, the amount of calcium in serum milk phase is reduced with increasing temperature (Anema 2009b; Koutina et al. 2014), which may be explained by reduction in calcium phosphate solubility with increasing temperature, so-called a reversed solubility, which will lead to calcium phosphate precipitation (Singh 2004). One of the main changes of milk properties during heat treatment to temperatures higher than 65 °C is the denaturation of whey proteins involving whey protein aggregation especially of β-lactoglobulin. β-Lactoglobulin creates complexes via sulfhydryl-disulfide interchange reactions with κ-casein or α-lactalbumin or with other β-lactoglobulin molecules (Vasbinder and de Kruif 2003). Calcium contributes to β-lactoglobulin complex formation through formation of salt bridges with calcium bound to negative groups of β-lactoglobulin (Simons et al. 2002). According to Anema et al. (1993), the extent of κ-casein dissociation from the casein micelles increases with an increase in total calcium content when milk is heated at 120 °C for 6 min (pH between 6.3 and 7.4).

Heat processing of calcium supplemented milk was found to lead to milk coagulation and to formation of a novel type of gel for which the term "calcium-milk coagulum" was coined (Ramasubramanian et al. 2012; Ramasubramanian et al. 2014). The addition of relatively high amounts of calcium salts will increase the ionic calcium concentration and decrease the pH which in combination with structural changes in whey proteins during heat treatment will lead to milk coagulation (Omoarukhe et al. 2010; Ramasubramanian et al. 2012; Vasbinder and de Kruif 2003). Still, the effect of pH on calcium gel formation under heat treatment is not well documented. The main objective of the present study is accordingly to describe the different structures formed by combinations of calcium addition and heat treatment to form calcium gels at pH typical for acidification of skim milk (6.6–4.6). Such a characterization will provide the understanding required by the dairy industry to develop the technology for producing novel dairy products.

2 Materials and methods

2.1 Chemicals

Calcium chloride dihydrate, sodium hydroxide solution, hydrochloric acid, and sodium azide of analytical grade were all from Sigma-Aldrich (St Louis, MO, USA). Fluorescein-5-isothiocyanate (FITC) was from Merck Millipore (Merck KGaA, Darmstadt, Germany). All aqueous solutions were made from deionized water (Milli-Q plus, Millipore Corporation, Bedford, MA, USA).

2.2 pH measurements

pH values in all samples were measured at ambient temperature (22 °C) by a pH meter (pH meter, 766-Calimatic, Knick Berlin-Zehlendorf, Germany) relative to international pH standards (Mettler, Toledo, Hasselager, Denmark).

2.3 Reconstitution of skim milk powder

Instant skimmed milk powder (milk protein 36%, lactose 52%, milk fat max. 1.25%, minerals 8%, and moisture 4%; Arla Foods Ingredients, Viby J, Denmark) was reconstituted in deionized water at a level of 10% (*w/w*) and was stirred overnight at ambient temperature (22 °C) to ensure complete hydration of casein micelles and equilibration of the mineral content. To avoid bacterial growth, sodium azide (0.02%) was added to the reconstituted skim milk. The instant skim milk powder contained native soy lecithin at 0.04% level on powder. The product has whey protein nitrogen index (WPNI) between 1.5 and 6.0 mg undenaturated whey protein nitrogen per gram of nonfat milk powder (medium heat process), and the percentage of native/denatured whey proteins is 50/50. The instant skim milk powder was used because it has better dispersing and reconstitution characteristics than standard skim milk powder. According to Tran Le et al. (2007), native lecithin will not influence the heat-induced protein aggregation. The heat-induced interactions between whey proteins and casein

micelles were highly influenced by hydrolysed and hydroxylated soybean lecithin (Tran Le et al. 2007).

2.4 Milk enrichment with calcium at different pH

The next day, the reconstituted skim milk was divided into smaller samples. Half of the samples was enriched with 30 mM calcium using calcium chloride dihydrate (ECa=enriched with calcium chloride) under stirring for 10 min, while the rest was not enriched (NCa=Non-enriched with calcium chloride). The volume of each sample was 100 mL. After 10 min, the pH of all the samples (ECa and NCa) was adjusted with 1.0 M HCl or 1.0 NaOH to 6.6, 6.0, 5.6, 5.2, 5.0, and 4.6. The dilution caused by the HCl or NaOH was kept constant for all samples by addition of deionized water when requested and then all samples were stirring for 10 min at ambient temperature. Skim milk samples with or without addition of 30 mM calcium chloride dihydrate were heated and then cooled in a water bath using a three-step heating process: first step, 22 to 90 °C in 20 min; second step, 90 °C for 10 min; and third step, 90 to 22 °C in 20 min. Consequently, all samples were left at 22 °C overnight before further analysis.

2.5 Calcium and phosphorus analysis

The separation between the micellar and the serum phase was done after centrifugation of all skim milk samples (Sorvall, RC 6 Plus Centrifuge, Axeb Lab Solutions, Albertslund, Denmark) at 10,000×g for 30 min at 22 °C, using centrifuge tubes fitted with 10-kDa ultrafiltration membranes (Vinaspin 20, GE Healthcare, Bio-Science, AB, Uppsala, Sweden). Minerals that remained in the supernatant will be referred to as serum phase fraction components. The separation was done before the heating process at 90 °C for 10 min and after the heating process at 90 °C for 10 min and overnight storage at 22 °C. Total calcium content was determined in the bulk milk preparation before being divided into samples with different pH levels. Serum contents of calcium were determined in all skim milk (NCa and ECa) and in their serum phase fractions using an atomic absorption spectrometric method (IDF 2007). Micellar calcium was considered as the difference between total and serum calcium. Total phosphorus content was determined in the bulk milk preparation before being divided into samples with different pH levels. Serum contents of phosphorus were determined in all skim milk samples (NCa and ECa) in the serum phase fractions using a standard absorption spectrometric method (IDF 2006). Micellar phosphorus was considered as the difference between total and serum phosphorus.

2.6 Rheological properties

The rheological properties (storage modulus G′) of the skim milk (NCa and ECa) as a function of time and temperature ramp were evaluated using a rheometer (Discovery Hybrid Rheometer HR-2, TA Instrument, Elstree, UK). The rheometer was equipped with concentric cylinder geometry (bob-cup; an outer radius of 14 mm and height of 42 mm) with a temperature-controlled system. The cylinder

was filled up with 10 mL from each sample. The heating process (temperature ramp) in the rheometer was first step, 22 to 90 °C in 20 min with a constant strain of 0.50% and frequency of 1 Hz; second step, 90 °C for 10 min with a constant strain of 0.50% and frequency of 1 Hz; and third step, 90 to 22 °C in 20 min with a constant strain of 0.50% and frequency of 1 Hz. The rheological properties (storage modulus G′) of the final skim milk samples (NCa and ECa), after the heating process at 90 °C for 10 min and overnight storage at 22 °C, as a function of frequency, were evaluated using a rheometer (Discovery Hybrid Rheometer HR-2, TA Instrument, Elstree, UK). The rheometer was equipped with concentric cylinder geometry (bob-cup; an outer radius of 14 mm and height of 42 mm) with a temperature-controlled system. The cylinder was filled up with 10 mL from each sample. A frequency sweep test was performed at 22 °C, and the frequency values were varied from 0.05 to 130 rad/s with a constant strain of 0.50%. All measurements were within the linear viscoelastic region.

2.7 Confocal laser scanning microscopy

In order to observe the protein structure of the final skim milk samples (NCa and ECa), after the heating process at 90 °C for 10 min and overnight storage at 22 °C, the protein-specific fluorescein-5-isothiocyanate (FITC; dissolved in acetone) was used as a fluorescent probe. Approximately 1.0 mL of each sample was placed on a 24×50 coverslip (Gerhard Menzel GmbH, Braunschweig, Germany), mixed with FITC and then was immediately inverted for confocal laser scanning microscopy (CLSM) analysis. The microstructures of all samples were observed by an inverted confocal scanning laser microscopy (Leica DMIRE2, Leica Microsystems, Heidelberg, Germany). The samples were viewed using an oil immersion ×100 lens having a pinhole diameter of 1 Airy unit. The emission filter was set at 500–650 nm for FITC which was excited at a wavelength 488 nm. Micrographs of the samples visualize proteins in green color. The micrographs were taken in size 512×512 pixels, having an average of two frames. Image analysis of CLSM micrographs was performed using ImageJ software (Research Service Branch, National Institute of Health, Maryland, USA). At least three micrographs were obtained for each sample and were analyzed using the ImageJ software. Firstly, the color of the images was spilt into three channels (red, green, and blue) and for further analysis, the green color was chosen because the proteins were visualized as green areas by the microscopy. Secondly, the density of the green color was adjusted and each image was converted using the "black background" function. Finally, the specific function "analyze particles" gave a final percentage of the green color area used as percentage of protein gel for each sample.

2.8 Statistical analysis

One-way ANOVA from the SPSS statistical software (version SPSS 19.0, 2010, IBM Danmark ApS, Kgs. Lyngby, Denmark) was used for handling the data. Paired comparison between means for each parameter was carried out using Tukey's test, when a significant probability was distinguished ($P<0.05$).

3 Results

3.1 Calcium and phosphorus equilibria

After addition of calcium chloride, the samples were stirring for 10 min before the pH adjustment. In our preliminary experiments, samples stirred for 24 h showed comparable final structure as samples stirred for 10 min and for practical reasons, the 10-min trials were used. Table 1 shows the concentration of micellar and serum calcium and micellar and serum phosphorus in skim milk before heating (Table 1a) and after the heating

Table 1 Concentration of micellar and serum calcium (mM) and micellar and serum phosphorus (mM) of skim milk samples at different pH at 22 °C

Sample	pH	Micellar Ca	Serum Ca	Micellar P	Serum P
a					
NCa	6.6	17.8±0.8^{a}	8.8±0.8^{a}	18.9±0.8^{a}	9.3±0.8^{a}
NCa	6.0	14.3±2.5^{b}	12.3±2.5^{b}	17.3±1.20 ^{a}	10.9±1.2^{a}
NCa	5.6	11.6±0.8^{b}	15.0±0.8^{b}	13.3±0.3^{b}	14.9±0.3^{b}
NCa	5.2	7.1±1.6^{c}	19.5±1.6^{c}	10.1±0.4^{c}	18.1±0.4^{c}
NCa	5.0	6.8±0.6^{c}	19.8±0.6^{c}	8.8±0.5^{c}	19.3±0.5^{c}
NCa	4.6	1.4±0.02^{d}	25.2±0.02^{d}	7.0±0.2^{d}	21.2±0.2^{d}
ECa	6.6	32.2±4.0^{a}	24.5±4.0^{a}	15.1±0.7^{a}	12.7±0.7^{a}
ECa	6.0	24.0±1.0^{a}	32.7±1.0^{a}	13.6±0.5^{a}	14.2±0.5^{a}
ECa	5.6	19.8±3.0^{b}	36.9±3.0^{b}	12.6±0.7^{b}	15.1±0.7^{b}
ECa	5.2	12.8±2.1^{b}	43.9±2.1^{b}	11.5±0.9^{b}	16.2±0.9^{b}
ECa	5.0	4.2±5.1^{c}	52.5±5.1^{c}	9.8±0.1^{c}	18.0±0.1^{c}
ECa	4.6	2.8±0.01^{c}	53.9±0.01^{c}	2.1±0.01^{d}	25.7±0.01^{d}
b					
NCa	6.6	16.0±0.1^{a}	10.6±0.1^{a}	14.6±0.4^{a}	13.6±0.4^{a}
NCa	6.0	13.6±0.9^{a}	13.0±0.9^{a}	12.5±0.3^{b}	15.7±0.3^{b}
NCa	5.6	10.0±0.4^{b}	16.6±0.4^{b}	10.5±0.5^{c}	17.7±0.5^{c}
NCa	5.2	5.9±0.6^{c}	20.9±0.6^{c}	8.7±0.1^{d}	19.5±0.1^{d}
NCa	5.0	4.0±0.6^{c}	22.6±0.6^{c}	7.7±0.2^{d}	20.5±0.2^{d}
NCa	4.6	1.3±1.1^{d}	25.3±1.1^{d}	7.5±0.2^{d}	20.7±0.2^{d}
ECa	6.6	28.5±0.6^{a}	28.2±0.6^{a}	22.4±1.2^{a}	5.4±1.2^{a}
ECa	6.0	20.5±1.6^{b}	36.1±1.6^{b}	18.8±0.7^{b}	9.0±0.7^{b}
ECa	5.6	13.6±0.5^{c}	43.0±0.5^{c}	16.0±0.6^{b}	11.7±0.6^{b}
ECa	5.2	9.4±1.2^{d}	47.3±1.2^{d}	11.8±0.7^{c}	16.0±0.7^{c}
ECa	5.0	6.5±0.2^{d}	50.2±0.2^{d}	8.9±0.5^{c}	18.8±0.5^{c}
ECa	4.6	5.0±0.5^{f}	51.7±0.5^{f}	5.9±1.2^{d}	21.9±1.2^{d}

(a) Before heating process and (b) after the heating process at 90 °C for 10 min and overnight storage at 22 °C. Means±standard deviation with different letters for each parameter at different pH differ significantly ($P<0.05$). Total calcium (mM) NCa 26.6±0.3 and ECa 56.7±2.6. Total phosphorus (mM) NCa 28.2±1.0 and ECa 27.8±0.1

NCa non-enriched with calcium chloride, *ECa* enriched with calcium chloride

process at 90 °C for 10 min and overnight storage at 22 °C (Table 1b), as mean±SD of two independent replicates. During milk acidification, the calcium and phosphorus equilibria between the micellar and the serum phase are disturbed and due to solubilization of colloidal calcium phosphate (CCP), more calcium and phosphorus are transferred from the micelles into the serum milk phase (van Hooydonk et al. 1986; Dalgleish and Law 1988). The increase in concentrations of serum calcium and phosphorus (Table 1), resulting from the pH decrease of skim milk (NCa and ECa) from 6.6 to 4.6, occurred with a concomitant decrease in the concentration of micellar calcium and phosphorus (Table 1). For decreasing pH, the addition of calcium chloride (30 mM) to enriched skim milk (ECa) skim milk (Table 1) caused an increase in both micellar and serum calcium which were more pronounced for the serum milk fraction at pH lower than 5.6. For skim milk samples enriched with calcium chloride (ECa) before heating (Table 1a), slightly more phosphorus was observed in the serum phase than for non-enriched (NCa) skim milk. For skim milk samples enriched with calcium chloride (ECa) after the heating process at 90 °C for 10 min and overnight storage at 22 °C (Table 1b), less phosphorus was observed in the serum phase than for non-enriched calcium chloride (NCa) skim milk.

3.2 Dynamic rheology of calcium induced gels

An example of the storage modulus (G′) evolution as a function of time and temperature ramp for a representative skim milk sample (ECa; pH 6.6) can be seen in Fig. 1. Table 2 shows the time period (min) and temperature (°C) of the gelation point of skim milk at different pH (NCa and ECa). As can be seen from Fig. 1, in the beginning of the dynamic measurements, G′ as a function of time, the system is still liquid as evidenced by a lag phase (straight line box). However, when the first aggregates start to be formed, a sudden increase (gelation point) can be observed (Fig. 1; dash line box) which growths more as a firmer gel is being produced until reaching a plateau (Fig. 1; dot line box) (Ercili Cura et al. 2009). In conclusion, all samples (NCa and ECa) reached the gelation point during the first step of the heating process, as can be seen in Table 2 (first step, 22 to 90 °C in 20 min). For non-enriched (NCa) samples, the temperature and the time to reach the gelation point were decreasing as pH was decreased (Table 2), while for skim milk samples enriched with calcium chloride (ECa), the time and the temperature of the

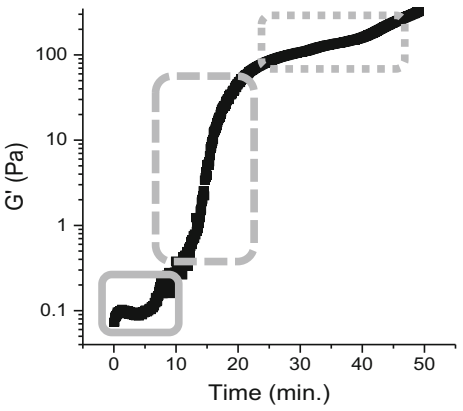

Fig. 1 Storage modulus (G′) as a function of time and temperature ramp of a representative skim milk sample (ECa pH 6.6). The three different boxes refer to the three different regions during the formation of a milk gel: *box with straight line*, initial lag phase; *box with dash line*, rapid increase phase; and *box with dot line*, plateau phase

Table 2 The time period (in minutes from the initiation of the first step) and temperature (°C) of the gelation point of the skim milk samples at different pH

Sample	pH	Time (minutes)	Temperature (°C)
NCa	6.6	No gel formation	No gel formation
NCa	6.0	No gel formation	No gel formation
NCa	5.6	17.0±0.1[a]	81.0±0.1[a]
NCa	5.2	10.0±0.1[b]	55.0±0.1[b]
NCa	5.0	7.8±0.4[c]	49.5±0.7[c]
NCa	4.6	Gelation prior to heating	Gelation prior to heating
ECa	6.6	13.0±0.1[a]	70.0±0.1[a]
ECa	6.0	12.8±0.5[a]	67.0±1.8[a]
ECa	5.6	12.7±0.4[a]	67.0±1.3[a]
ECa	5.2	12.2±0.4[a]	65.0±1.2[a]
ECa	5.0	11.7±1.1[a]	63.0±3.7[a]
ECa	4.6	5.5±0.1[b]	42.0±0.1[b]

The heating process in the rheometer was first step, 22 to 90 °C in 20 min; second step, 90 °C for 10 min; and third step, 90 to 22 °C in 20 min. Means±standard deviation with different letters for each parameter at different pH differ significantly ($P<0.05$)

NCa non-enriched with calcium chloride, *ECa* enriched with calcium chloride

gelation point were between 13 and 12 min and 70 and 60 °C for pH values between 6.6 and 5.0 and 5.5 min and 42 °C for pH 4.6.

3.3 Rheology and microscopy of calcium-induced gels

Figure 2 shows G′ as a function of frequency (rheology properties) as the mean±SD of two independent replicates, and Fig. 3 shows representative microscopy images of skim milk gels at different pH at 22 °C (NCa and ECa).

Non-enriched calcium chloride (NCa) samples having pH 6.6 and 6.0 were not able to form a gel because at this high pH, casein micelles have high negative charge and repulsion forces prohibit formation of gel networks (Zoon et al. 1989). As pH is reduced from 6.0 to 5.4, dissociation of the CCP occurs and caseins will start to form loosely entangled aggregates (Fig. 3). The microscopy images (Fig. 3a) of non-enriched calcium chloride (NCa) samples show a network that becomes denser as the pH is decreased, which was confirmed by image analysis showing that approximately 20% of partial area at pH 6.6 increased to 29% partial area at pH 4.6. These samples all had macroscopic properties as gels.

Skim milk samples enriched with calcium chloride (ECa) is from Fig. 3 seen to form a gel network at all pH values investigated, and from the rheological data, it is further concluded that the gels become stronger with decreasing pH (Fig. 2). In addition, the rheological data (Fig. 2) shows that enriched with calcium chloride samples (ECa) gave stronger gels (higher G′ values) than non-enriched calcium chloride (NCa) samples from pH 6.6 to 5.2. In contrast, at pH 5.0, the non-enriched calcium chloride (NCa) samples had higher G′ values than enriched with calcium chloride samples (ECa), while at pH 4.6, the G′

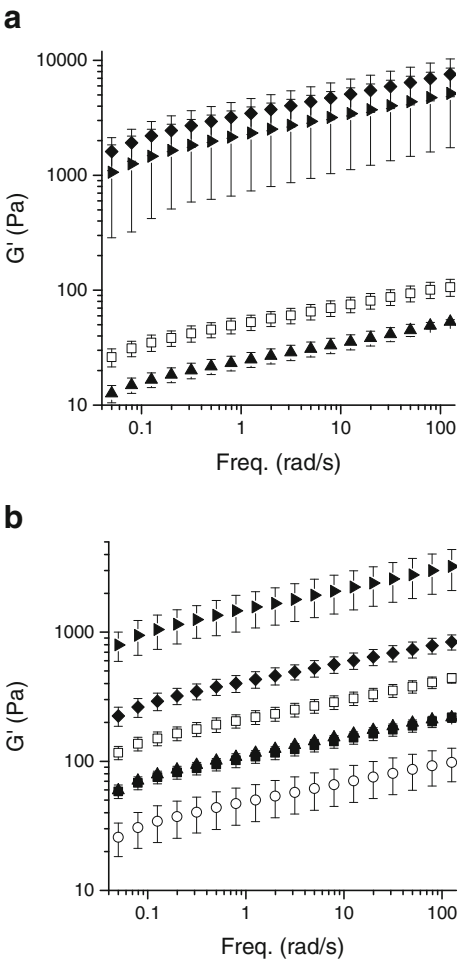

Fig. 2 Storage modulus (G′) as a function of frequency of skim milk at different pH at 22 °C. **a** NCa=non-enriched with calcium chloride. **b** ECa=enriched with calcium chloride. The symbols in the two diagrams referring to the following pH values: pH 6.5 (*black square*), 6.0 (*white circle*), 5.6 (*black triangle*), 5.2 (*white square*), 5.0 (*black diamond*), and 4.6 (*triangle pointing to the right*). For the NCa=non-enriched with calcium chloride (**a**), the samples with pH 6.5 and 6.0 are not included in the figure

values of non-enriched calcium chloride (NCa) and enriched with calcium chloride (ECa) samples were almost the same. The microscopy images (Fig. 3b) of enriched with calcium chloride (ECa) samples show a network that becomes less dense as pH is decreasing, and image analysis confirmed this tendency with partial areas of 42% at pH 6.6 and 28% at pH 4.6. These samples all had macroscopic properties as gels.

4 Discussion

4.1 Calcium and phosphorus distribution in calcium gels at different pH

As is clearly seen from Table 1, both the calcium and phosphorus distribution between the micellar and serum phase are highly affected by acidification of milk, confirming the results of previous studies (Anema 2009a; Anema 2009b; Dalgleish and Law 1989;

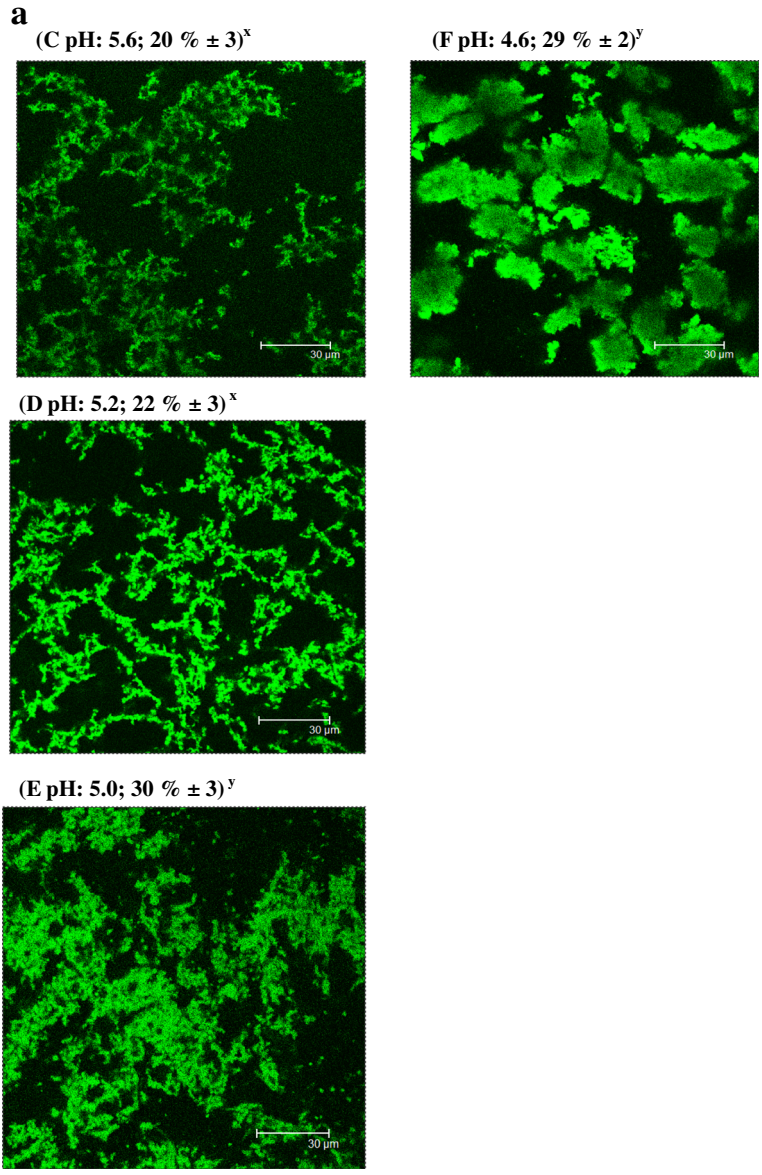

Fig. 3 CLSM micrographs showing the microstructure of skim milk samples at different pH (*A*, pH 6.6; *B*, pH 6.0; *C*, pH 5.6; *D*, pH 5.2; *E*, pH 5.0; *F*, pH 4.6) at 22 °C; **a** NCa=non-enriched with calcium chloride; **b** ECa=enriched with calcium chloride. The FITC-stained protein appears green in all micrographs. *Information inside the parenthesis* indicate the pH of each sample encode with letters, and the particle area from image analysis using ImageJ software, as mean from three independent samples (%)±standard deviation. Means with different letters differ significantly ($P<0.05$). For the NCa=non-enriched with calcium chloride (**a**), the samples with pH 6.5 and 6.0 are not included in the figure

Koutina et al. 2014; Mekmene et al. 2010; van Hooydonk et al. 1986; Visser et al. 1986). In addition, enrichment of milk with calcium chloride is seen to increase both

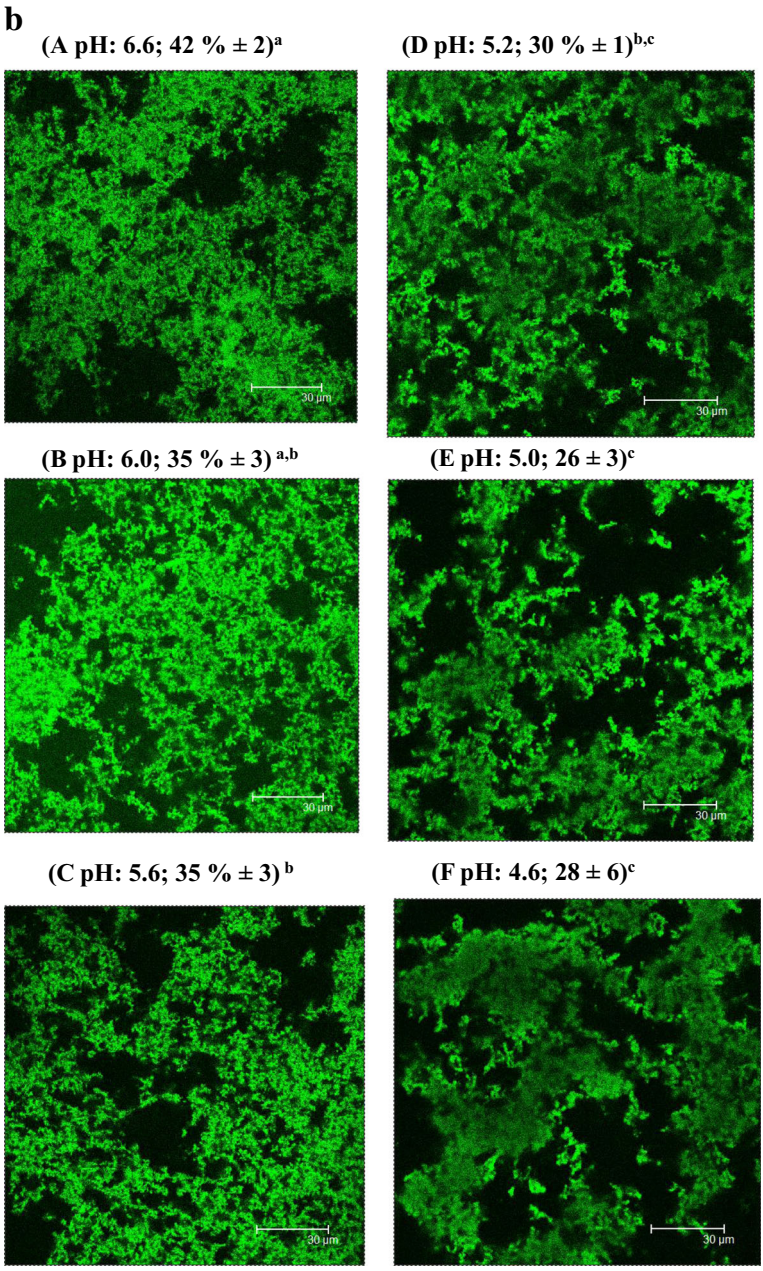

Fig. 3 (continued)

micellar and serum calcium, but for pH lower than 5.6, the majority of added calcium remained in the serum phase.

As is further seen from Table 1a, the addition of calcium chloride to skim milk (ECa) causes a slight increase of phosphorus in the serum phase, while as seen from Table 1b, the addition of calcium chloride (ECa) cause a decrease of

phosphorus in the serum phase, after heating (Famelart et al. 1999; Koutina et al. 2015; Udabage et al. 2000). The decreasing concentration of phosphorus in the serum phase after addition of calcium chloride and heating (Table 1b) can be understood from the low solubility of calcium phosphate in the serum phase upon heating and the tendency to form supersaturated solutions (Mekmene et al. 2010). The decreased amount of phosphorus in the serum phase following calcium addition and heating seems to indicate that the added calcium binds serum phosphorus and that calcium and phosphorus together enter the micellar phase causing casein micelles to aggregate. The difference in mineral distribution between skim milk (ECa) before heating (Table 1a) and after the heating process at 90 °C for 10 min and overnight storage at 22 °C (Table 1b) can be explained by the shorter time for equilibration in the non-heated skim milk (less than 30 min) compared to heated samples (overnight storage). The decreasing solubility of calcium phosphate at increasing temperature further contributes to this effect.

4.2 Rheology and microscopy of calcium gels at different pH

For non-enriched calcium chloride (NCa) samples, the temperature and the time to reach the gelation point were found to decrease as pH decreased (Table 2). The combination of heating with acidification results in formation of aggregates leading to formation of a final gel network. Heat is known to denaturate whey proteins and further to result in binding of whey proteins to κ-casein on the surface of casein micelles (Vasbinder and de Kruif 2003). These binding interactions will not interrupt the removal of CCP from the casein micelles during acidification, but the dissociation of caseins from the micelles will be reduced especially at temperature around 20 °C, as was used in this study (Singh et al. 1996; Law 1996; Dalgleish and Law 1988).

For enriched with calcium chloride (ECa) samples, the time and the temperature of the gelation point was around 13–12 min and 70–60 °C for pH values between 6.6 and 5.0 and 5.5 min and 42 °C for pH 4.6. Addition of calcium chloride is known to make whey proteins more sensitive to denaturation and aggregation (Pappas and Rothwell 1991), and for all skim milk samples enriched with calcium chloride (ECa), the gelation point was reached at temperatures between 60 and 70 °C, in which temperature range the whey proteins are known to denaturate (de Wit and Klarenbeek 1984). At pH 4.6, the gelation point was reached faster and at lower temperature compared to higher pH probably because the high affinity for casein to casein bonds results in casein gelation as this low pH corresponding to the isoelectric point of caseins (Lucey and Lee 2004; Walstra and Jenness 1984). The pH decrease will increase the solubilization of CCP and calcium and phosphorus will dissolve from the casein micelles causing partial rearrangement of the inner structure of casein micelles to form a final acid gel network (Lucey 2002).

For skim milk samples not enriched with calcium chloride (NCa), the changes in microstructure were reflected in an increase in G′ value for the final milk gels (Figs. 2 and 3). At pH between 5.3 and 4.9 loosely entangled aggregates of proteins form more compact particles, while at pH lower than 4.8, caseins aggregated rapidly into a gel network consisting of chains of compact spherical particles (Famelart et al. 2004). In addition, acid gels form a particular heterogeneous structure consisting of fairly large clusters with holes as seen also in Fig. 3a F (Roefs et al. 1990). These observations are

confirmed by microscopy in the present study as seen from the microscopy images and further reflected in the rheological profile of the skim milk samples not enriched with calcium chloride (NCa).

For the skim milk samples enriched with calcium chloride (ECa; Figs. 2 and 3), the addition of calcium chloride in combination with heat treatment causes the formation of a gel network independent of pH (Ramasubramanian et al. 2013; Ramasubramanian et al. 2014). Heating of milk causes denaturation of whey proteins and attachment to the surface of the casein micelles through the κ-casein (Vasbinder and de Kruif 2003). Consequently, denatured whey proteins become sensitive to aggregation as the net charge of the proteins is decreased leading to less repulsion. The denatured whey proteins form linkages to whey proteins already bound to casein micelles which increasing the number and the strength of protein bonds in particles leading to a final gel network (Lucey et al. 1997). In addition, heated whey proteins are sensitive to aggregation in the presence of calcium ions causing the formation of a firm gel network as was confirmed by the higher G′ values (Fig. 2) of skim milk samples enriched with calcium chloride (ECa) compared to non-enriched calcium chloride (NCa) samples in the present study (Hongsprabhas and Barbut 1996; Simons et al. 2002).

At high pH (6.6–5.6), a fine gel structure was observed with negligible whey separation for enriched with calcium chloride (ECa) samples. Moreover, for enriched with calcium chloride (ECa) samples, the majority of added calcium remained in the serum milk phase at pH 5.2–4.6, while at pH higher than 5.2, the added calcium was distributed more uniformly between the micellar and serum phases (Table 1). Addition of calcium in milk will accordingly cause an increase in the level of CCP available as a micellar-connecting agent forming calcium bridges between caseins. At pH around and below 5.2, CCP become completely solubilized and the majority of added calcium remains in the serum phase causing fewer rearrangements for the formation of a gel network. Consequently, at pH lower than 5.6, the effect of pH is dominating in relation to calcium addition for gel formation during milk heating.

5 Conclusion

A fine gel network has been demonstrated to be formed by addition of 30 mM calcium chloride to skim milk followed by pH adjustment to values between 6.6 and 4.6 and heat treatment at 90 °C for 10 min before storage at 22 °C overnight. In contrast for pH lower than 5.6, the effect of pH dominates over the effect of calcium chloride addition on protein gel formation. The formation of a gel network with little whey separation at relative high pH (6.6 to 5.6) was obtained by the combination of calcium addition and heat treatment. Such types of gel should be further investigated for production of new yogurt-like dairy products with high pH.

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