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ORIGINAL PAPER

Post-processing of concentrated fermented milk: influence of temperature and holding time on the formation of particle clusters

Christian Hahn · Martin Sramek · Stefan Nöbel · Jörg Hinrichs

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Abstract Graininess is a common structural defect in microgel suspensions, e.g., yogurt or fresh cheese. The aim of this study was to investigate the impact of the temperature (23–54 °C) and the holding time (1–300 min) on the formation of particle clusters in concentrated, fermented milk (protein content 8.2% (*w*/*w*)) during post-processing. Since graininess is correlated with the presence of large protein aggregates, temperature treatment during post-processing was varied systematically to promote particle growth and the particle size $d_{75.3}$ was measured. The samples revealed a polydisperse particle size distribution present at all temperatures and holding times. With longer holding times, rearrangement led to larger particle clusters while the number of smaller particles decreased. The increase in the $d_{75.3}$ was fitted with a power law function while higher temperatures promoted both the aggregation rate and the particle size. By using the Arrhenius equation, the activation energy, E_A, was calculated (26 kJ.mol^{-1}), which was in agreement with the aggregation kinetics occurring at smaller scales, e.g., the acid-induced aggregation of casein micelles and the temperature-induced aggregation of casein submicelles. According to the activation energy, the particle growth in microgel suspensions was proposed to be predominantly diffusion-limited. The results of this study imply that particle size during post-processing is adjustable. Furthermore, the particle size increases with increasing temperature load, thus, rapid cooling reduces the

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aggregation rate and stops particle growth. The results give manufacturers a useful tool to control the graininess that affects the structure and the sensory properties.

浓缩发酵乳后处理过程中温度和保持时间对颗粒簇形成的影响

摘要 颗粒态是凝胶微粒类产品(酸奶或新鲜干酪)中普遍存在的质构缺陷。加工后期的温度(23~54 °C)和保持时间(1~300 min)影响浓缩发酵乳(蛋白含量为8.2%)中颗粒簇的形成。颗粒是由蛋白质聚集后形成的凝聚物。本文系统地测定了加工后期随着温度的逐渐升高,蛋白颗粒直径的增加。结果表明在所选定的温度和时间范围内,颗粒直径的分布较分散。随着保持时间的延长,由于发生了颗粒的重新分配作用,较大颗粒簇的数量增加,而小颗粒的数量减少。$d_{75.3}$的增加是能量的函数,也就是高温既能增加颗粒的凝聚速率又能增加颗粒粒度。根据阿雷尼乌斯方程计算出活化能E_A为26 kJ.mol^{-1},其与在酪蛋白胶束的酸凝聚和酪蛋白亚胶束的热凝聚过程中发生的凝聚反应动力学一致。根据活化能,在凝胶微粒类产品中颗粒的产生是由于颗粒扩散运动受到限制的原因。研究结果说明后加工过程完全可以控制颗粒大小。此外,颗粒大小与凝胶微粒所承受的温度呈正相关,因此,快速冷却可以降低颗粒凝聚速率和终止颗粒的增长。该研究结果可以在工业生产中用于控制凝胶颗粒的形成和保证产品的质构和感官质量。

Keywords Fresh cheese · Growth kinetics · Particle size · Graininess · Aggregation

关键词 新鲜干酪 · 生长动力学 · 颗粒大小 · 颗粒状 · 凝聚

1 Introduction

Most types of cultured products are supposed to have a smooth and uniform body free from lumps or graininess. Lumpiness refers to the presence of large protein aggregates ranging from 1 to 5 mm in the yogurt or fresh cheese (Lucey and Singh 1997). During bacterial fermentation, the lactic acid produced by the starter culture reduces the natural pH of bovine milk (6.7). Thus, the native casein micelles in the range of 100–300 nm successively lose their repulsion forces, aggregate to clusters, and form a three-dimensional network. In the milk gel, the clusters fuse to roughly homogeneous strands and cannot be (well) distinguished anymore (van Vliet et al. 2004). According to the rheological properties, fermented milks can be regarded as weak gel networks (Lucey et al. 1997a; van Vliet et al. 1991). By stirring, this gel is broken down into more or less stable microgel particles suspended in the whey that conserve the properties of the initial gel (Renan et al. 2009) and that have sizes ranging from 1 to 100 μm (Ciron et al. 2010; Weidendorfer and Hinrichs 2010). For some cultured products like fresh cheese or curd cheese, further processing results in whey separation to obtain a concentrated microgel suspension. Depending on the process parameters applied, this concentration step results in a classification of the particle clusters due to the mechanical load and is prone to promote particle growth, as first described by Bäurle et al. (1984).

However, the particle size affects not only the microstructural and rheological properties but also the sensory perception (Ciron et al. 2010; Janhøj et al. 2006; Kealy 2006). For instance, the sensory perception of creaminess in low-fat yogurt was found to be dependent on the oral viscosity and oral smoothness. Particles

exceeding approximately 150 μm negatively affected the smooth perception and were sensorically recognized, even in marginal volumetric fractions (Cayot et al. 2008; Sainani et al. 2004). Sainani et al. (2004) induced the formation of large particle clusters during cream cheese production by tempering (55 °C for 30 min), isolated them by consecutive washing with distilled water at 25 °C and 50 °C repeatedly four to five times after storage at 10 °C, and studied the effect on the graininess. This implies that at least some large particle clusters are resistant to stirring and diluting in distilled water and can produce the grainy defect. Therefore, it is crucial to generate knowledge about the underlying mechanism of particle growth during post-processing which, in turn, can enable manufacturers to control microgel particle growth in fermented milk products.

The manufacture of concentrated fermented milk is subdivided into two main processing steps: fermentation and concentration (Hinrichs 2001). Up to now, some work describing factors that promote the particle growth in dairy products during pre-processing and fermentation has been published: regarding a decreasing fat content and an increasing protein content (Ciron et al. 2010; Janhøj et al. 2009; Johansen et al. 2008; Rasmussen et al. 2007), a decreasing dry matter of milk (Küçükçetin et al. 2008), a decreasing heating temperature and a decreasing casein to whey protein ratio (Küçükçetin 2008; Lucey and Singh 1997; Puvanenthiran et al. 2002), the choice of cultures (Küçükçetin et al. 2009; Sodini et al. 2005), the fermentation conditions (Aichinger et al. 2003; Lucey and Singh 1997), the final pH (Johansen et al. 2008), or the rennet activity (Aichinger et al. 2003). At the present time, stabilizers are often empirically added in order to correct structural defects like graininess or syneresis (Lucey and Singh 1997). Nevertheless, adding starch to stirred skim milk yogurt has also been shown as being capable to create a grainy texture (Williams et al. 2004). Less work has been published about the factors promoting the particle growth during the post-processing of the fermented milk and involving the concentration step, e.g., with regards to the temperature during membrane filtration (Bäurle et al. 1984) or temperature–time load of, e.g., cheese spread (Modler et al. 1989), camembert (Stehle 1980), and cottage cheese (Rosenthal et al. 1996). However, what is essential to control the particle growth beyond the mentioned process parameters is to understand the underlying mechanism of particle growth. In this context, it was the aim of the present study to investigate the mechanism of particle growth in microgel suspensions during post-processing based upon the main influencing parameters found in literature. For this study, fresh cheese was chosen as microgel suspension.

2 Materials and methods

2.1 Materials

Raw milk was obtained freshly from the Meiereihof (Universität Hohenheim) and skimmed to a fat content <0.1% (*w*/*w*). After pasteurization at 74 °C for 30 s with a plate heat exchanger (1,000 $L.h^{-1}$, Südmo Holding GmbH, Riesbürg, Germany), the milk was cooled to 6 °C and stored overnight (6 °C). The skimmed milk was standardized with a permeate solution (5.2% *w*/*w*) to adjust the protein content at 3.4±0.1% (*w*/*w*),

gently stirred for 30 min and, afterwards, continuously heated at 95 °C for 4.3 min in a tubular pilot plant (150 $L.h^{-1}$, ASEPTO-Therm, Asepto GmbH, Dinkelscherben, Germany). The permeate solution was reconstituted from permeate powder Bayolan PT (BMI eG, Landshut, Germany) with distilled water. The heated milk (180 L) was cooled to the fermentation temperature (20 °C), pumped directly in a 200-L sterile tank (Südmo Holding GmbH, Riesbürg, Germany), and allowed to equilibrate for 1.5 h.

2.2 Fresh cheese production

Fermentation was carried out overnight at 20 °C by suspending 0.02% (*w*/*w*) F-DVS CC06 (Chr. Hansen GmbH, Nienburg, Germany), provided as frozen pellets and containing *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* in the tempered milk. According to the manufacturer, the cultures (type O) produced neither CO_2 nor exopolysaccharides. When the pH reached a pH of 6.50, 1 mL per 100 L Chy-Max™ (Chr. Hansen GmbH, Nienburg, Germany; minimum activity 190 IMCU per mL) was added, and the fermentation proceeded until the pH reached 4.50±0.03 (n=12). The gel was gently mixed with a stirring device (Stelzer Rührtechnik International GmbH, Warburg, Germany), which was mounted in the sterile tank and consisted of three Zeta mixer elements (diameter 35 mm) fixed to the agitator shaft with a distance of 25 mm, while increasing the rotational number from 30 to 90 rpm within 10 min. The microgel suspension was pumped through a tubular heat exchanger with an impeller pump (Kiesel GmbH, Heilbronn, Typ IP 2), tempered to 38 °C (volume flow 300 $L.h^{-1}$), and directly transferred to the storage tank of the cross flow membrane filtration device (Pall Seitz Schenk, Membralox, cut off 0.1 μm, Germany) by means of a tube (inner diameter 16 mm). The microgel suspension was concentrated to a final protein content of 8.22±0.23% *w*/*w* (n=12), measured using the method of DUMAS. Filtration temperature, tangential velocity, and transmembrane pressure were kept constant at 38±1 °C, 7.0±0.2 $m.s^{-1}$, and 100± 10 kPa, respectively. Due to the mechanical load during filtration, a classification of the particles regarding the particle size may have taken place. Thus, the measured particle size reflected the whole parameters of processing including fermentation and concentration. The concentrated microgel suspensions were filled into 100 mL cylindrical glasses and tempered at 23.0±0.5 °C, 38.0±0.5 °C, 45.0±0.5 °C, or 54.0±0.5 °C for 1, 30, 60, 100, or 300 min to facilitate particle growth, which was stopped by putting the samples into a water bath (6.0±0.3 °C). The samples were stored overnight (6.0±0.3 °C) until the particle size distribution was measured. The experiments were conducted five times for each temperature.

2.3 Particle size measurement

The aim of the present study was to investigate the formation of large particle clusters potentially resistant to stirring and likely to induce the grainy defect, as described by Sainani et al. (2004). The particle size distribution of differently tempered microgel suspensions was measured by static laser light scattering with a Small Volume Module Plus LS230 (Beckman-Coulter Inc., Miami, USA), modified according to Ciron et al. (2010). The samples (6 °C) were gently stirred in the glass five times with a spoon, and 3.0 g was diluted in 47 g of distilled water; additionally, to analyze the effect of

the solvent, permeate was used to dilute the samples. In contrast to the reconstituted permeate solution (5.2% *w*/*w*), which was used to standardize the protein content in the skimmed milk, this permeate was obtained after the fermentation from the cross flow membrane filtration device, and stored at 6.0±0.3 °C until the measurement was carried out. Thus, the permeate represented the aqueous phase of the microgel particles. After stirring on a magnetic stirrer (15 min, 150 rpm), the suspensions were transferred to a measuring cell containing distilled water. When the obscuration reached 14–16%, three consecutive runs (each 60 s) were conducted, and the $d_{75.3}$ was taken from the average curve (protein and water refractive index 1.75 and 1.33, respectively). The volume-weighted diameter $d_{75.3}$ was chosen to reveal the particle growth of large particle clusters and represents the diameter below which 75% and beyond which 25% of the volume of the particles is found. Table 1 shows the $d_{75.3}$ when either distilled water or permeate taken from the membrane filtration was used for dilution and measuring purpose. There was no significant difference in the $d_{75.3}$ for both solvents. In addition, the standard deviation of the process for all samples was higher than for each measurement. Note that the particle size distribution reflects both the temperature treatment and the mechanical impact during concentration and measuring and is therefore related to those particle clusters formed during tempering and being resistant to stirring.

2.4 Confocal laser scanning microscopy

To visualize the temperature-induced particle growth, particles were washed out by gently mixing 0.5 g of the tempered microgel suspensions with 9.5 g distilled water in a Petri dish. Rhodamine B (Merck, Darmstadt, Germany) was used as the protein dye (λ_{ab}=543 nm, λ_{em}=590 nm), and a Rhodamine B solution (0.016% *w*/*w*) was prepared with distilled water (Heilig et al. 2009). The washed out particles (650 μL) were transferred in a custom-made plexiglass specimen holder, and 20 μL Rhodamine B solution was added and evenly distributed. The specimen was covered with a cover slip, fixed with nail polish, and all samples were stored for at least 1 h in a light-proof refrigerator at 10 °C before pictures were taken at 10 °C. For confocal laser scanning microscopy (CLSM), a microscope (Type: Eclipse-C1,

Table 1 Particle size $d_{75.3}$ of the microgel suspension taken directly after the concentration step depending on the solvent used for the sample preparation and the measurement

$d_{75.3}$	Distilled water (μm)		Permeate (μm)	
	Measurement 1	Measurement 2	Measurement 1	Measurement 2
Run 1	48.6	48.2	49.6	47.6
Run 2	44.4	44.7	45.3	44.0
Run 3	41.6	41.5	41.8	40.5
	44.9±3.6	44.8±3.4	45.6±4.0	44.0±3.6
Mean value of all productions				35.8±7.5

The permeate was taken from the membrane filtration and stored at 6 °C before the measurements were carried out at room temperature (~23 °C). The values represent the mean value and the standard deviation

Nikon GmbH, Düsseldorf, Germany) equipped with a 20× and 63× oil immersion objective was used. Microscope settings were controlled with the software Nikon-E-CZ1 (Nikon GmbH, Düsseldorf, Germany). The temperature of the samples was kept constant at 10 °C by a water-cooled tempering unit, image resolution and pinhole were set at 1,024×1,024 pixels and 30 μm, respectively, and the average mode of two pictures was used.

3 Results

According to literature, the main parameters affecting particle growth are protein content, temperature, and holding time. For this study, the protein content was kept constant at 8.22% (*w*/*w*). During the first step, the temperature and the holding time during post-processing were varied on three and five levels, respectively, and the kinetic data (k_T, E_A) were calculated. During the second step, independent of the first experiments, the microgel suspension was tempered on a fourth temperature level to confirm the kinetic data from step one.

3.1 Influence of the holding time

The influence of the holding time on particle growth in microgel suspensions is exemplarily shown in Fig. 1 for the microgel suspensions tempered at 38 °C for 1 and 300 min. The microgel suspension tempered for 1 min revealed a polydisperse particle size distribution composed of two distinct and one evolving peak which we subdivided in three particle classes: 0–40 (class I), 40–100 (class II), and >100 μm (class III). The size range of these three particle classes was nearly independent of the holding time for all temperatures (not shown). However, the relative volume in these particle classes changed with the holding time; with longer holding times, the relative volume of class I decreased. In contrast, the relative volume of class II and class III increased for the 300 min tempered microgel suspension, indicating that large particle clusters have been grown at the expense of smaller ones.

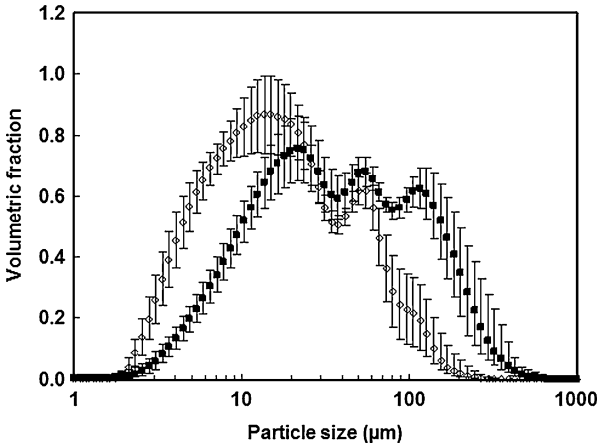

Fig. 1 Particle size distribution of microgel suspensions depending on the holding time. The protein content was 8.22% (*w*/*w*), tempered at 38 °C for 1 (*diamond*) and 300 min (*filled squares*). The particle size distribution reveals three particle classes independent of the holding time: 0–40 (class I), 40–100 (class II), and >100 μm (class III). *Values* and *error bars* represent the mean value and the standard deviation of five independent trials

Figure 2 depicts the washed out particles visualized by confocal laser scanning microscopy. The size range of the washed out particles supported the particle size measurement (Fig. 1). A closer look showed that the washed out particles consisted of smaller spherical building blocks having a diameter of 1–5 μm; these were suggested to be the aggregated casein micelles (Chardot et al. 2002). In addition, especially the larger particle clusters (~100 μm) seemed to be composed of several smaller particle clusters (10–20 μm), which in turn were composed of the building blocks (1–5 μm), supporting the assumption that smaller particles are the sub-elements of larger particle clusters. This is in accordance with Mellema et al. (2002) who stated that building blocks consisting of fused casein micelles aggregate to particle clusters. Developing this concept further for our microgel suspensions, we propose that after stirring microgel particles (particle clusters) are generated, which potentially may aggregate during post-processing.

3.2 Influence of temperature

The impact of temperature on particle growth is shown in Fig. 3. For all temperatures applied, there was the same pattern of particle growth; at the beginning, the $d_{75.3}$ increased rapidly with the holding time, followed by a gradually decelerated increase of the $d_{75.3}$. The higher the temperature was, the higher was both the increase of the $d_{75.3}$ with time and the final particle size. In detail, the $d_{(75.3)t=1\ \mathrm{min}}$ for all temperatures was 35.8±7.5 μm, and the $d_{(75.3)t=300\ \mathrm{min}}$ gained 61±16, 92±24, and 174±16 μm at the temperatures 23 °C, 38 °C, and 54 °C, respectively.

Figure 4 shows the corresponding washed out particles. The tempering tended to result in both a reduction in the number of particles and an increase in particle size with the higher temperatures having a greater impact. However, the particles tempered at 54 °C (Fig. 4B) seemed to differ in their inner structure. While particles at 38 °C (C) and 23 °C (D) revealed different parts that stayed aggregated after 300 min despite of the washing out step, the particles at 54 °C (B) were bigger in size and looked more homogeneous. Additionally, with increasing temperature two different areas of the particle clusters showing different emitted

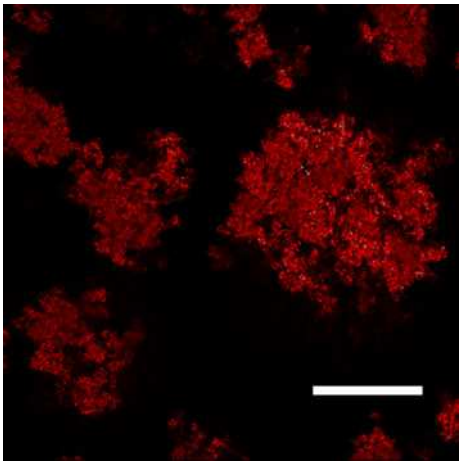

Fig. 2 Particles in tempered microgel suspensions representing three particle classes: 0–40 (class I), 40–100 (class II), and >100 μm (class III). Particles were washed out after tempering at 38 °C for 300 min and storage at 6 °C with distilled water and visualized by confocal laser scanning microscopy with Rhodamine B as protein dye. Magnification 63×; *scale bar* represents 50 μm

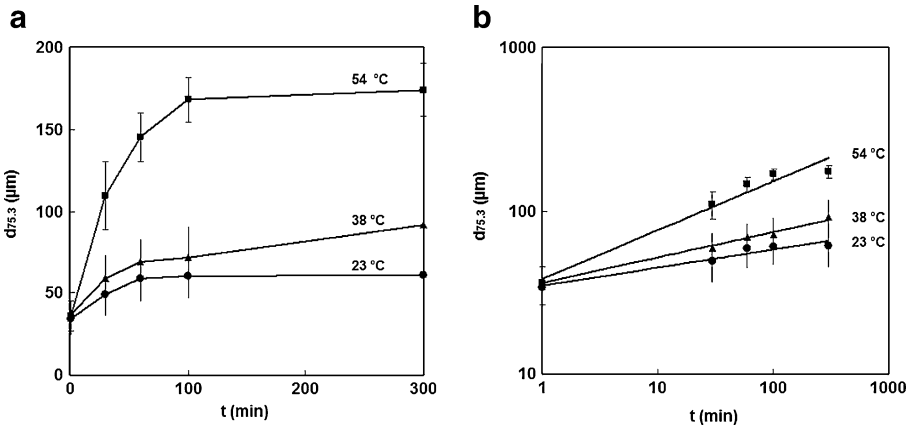

Fig. 3 Particle size $d_{75.3}$ in microgel suspensions depending on temperature and holding time with **a** linear and **b** log–log scale. The protein content was 8.22% (*w*/*w*). *Values* and *error bars* represent the mean value and standard deviation of five independent trials

intensities evolved: an outer and an inner zone with a higher and a lower emitted intensity, respectively. Note that all samples were stored at least for 1 h at 10 °C to allow the specimen to equilibrate. Figure 5 depicts exemplarily the relative number distribution (absolute is not possible due to the measuring principle) of the samples tempered at 38 °C for 1 and 300 min corresponding to Fig. 1. Higher holding times led to a decrease in the relative number of smaller particles and an increase in the relative number of larger particle clusters. Since the volume is proportional to the cube of the particle diameter, many small particles make up the same volume than one large particle cluster. This implies that during aggregation the absolute number of particles decreases.

Depending on the holding time (1 min $\leq t \leq$ 300 min), the particle growth was fitted with a power law function (Fig. 3) as shown in Eq. (1). The R^2 for 23 °C, 38 °C, and 54 °C was 0.950, 0.989, and 0.962, respectively. The exponents for each temperature represent the velocity constants, k_T, which were analyzed by using the Arrhenius in Eq. (2).

$$d_{(75.3)_t} = d_{(75.3)_{t=1}} \cdot t^{k_T} \tag{1}$$

$$k_T = k_{ref} \cdot \exp\left(-\frac{E_A}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \tag{2}$$

$d_{75.3}$: volume-weighted diameter below which 75% of the volume of the particles is found; t: holding time (min); k_T: velocity constant; k_{ref}: velocity constant at reference temperature T_{ref}=311 K; E_A: activation energy (J.mol^{-1}); R=8.314 J.mol^{-1}.K^{-1} (universal gas constant); T: absolute temperature (K); T_{ref}: reference temperature (K).

The velocity constants $k_{296\ °C}$, $k_{311\ °C}$, and $k_{327\ °C}$ were plotted according to the Arrhenius equation (Fig. 6, closed symbols). An activation energy of E_A=26±5.1 kJ. mol^{-1} was calculated. In addition, the $k_{318\ °C}$ was determined in two separate experiments (Fig. 6, open symbol). The $k_{318\ °C}$ value fitted very well in the temperature dependence graph (Fig. 6).

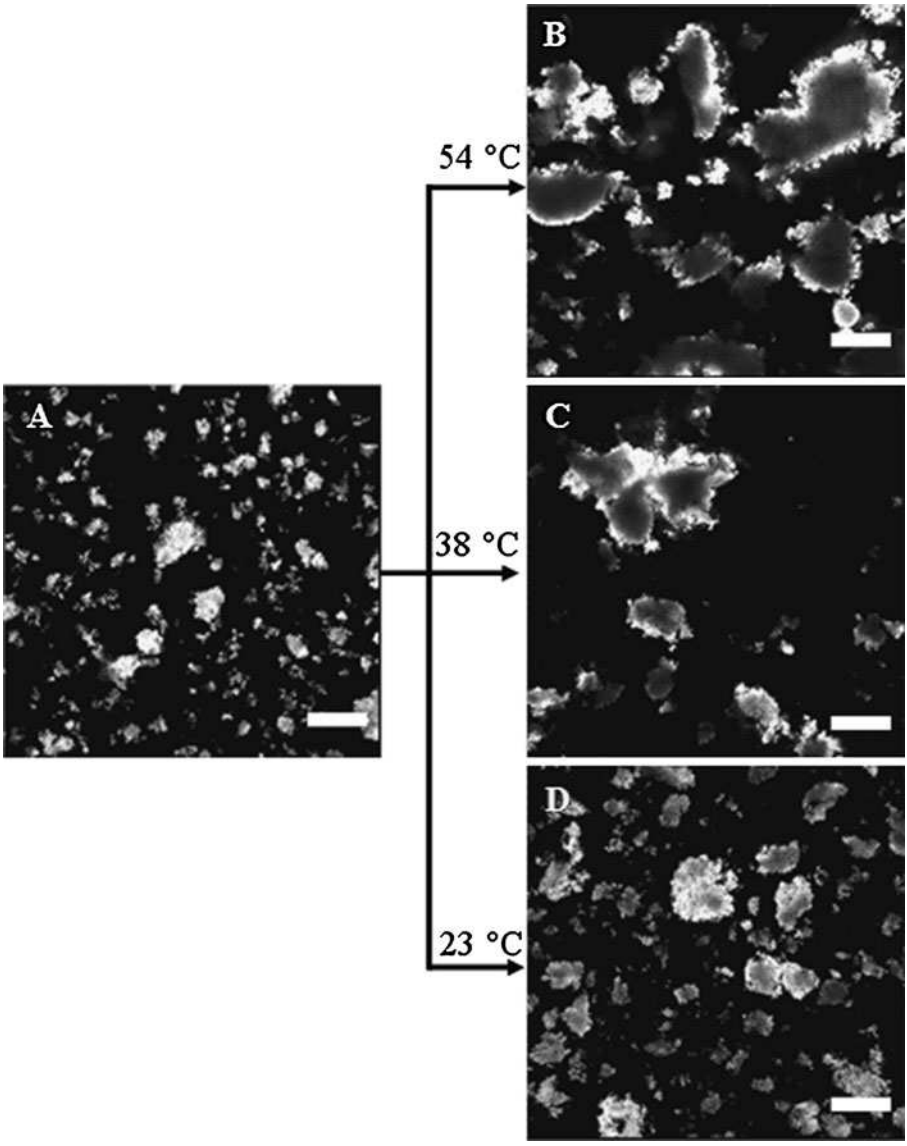

Fig. 4 Influence of temperature on the particle size and number of particles in microgel suspensions. The protein content was 8.22% (*w*/*w*); microgel suspensions were tempered for **A** 1 or 300 min at **B** 54 °C, **C** 38 °C, or **D** 23 °C. Particles were washed out with distilled water and visualized by confocal laser scanning microscopy with Rhodamine B as protein dye. Magnification 20×; *scale bar* represents 50 μm

4 Discussion

Milk gels are particle gels (Horne 1999) that show a fractal gel structure (Horne 1999; Mellema et al. 2002). This involves that the fractal clusters formed during undisturbed aggregation are self-similar and, thus, resemble each other on different length scales (Mellema et al. 2002). Consequently, the structure of the casein micelles greatly determines the properties of the gel (van Vliet et al. 2004), which in turn was shown to

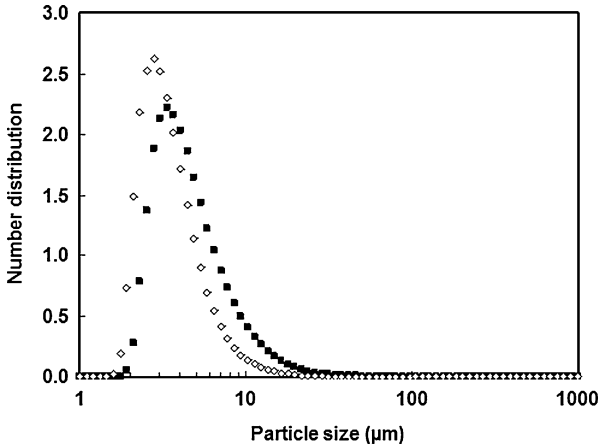

Fig. 5 Number distribution of microgel suspensions depending on the holding time. The protein content was 8.22% (*w*/*w*), tempered at 38 °C for 1 (*diamond*) and 300 min (*filled squares*). *Values* represent the mean value of five independent trials

determine both the properties of the gel fragments after stirring and of the stirred yogurt as the gel fragments were supposed to conserve the structure of the initial gel (Renan et al. 2009). According to this concept, we propose to carry over the aggregation mechanism of casein micelles to clusters—or in general of charged, colloidal particles—to the aggregation of these clusters in fresh cheese.

For the aggregation of casein micelles during acidification, four characteristic particle classes (0.1–1, 1–10, 10–100, and 100–1,000 μm) were reported (Chardot et al. 2002). With decreasing pH, the casein micelles gradually disappeared and larger aggregates of clusters successively emerged. A predominantly diffusion-controlled process was at least proposed for higher dilutions. In the network consisting of aggregated particle clusters, the protein bonds are dynamic (van Vliet et al. 1991), which could lead to rearrangement of particles and clusters on all length scales resulting in particle fusion and an increase of the size of the building blocks (Mellema et al. 2002). Higher temperatures may enhance both extensive particle rearrangement during gel formation and the formation of dense clusters of aggregated particles (Lucey et al. 1997b). The structure before and immediately after stirring was shown to be crucial for the generated gel fragments upon shearing (Renan et al. 2009). The following discussion

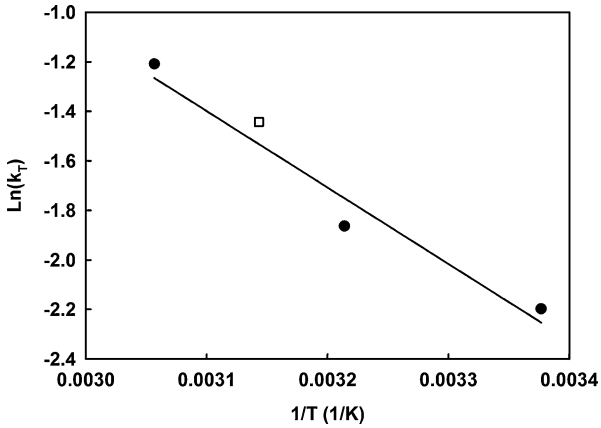

Fig. 6 Velocity constants plotted according to Arrhenius equation for microgel suspensions. The protein content was 8.22% (*w*/*w*). The activation energy was calculated by means of the temperatures 23 °C, 38 °C, and 54 °C (*filled circle*). To verify the relation a microgel suspension was tempered at 45 °C for 1–300 min (*square*). *Values* represent the mean value of five (*filled circle*) and two (*square*) independent trials

of the effects of the holding time and the temperature on the particle growth is based on the idea that (spherical) gel fragments consisting of aggregated particle clusters are suspended in whey.

4.1 Influence of the holding time

Following the particle size distribution, two interesting observations for the microgel suspensions were made: at first, the microgel suspensions consisted of three particle classes independent of the holding time, and secondly, with higher holding times, rearrangement led to the growth of large particle clusters at the expense of smaller ones (Fig. 1). The latter mentioned growth behavior of the particles was also observed for supersaturated solid solutions (Lifshitz and Slyozov 1961), hardly soluble precipitates (Wagner 1961), the aggregation of colloidal particles (Smoluchowski 1917), and for rennet-induced casein particle gels (Mellema et al. 2002).

Lifshitz and Slyozov (1961) postulated a two-stage mechanism for the particle growth: the formation of nuclei due to concentration fluctuations leading to a minor degree of supersaturation, followed by diffusion-driven particle growth. In order to apply this two-stage mechanism to our microgel suspensions, we have to consider the aggregation in the two main processing steps: fermentation and post-processing. During fermentation, the colloidal dispersion of spherical casein micelles (0.1–0.3 μm) within the skim milk aggregate and form a gel. This gel is broken down into microgel particles by stirring, resulting, in turn, in the aggregation of these mircogel particles during post-processing (Fig. 2).

The reason why colloidal particles can lose their stability and can aggregate is explained by the DLVO theory; the application for milk systems and different theoretical approaches for the aggregation are discussed elsewhere (Horne 1999). For instance, native casein micelles additionally show a steric repulsion, which is reduced by the action of chymosin. In the present study, both reduction of the pH and the addition of chymosin were carried out. Charged particles interact through a liquid medium by means of short-range attractive forces (van der Waal) and long-range electrostatic repulsive forces. When particles approach each other in stable suspensions (e.g., skim milk), the repulsive forces increase to a maximum and prevent aggregation as the kinetic energy is lower than the potential energy between the particles. Aggregation takes place when either the kinetic energy is increased by increasing the temperature or by lowering the repulsive forces, which do not affect the short-range attractive forces (Stauff 1960).

We propose that this concept of aggregation also applies for the presented microgel suspensions, only on larger scales. At the final pH of 4.5, repulsive forces are reduced and mainly attractive hydrophobic interactions exist (Hinrichs and Keim 2007; Horne 1999). The microgel suspensions revealed a polydisperse particle size distribution (Fig. 1); by assuming that bigger particles are more prone to the attachment of smaller particles, nuclei for the following aggregation were already built during fermentation and concentration and, thus, before tempering. Lifshitz and Slyozov (1961) proposed such a critical dimension for the volume of particles that particles having a greater volume grew at the expense of smaller ones. The critical dimension was postulated to increase asymptotically with time ($t^{1/3}$), meaning that some particles only grow to be dissolved later. This, in turn, reduces the number of

particles continuously. This phenomenon has been investigated in detail for the aggregation of suspended gold particles (Smoluchowski 1917). The initial stability of the gold particles was reduced by the (preferably fast) addition of an electrolyte which provided each gold particle with a region of attraction. It was proposed that the gold particles move unaffected as a result of Brownian movement, unless they enter the region of attraction of another particle, leading to an instantaneous aggregation. The newly formed aggregates proceed in the Brownian movement with a decreasing velocity in accordance with their bigger size. Starting with a monodisperse particle size distribution, the relative number of primary particles per volume decreased continuously with time. Consequently, the relative number of the aggregated twofold particles, being composed of the primary particles, increased rapidly at the beginning, came to a maximum, and then decreased rapidly as threefold and higher aggregates were generated. Both the aggregation rate and the relative number of particles at the maximum decreased with the higher degree of aggregation. At the same time, this maximum appeared after a longer period of observation. During aggregation, the relative number of all particles decreased, which is in agreement with the findings in the present study (Figs. 4 and 5). These principles of growth kinetics were suggested to apply for spherical particles and are independent of the particle type, the particle size, the concentration, the type of medium, or the temperature. Furthermore, the principles are also adaptable to slow aggregation, meaning that there is only a partial discharge and not every particle contact leads to aggregation (which demands longer observation times). These findings support our results for the aggregation of microgel particles in fresh cheese: there were different particle classes (Fig. 1); the particles were spherical and rearrangement led to the growth of large particle clusters at the expense of smaller ones, thereby reducing the number (Figs. 4 and 5).

4.2 Influence of the temperature

In this study, the particle size grew asymptotically with time with an initial rapid increase which was in agreement with the findings described for the formation of ice crystals (Trgo et al. 1999) and macroporous silica structures on colloidal particles (Lee et al. 2006) or for the hydrothermal growth of nanocrystals (Huang et al. 2003). The higher the temperature was, the higher was both the aggregation rate (k_T, Fig. 6) and the particle size ($d_{75.3}$, Fig. 3). These findings are in agreement with the acid-induced aggregation of casein micelles at different temperatures (Haque et al. 2001; Lee and Lucey 2004; Lucey et al. 1997b; Vétier et al. 1997), and the temperature-induced aggregation of casein submicelles (20–80 °C; Panouillé et al. 2004) or the growth kinetics of nanocrystals (Lee et al. 2006). Thus, temperature plays an important role in the rearrangement of particles which was proposed to be related to the increased mobility due to lower bond energy (Mellema et al. 2002). This, in turn, should increase the collision frequency between particles and, consequently, the number of successful collisions leading to extensive particle rearrangement with increasing temperature (Lucey et al. 1997b).

Besides the aggregation rate and the particle size, the temperature was also proposed to be important for the inner structure of the particles formed. Hydrophobic interactions were shown to be predominant in such acid and acid–rennet microgel suspensions like yogurt or fresh cheese (Hinrichs and Keim 2007). Lucey et al.

(1997b) reported that at low temperatures casein particles have a higher voluminosity and are probably more deformable, which would allow particles to aggregate with a larger number of protein–protein bonds, thereby causing less rearrangement. In contrast, higher temperatures increase hydrophobic interactions which in principle leads to a stronger driving force for particle fusion (Mellema et al. 2002). Thus, the building blocks, the volume fraction and the region of contact between two particles may be smaller at higher temperatures. On the other hand, higher temperatures were shown to promote extensive particle rearrangement resulting in a change in the mutual position of particles and in the formation of dense clusters of aggregated particles which increases the density of aggregates because the number of particle–particle junctions is increased (Mellema et al. 2002). In accordance with that, Lee and Lucey (2004) reported that higher incubation temperatures led to a higher thermal motion of protein particles which may alter the aggregation process and results in a more compact conformation and a contraction of casein particles. Therefore, increasing the hydrophobic interactions at higher temperatures was proposed to have two opposite effects: on the macroscopic level, interparticle bonds may be increased, while on the microscopic level, intraparticle bonds lead to particle shrinkage and, thus, a reduction of the possibility to form interparticle bonds (Renan et al. 2009). In addition, at a constant temperature, the shrinkage of the particles leads to a denser structure, which, in a diffusion-limited process, may decrease the rate of bond formation between two particles. Applying these findings to our results supported the idea that more ramified particles with higher stiffness of junctions were rapidly formed during tempering (especially at 54 °C), and longer holding times led to shrinkage and a more compact and dense particle structure (Fig. 4). This may explain the outer and inner zone of some large particle clusters showing different emitted intensities in the CLSM (Fig. 4B). If higher temperatures led to larger particle clusters with a lower voluminosity and a more compact and dense structure, i.e., higher protein content in those particles than in the whole microgel suspension (Sainani et al. 2004), the diffusion rate of the dye would be lower. In addition, we observed that the washed out particles in the microgel suspension tempered at 54 °C were more resistant to mechanical forces, e.g., when pressing them with the tongue against the palate.

Some researchers explored the aggregation kinetics of casein micelles during fermentation. Castillo et al. (2006) explored the aggregation kinetics in milk coagulated by a combination of bacterial fermentation and chymosin. The activation energy, E_A, was calculated according to the Arrhenius equation and E_A decreased from 83.1 to 72.6 kJ.mol^{-1} with increasing inoculum concentration. Since the pH at the beginning of the aggregation process decreased with increasing inoculum concentration and the pH was in the range of 5.97–5.76, this result was attributed to a decreasing energy barrier against aggregation by a lower pH.

Takeuchi and Cunha (2008) studied the influence of the incubation temperature (4–40 °C) and the sodium caseinate concentration (2–6% *w*/*w*) during acidification to a final pH value of around 4.6. By monitoring the stress at rupture during gelation as a function of time, the k_{tens} was determined as a measure of the gel network formation rate due to aggregation of the caseinate particles. Thus, the mathematical calculation of k_{tens} followed the same approach as k_T in the present study. However, as the k_{tens} and the k_T used the stress at rupture and the $d_{75.3}$, respectively, these parameters represented the bulk properties and the inner structure, which, in turn,

affects the bulk properties. According to these authors, higher temperatures promoted the acidification and the gelation rate, and thus, k_{tens} which is in agreement with our findings (Fig. 6). From the reported k_{tens}, we calculated an activation energy, E_A, of about 20–36 kJ.mol^{-1} depending on the protein content.

These observations supported the results of Panouillé et al. (2004), who explored the temperature-induced aggregation and gelation of casein. As the first step, natural casein micelles were dissociated by removing colloidal calcium phosphate with chelating salts into so-called submicelles. Subsequently, in a second step, heat-induced aggregation and gelation was studied. Based upon the gelation time (t_g), an E_A of about 40 kJ.mol^{-1} was calculated by using the Arrhenius equation.

Interestingly, the activation energy for neither the aggregation of the casein micelles nor for the casein submicelles was further interpreted regarding the mechanism of the aggregation. According to literature, the temperature dependency of physical quality changes can be captured in mathematical models containing such characteristic kinetic parameters as the activation energies and the rate constants, which may give an insight in the mechanism of the underlying process (van Boekel 2008). In general, several levels of E_A exist (Kessler 2002): 10–20 (diffusion), 50–150 (chemical reaction), and 250–400 kJ.mol^{-1} (inactivation of microorganisms and enzymes). In detail, two main types of aggregation were described for the particle growth in suspensions (Smoluchowski 1917; Wagner 1961): a diffusion-limited and a reaction-limited. Diffusion-limited particle growth exists when only negligible repulsive forces between particles occur, and almost every contact leads to aggregation. The diffusion process controls the overall aggregation rate. In contrast, reaction-limited particle growth exists when repulsive forces are not negligible, and the aggregation needs time to overcome these repulsive forces.

In our study, an activation energy of E_A=26 kJ.mol^{-1} was calculated for the particle growth in microgel suspensions at a pH of 4.5. This was in accordance with the acid-induced aggregation of casein micelles and the heat-induced aggregation of casein submicelles. Based upon the findings described in literature, we propose that the particle growth in the studied microgel suspensions was a predominant diffusion-limited process. Since diffusion is the overall effect of the Brownian movement of each particle (Smoluchowski 1917) and, according to the Stokes–Einstein relation, the diffusion constant is inversely related to the particle size; the diffusion process does not stop with increasing particle size but slows down.

Hence, in the presented study, long observation times (1–300 min) at higher temperatures (23–54 °C) were applied. To further simplify the issue, larger particles can be assumed to represent stationary nuclei for particle attachment, while smaller particles are slowly transferred to these nuclei. This implies that the number of small particles declines as the particle size of large particles grows (Fig. 5). The result of this is a decreasing change in the particle size with time which is reflected in the asymptotical growth behavior.

5 Conclusion

From the presented results, the following conclusions are proposed. The aggregation of spherical particle clusters in microgel suspensions, e.g., in fresh cheese, demands,

first, the transport and, secondly, the aggregation of particles as soon as they encounter each other. In the presented study, higher aggregation rates (k_T) were observed with increasing temperature. Based upon the activation energy (26 kJ.mol^{-1}) and the fact that higher temperatures promote both the diffusion process and hydrophobic interactions, the particle growth was postulated to be diffusion-limited for the studied microgel suspensions. During the aggregation process, rearrangement led to the growth of large particles at the expense of smaller ones. Thus, the overall amount of particles was reduced. To prevent particle growth, the results imply that a fast reduction in the temperature is necessary, or a hydrocolloid may be added to reduce the diffusion rate of the particles (running research). The presented results enable fresh cheese manufacturers to control particle growth in such a way that defined particle sizes and, therefore, desired structures are adjustable.

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