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To cite this version:

Katiuchia P. Takeuchi, Rosiane L. Cunha. Influence of ageing time on sodium caseinate gelation induced by glucono-δ-lactone at different temperatures. Dairy Science & Technology, 2008, 88 (6), pp.667-681. hal-00895686

HAL Id: hal-00895686

https://hal.science/hal-00895686v1

Submitted on 11 May 2020

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Dairy Sci. Technol. 88 (2008) 667–681
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DOI: 10.1051/dst:2008031

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Original article

Influence of ageing time on sodium caseinate gelation induced by glucono-δ-lactone at different temperatures

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Received 15 November 2007 – Accepted 29 October 2008

Abstract – The aim of this work was to study the influence of a wide range of incubation temperatures (4 to 40 °C) and sodium caseinate concentrations (2 to 6% (w/w)) on acid-gel properties during and after long ageing times, formed under different acidification rates promoted by gradual hydrolysis of glucono-δ-lactone (GDL) into gluconic acid. The kinetics of acidification and gelation were followed from pH 6.7 to a final pH value around 4.6 by evaluation of the pH and mechanical properties using uniaxial compression measurements (stress and strain at rupture). As a general trend, faster acidification rates led to faster gel network formation, and lower incubation temperatures led to a higher final pH, while increasing caseinate concentration promoted a small increase in the pH value. Stress at rupture of gels induced by fast acidification rates did not exhibit a weaker gel network, showing the contribution of rearrangements of the network gel at final pH to the electrostatic balance, besides the fact that the hydrophobic interactions and hydrogen bonds were important forces involved in microstructure stabilization. Rearrangement of the gel network was mainly observed at pH values close to 4.6 and was more pronounced at longer ageing times of incubation. The stronger gels were obtained at incubation temperatures of 10 °C followed by 25, 4 and 40 °C, which was an indication of the behavior of the gel network microstructure, since at the highest temperature the pores were larger and syneresis more pronounced. The results of this study suggest that the incubation temperature, protein concentration and rearrangement at final pH have a great influence on the balance between the attractive and repulsive forces between protein and water, contributing to development of optimized texture and water-holding capacity of acid-gels of sodium caseinate.

sodium caseinate / glucono-δ-lactone / acidification rate / mechanical properties / syneresis

摘要 – 不同温度和成熟时间对葡萄糖酸-δ-内酯引起的酪蛋白酸钠凝胶的影响。葡萄糖酸-δ-内酯水解为葡萄糖酸, 形成了酪蛋白的酸凝胶。本文研究了温度 (4~40 °C) 和酪蛋白酸钠浓度 (2%~6%,w/w) 对在不同酸化速率下形成酸凝胶和长时间成熟后的酸凝胶特性的影响。利用单轴压缩测量法 (应力和极限应变) 分析了 pH 6.7 到终点 pH (约 4.6) 范围内酸化和凝胶作用的动力学特性。一般来说, 快速酸化有利于凝胶网络的快速形成, 低温会导致终点 pH 值较高; 而增加酪蛋白酸钠浓度会使 pH 值略有升高。由于快速酸化引起的凝胶极限应力并没有显示出较弱的凝胶网络, 可见, 除了疏水基团的相互作用和氢键是稳定凝胶微观结构的作用力外, 重要的原因是在终点 pH 值下静电平衡导致凝胶网络的重新排列。在 pH 值接近 4.6 时可以观察到凝胶网络重排现象, 特别是较长时间成熟的凝胶网络重排现象非常明显。在 10 °C 时形成的凝胶最硬, 其次是 25 °C 、 4 °C 和 40 °C, 这说明温度直接影响凝胶网的微观结构,

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因为在最高温度下凝胶孔洞较大, 脱水收缩作用较显著。实验表明: 温度、蛋白质浓度和终点 pH 值下凝胶网络的重排对蛋白质与水之间的吸引和排斥平衡有很大的影响, 正是这种作用使得酪蛋白酸钠凝胶具有最佳的质构和持水能力。

酪蛋白酸钠 / 葡萄糖酸-δ-内酯 / 酸化速率 / 力学性质 / 脱水收缩

Résumé – Influence de la durée de maturation sur la gélification du caséinate de sodium provoquée par la glucono-delta-lactone à différentes températures. Ce travail a pour objectif d'étudier, au cours de longues durées de maturation, l'influence d'une grande plage de températures d'incubation (4–40 °C) et de concentrations en caséinate de sodium (2–6 % w/w), sur les propriétés des gels acides formés à différentes vitesses d'acidification obtenues par l'hydrolyse progressive de la glucono-δ-lactone (GDL) en acide gluconique. Les cinétiques d'acidification et de gélification ont été suivies par mesure du pH à partir de pH 6,7 jusqu'à un pH final avoisinant 4,6 et par l'évaluation des propriétés mécaniques en utilisant des mesures de compression uniaxiale (contrainte et déformation à la rupture). De façon générale, on observe qu'une acidification plus rapide conduit à une formation de gel plus rapide. Une température d'incubation plus basse conduit à un pH final plus élevé. L'augmentation de la concentration en caséinate de sodium favorise une légère augmentation du pH. La contrainte à la rupture des gels formés par acidification rapide n'a pas mis en évidence des réseaux de gel plus faibles, montrant la contribution des réarrangements du gel au pH final à l'équilibre électrostatique, malgré le fait que les interactions hydrophobes et les liaisons hydrogène soient d'importantes forces impliquées dans la stabilisation de la microstructure. Le réarrangement du gel a été principalement observé à des valeurs de pH proches de 4,6 et celui-ci était plus marqué pour les durées de maturation plus longues lors de l'incubation. Des gels plus fermes étaient obtenus avec une température d'incubation de 10 °C, puis respectivement de 25, 4 et 40 °C, ce qui donne une indication du comportement du réseau puisqu'à des températures plus élevées les pores étaient plus grands et la synérèse plus prononcée. Les résultats obtenus au cours de cette étude indiquent que la température d'incubation, la concentration en protéines et le réarrangement au pH final ont une grande influence sur l'équilibre entre les forces d'attraction et de répulsion entre les protéines et l'eau, contribuant au développement d'une texture et d'une capacité de rétention d'eau des gels acides de caséinate de sodium optimisées.

caséinate de sodium / glucono-δ-lactone / taux d'acidification / propriétés mécaniques / synérèse

1. INTRODUCTION

Acid gelation of milk proteins is of importance during the processing of a number of dairy products such as yoghurt-like desserts. Acid-casein gels may be formed from a variety of primary particles, ranging from single protein molecules or protein oligomers (caseinate nanoparticles) to native micellar casein particles [23]. Sodium caseinate is an ingredient widely used in a range of food formulations because of its nutritional value and functional properties, such as its gelling capacity [3], and emulsifying. It is prepared by acid precipitation of milk casein at its isoelectric point. Sodium hydroxide is added to redissolve the protein and to restore the medium to neutrality. Under these conditions, caseinates form aggregates or sub-micelles because of the high proportion of hydrophobic amino acid side chains that self-associate in aqueous solutions [10]. Further association of sub-micelles to form casein micelles is prevented by removal of most of the calcium into the supernatant during the acid precipitation [22]. At the natural pH of milk (6.7) the casein micelles and probably the sub-micelles are stabilized to a large extent by the "hairy" surface layer of the negatively charged κ-casein [8, 13, 32], but a reduction in pH neutralizes the carboxylic acid groups and charged side chains of the amino acids,

leading to aggregation of the protein [8, 13, 32] at pH values around the isoelectric point of casein, close to 4.6.

Besides the final pH, the acidification kinetics process can also influence the physical attributes of the final product, such as the overall visual appearance, microstructure and rheological properties. Such properties contribute to the overall sensory perception and functionality of the products [9, 14]. On the other hand, independent of the acidification process, the temperature has a great influence on protein-protein interactions [12] because at elevated temperatures thermal vibrations enable the protein molecules to slide past one another, but upon cooling the thermal vibrations decrease and the prevailing protein attractive forces lead to entanglements. The complete movement of whole chains away from one another is thus prevented and the structure solidifies as a gel [6].

Milk can be acidified by bacterial cultures (lactic fermentation) or by the addition of glucono-δ-lactone (GDL), which is hydrolyzed into gluconic acid with a resulting reduction in pH. The use of GDL would avoid some of the difficulties associated with starter bacteria, including variable activity and variation in the type of culture used. Gels made with these two types of acidifying precursors differ in their rheological properties [18], partly as a function of the acidification rate. Acidification using GDL can be performed at different rates, which can lead to different final pHs, depending on the temperature, other components present (proteins, sugars, salts) or the acid precursor concentration [3]. In GDL gels, the isoelectric pH value (pH 4.6) of caseins in milk can be reached faster and remains stable, thus allowing longer ageing close to this point. This phenomenon contributes to the continuous fusion and rearrangement of the casein particles.

Several studies have been performed using GDL as an acid precursor to obtain acid milk gels [3, 8, 11, 15, 17–21, 29, 30], but most of them did not show a clear consideration of the acidification rate and its consequences on the reorganization process of the gel network. In addition, these studies used different GDL concentrations and incubation temperatures, so different acidification rates were obtained, although the results were analyzed at the same final pH, and the kinetic effects were not considered. Braga et al. [3] evaluated the influence of acidification rate and the final pH on the properties of sodium caseinate gels by adding different GDL concentrations at 10 °C, and reported that the acidification rate had a significant effect on the final mechanical properties of gels, but did not affect the rheological properties measured during the gelation process. Vétier et al. [31] studied the influence of temperature and acidification rate on the fractal structure of GDL-acidified casein aggregates using scattering and turbidimetric techniques. Different acidification rates promoted by the addition of 0.40 to 1.50% (w/v) of GDL in a fifty-fold diluted reconstituted skim milk were only analyzed at 20 °C, and the effect of aggregation kinetics on the fractal dimension was not observed. However, the effect of temperature (10–40 °C) at the same acidification rate showed casein aggregates with a more completely filled structure (higher fractal dimension) at lower incubation temperatures than at higher temperatures.

However, we did not find studies about evaluation of gel network strengthening of acid-induced sodium caseinate gels during their formation and up to reaching the final pH or steady state of pH, considering the effect of temperature and network rearrangement at longer ageing time as fundamental parameters for the relation of structure-mechanical properties. The goal of this work was to study the influence of a wide range of incubation temperatures (4 to 40 °C) and sodium caseinate concentrations (2 to 6% (w/w)) on the gel properties

during and after long ageing times, taking into account the crucial role of this protein ingredient on the texture improvement, especially of acidified dairy products. With this aim, mechanical properties (stress and strain at rupture), the microstructure and syneresis of gels obtained at different acidification rates but a similar final pH value (around 4.6) were analyzed.

2. MATERIALS AND METHODS

2.1. Materials

The ingredients used to prepare the model systems were casein from bovine milk (C7078, Lot 100K0223, Sigma Chemical Co., St Louis, MO, USA) and glucono-δ-lactone (Sigma). Acid digestion (nitric and chloride acid) followed by Inductively Coupled Plasma (ICP) Emission Spectroscopy (Perkin Elmer – Optima 3000 DV, Waltham, MA, USA) was done to characterize the casein powder and the following ion composition was obtained: Na^+ = 0.16%, Ca^{2+} = 0.14% and K^+ = 0.08%. The moisture (6.5 ± 0.1%) and ash (0.84 ± 0.08%) contents were determined using the gravimetric method and the protein concentration of the powder was 88.2 ± 0.9% as determined by the Kjeldahl (N × 6.38) procedure [1].

2.2. Solutions and gel preparation

Sodium caseinate solutions were prepared by suspending the casein powder in deionized water at a maximum temperature of 50 °C, with magnetic stirring for 4 h. Sodium hydroxide (10 mol·L^{-1}) was added to maintain the pH at 6.7 during the preparation of sodium caseinate stock solutions (10% w/w), which were subsequently diluted with deionized water to concentrations of 2, 4 and 6% (w/w). GDL powder was quickly dissolved (~ 1 min) in a small amount of deionized water, previously equilibrated at 4, 10, 25 or 40 °C before being added to the sodium caseinate solutions, equilibrated at the same temperatures. Both solutions were quickly mixed (about 2 min) with magnetic stirring to avoid early casein aggregation. Glucono-δ-lactone (GDL) was added according to a GDL/caseinate ratio corresponding to 0.135 (w/w) [3] to reach a final pH of about 4.6 at 10 °C. Mixed solutions (sodium caseinate and GDL) were poured into cylindrical plastic tubes (30 mm in diameter and 30 mm height) and stored at incubation temperatures of 4, 10, 25 and 40 °C before analyzing the mechanical properties. For the pH measurements, aliquots of mixed solutions were stored under the same conditions described above. The pH of the samples was monitored throughout the acidification process, from pH 6.7 to about 4.6. The fall in pH of the sodium caseinate systems due to GDL hydrolysis was measured using a Digimed Analitica (Mod. DME-CF1, São Paulo, Brazil) pH meter for soft solids. The gels were prepared in duplicate. The analysis of syneresis and microstructure by scanning electron microscopy was evaluated after reaching the final pH or steady state of pH.

2.3. Mechanical properties

The mechanical properties of the gels were determined by uniaxial compression using a TA-XTIIi Texture Analyzer (Stable Microsystems Ltd., Godalming, UK) equipped with an acrylic cylindrical plate (40 mm diameter). The surfaces in contact with the gel were lubricated with silicon oil to avoid friction between the samples and probe. The mechanical properties during gelation were obtained from the first self-supporting gels (i.e. when it was possible to remove the gels from the container and they maintained their shape) up to gels at the steady state of mechanical properties, which means that these properties did not change significantly after this point ("pseudo-equilibrium"). The rupture tests were quickly and carefully performed

at the gel incubation temperatures in order to avoid temperature alterations greater than ± 1 °C. Self-supporting gels were submitted to 80% compression of their initial height with a crosshead speed of 1 mm·s^{-1}. All measurements were done in quintuplicate. The raw data were transformed into true or Hencky strain (ε_H) and stress (σ) values [27]. Stress (σ_{rupt}) and strain (ε_{rupt}) at rupture were determined from the maximum point of the true stress-strain curve.

A first-order kinetics equation (Eq. (1)) was used to fit the stress at rupture data as a function of time after the addition of GDL to the sodium caseinate solutions at each incubation temperature:

$$\sigma_{rupt}(t) = \sigma_{ss} + A \,.\, \exp(-k_{tens} \,.\, t) \qquad (1)$$

where $\sigma_{rupt}(t)$ is the stress at rupture measured as a function of time, σ_{ss} is the value of stress at rupture at steady state, t is the time after GDL addition, k_{tens} is the gel network formation rate and A is a fitting parameter.

The pH at the beginning of network rearrangement was arbitrarily defined as the point where the variation in the stress at rupture values as a function of pH ($d\sigma_{rup}/dpH$) was lower than 1. As a consequence, the stress and time at the beginning of network rearrangement were the corresponding values when $d\sigma_{rupt}/dpH < 1$. Final pH, time and stress at rupture corresponded to the last result of the kinetics data.

2.4. Scanning electron microscopy

Pieces of gel (approximately 10 mm × 2 mm × 2 mm) were fixed overnight in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol·L^{-1}) at pH 7.2. After rinsing in cacodylate buffer (0.1 mol·L^{-1}), the samples were fractured under liquid nitrogen, followed by another rinse with cacodylate buffer. The fractured samples were postfixed overnight in 1% buffered osmium tetroxide. Fractured samples were rinsed twice in cacodylate buffer and then dehydrated in a graded ethanol series (30, 50, 70 and 90% v/v). Dehydration was continued in 100% ethanol (three changes in 1 h) followed by critical point drying (Critical Point Dryer CPD03, Balzers, Alzenau, Germany). The dried samples were mounted on aluminium stubs and coated with gold in a Sputter Coater SCD 050, Balzers (Alzenau, Germany). At least five images of typical structures at a magnification of 2000 × were recorded using a JEOL JSM 5800 LV (Tokyo, Japan) apparatus operating at 10 kV.

2.5. Syneresis

After reaching the steady state of pH, the gels were carefully removed from their containers and an aliquot of gel (about 2.0 g) placed in beakers containing a double disc of filter paper (superficial density: 80 g·m^{-2}, thickness: 205 μm, mean pore diameter: 1.4 μm) to support the gel. The beakers were placed in a closed recipient and kept at the incubation temperatures for 3 h. The amount of water released from the gel (absorbed by the filter) was evaluated and the syneresis values were determined (Eq. (2)). Syneresis values were evaluated after 95 h of GDL addition for the lower temperatures (4 and 10 °C) and 42 to 45 h for the higher temperatures; 40 and 25 °C, respectively:

$$\text{Syneresis (\%)} = \left(\frac{m_{released}}{m_{initial}}\right) \times 100 \qquad (2)$$

where $m_{initial}$ (g) and $m_{released}$ (g) are the amount of water initially present in the gel (calculated from the centesimal composition of the gels) and the amount released after 3 h at each incubation temperature (4, 10, 25 and 40 °C), respectively. All the measurements were made in quintuplicate.

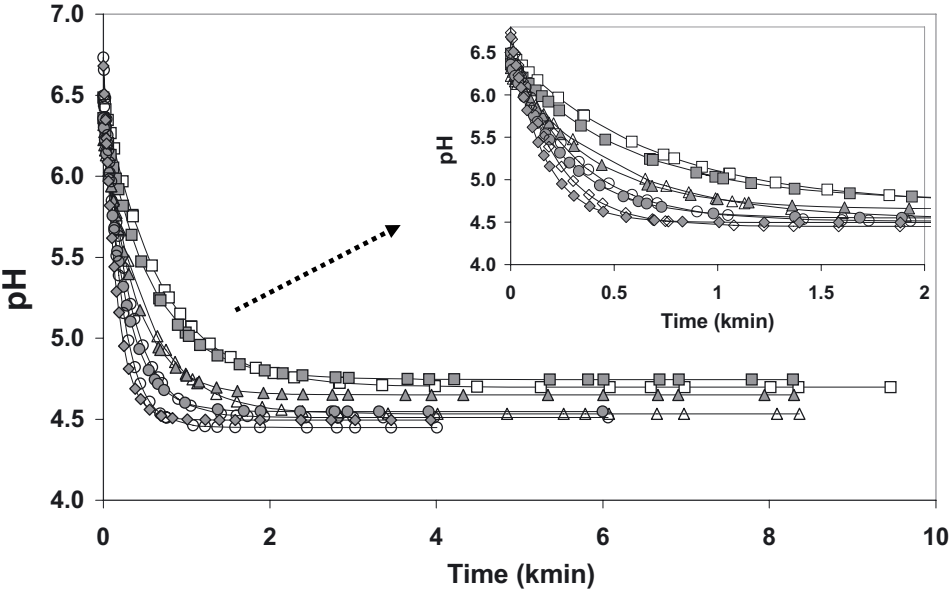

Figure 1. Reduction in pH of the 2 and 6% (w/w) sodium caseinate (CS) solutions with GDL addition. The samples were acidified at incubation temperatures of 4 (□), 10 (△), 25 (○) and 40 °C (◊). The open and solid symbols show CS at 2% and 6% (w/w), respectively. The inset shows a zoom of the initial pH fall up to 2000 min to visualize better the acidification rates.

2.6. Statistical analysis

The differences between the means of syneresis values for the various treatments were assessed using the Tukey procedure with the Statistica software package (StatSoft Inc., v. 5.0, Tulsa, OK, USA) and $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Gelation kinetics

The fall in pH as a function of time can be observed in Figure 1, which shows a fast reduction in pH during the first 500 min, followed by a decrease in the acidification rate until the steady state value was reached. Only the results of the 2% and 6% sodium caseinate gels are presented, since the 4% (w/w) gels showed the same trend. The pattern of pH reduction was quite similar for all systems, with some differences in the pH at steady state and the acidification rate (Fig. 1). Higher incubation temperatures led to an increase in the acidification rate and decrease in final pH, probably due to an intensification of the dissociation velocity of the GDL ester into gluconic acid [3]. The final pH varied between 4.45 and 4.55 for the higher incubation temperatures (40 and 25 °C), reached in approximately 2000 min (about 34 h), and between 4.53 and 4.74 for the lower storage temperatures (10 and 4 °C), taking close to 4000 min (about 67 h). On the other hand, an increase in sodium caseinate concentration showed a slight increase in the final pH values. This could be related to the buffer role of the protein, promoting a smaller fall in pH with a larger amount of protein, since more amino acid NH residues were able to attract the H^+

Table I. Fitting parameters obtained from Equation (1) for the stress at rupture behavior of sodium caseinate (CS) gels during acidification with GDL at different incubation temperatures (4, 10, 25 and 40 ˚C).

Temperature	CS (% w/w)	σ_{ss}(kPa)	A	k_{tens} ($10^{-4}\cdot min^{-1}$)	R^2
4 ˚C	**2**	0.44	–0.38	3.54	0.98
	4	2.23	–4.17	5.06	0.98
	6	4.09	–33.65	16.43	0.99
10 ˚C	**2**	0.54	–1.19	10.44	0.97
	4	2.37	–5.82	12.86	0.98
	6	5.22	–25.41	14.24	0.98
25 ˚C	**2**	0.78	–0.47	12.66	0.97
	4	1.74	–1.42	15.68	0.98
	6	4.36	–3.22	19.54	0.98
40 ˚C	**4**	0.87	–0.76	34.44	0.97
	6	1.42	–1.29	43.59	0.96

ions liberated from the gluconic acid [26]. This combined effect of protein concentration and temperature could be an important tool to control the final pH of protein-based food formulations.

An increase in the incubation temperature and the caseinate concentration also promoted a rise in the gel network formation rate (k_{tens}) (Tab. I), in the same way as the acidification velocity.

Lucey et al. [20] reported that low values of storage modulus (G′) or elasticity for sodium caseinate gels formed at higher temperatures were observed, which could be due to extensive rearrangements during gel formation as fewer bonds between the particles are formed. This resulted in the formation of dense clusters of aggregated particles, which in turn aggregated to form a gel. From these dense clusters many particles would hardly contribute to the rigidity of the network, resulting in a weak gel. At lower temperatures fewer hydrophobic interactions are present, which would allow particles to aggregate with a larger number of bonds between two particles and serum caseins, thereby causing fewer rearrangements during gel formation.

Bringe and Kinsella [4] also reported a direct relationship between acidification and the gel network formation rates for the acid coagulation of skimmed milk. The faster dissociation of GDL that occurred at higher incubation temperatures was associated with earlier network formation under such conditions. However, an increase in acidification rate did not necessarily imply lower values for stress at rupture at steady state (σ_{ss}) or weaker gel networks (Tab. I).

Incubation temperatures of up to 25 ˚C exhibited increased values for stress at rupture at steady state (σ_{ss}) for sodium caseinate gels, but at 40 ˚C a considerable decline was observed. Samples containing 2% (w/w) of CS and incubated at 40 ˚C were non-self-supporting gels, and therefore the stress at rupture of these samples was not calculated. Such an effect could be partly associated with the weaker network formed with a lesser amount of protein. Indeed, the gels containing 2% (w/w) of protein had a much lower strength than those at higher protein concentrations for all incubation temperatures. However, the pronounced decrease in network force observed only at 40 ˚C could also be somewhat attributed to altered properties of the casein at the higher temperatures due to hydrophobic interactions: reduced voluminosity, less deformability, and a lesser amount of casein released into the serum [7].

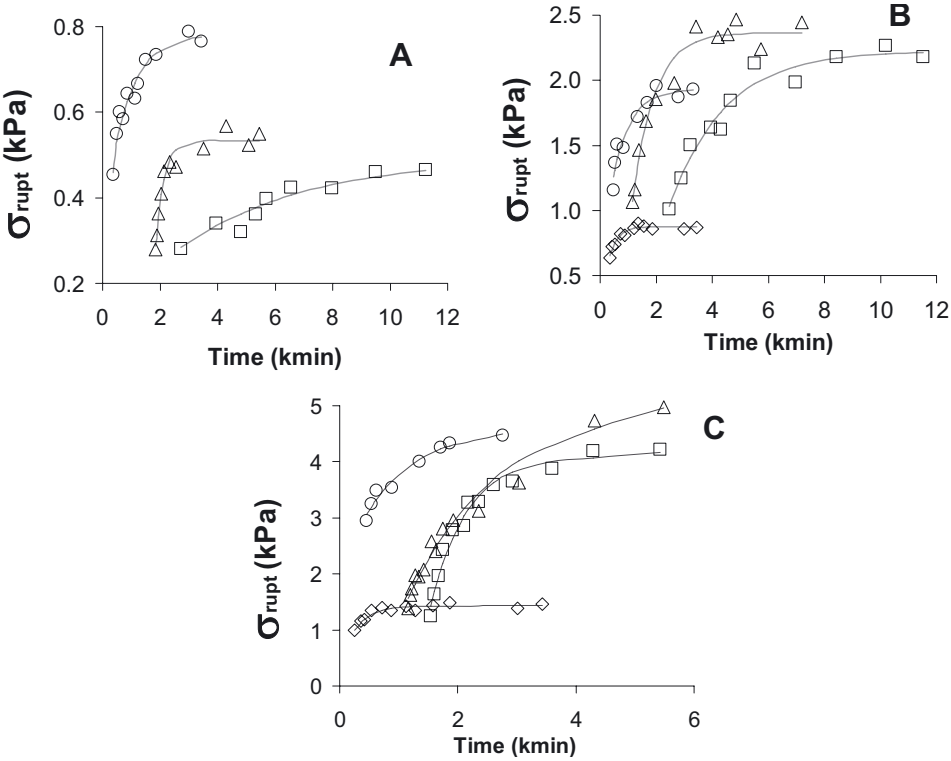

Figure 2. Gel network strengthening during acidification of sodium caseinate solutions at 2 (A), 4 (B) and 6% (w/w) (C) with GDL at incubation temperatures of 4 (□), 10 (△), 25 (○) and 40 ˚C (◊).

Stress at rupture is related to gel network strength and thus this property was followed as a function of the acidification time (Fig. 2). Harder (higher stress at rupture values) gels were observed at higher sodium caseinate concentrations, as occurred with the gel network formation rate (k_{tens}), and both these effects could be attributed to a greater amount of protein molecules being used to form the gel. This influence of concentration on stress at rupture was observed for all systems and was greater at 4, 10 and 25 ˚C than at 40 ˚C (Tab. I).

Gels formed at lower temperatures took more time to reach steady state because of the slower acidification rates, and the gel network formed was probably more organized than at higher gelation temperatures [4]. Indeed, gels formed at lower gelation temperatures showed higher stress at rupture values. At higher temperatures the gelation process occurs under high kinetic energy, leading to a more chaotic network, but this effect was clearly observed only at 40 ˚C. The low values for stress at rupture or hardness of the gels formed at 40 ˚C may be due to extensive local particle rearrangements during gel formation, resulting in the formation of dense clusters of aggregated particles and a less continuous network [33]. Arshad et al. [2] reported similar results for skimmed milk gels formed by acidification with GDL.

During the pH decline strengthening of the gel network occurred (Fig. 3), due to

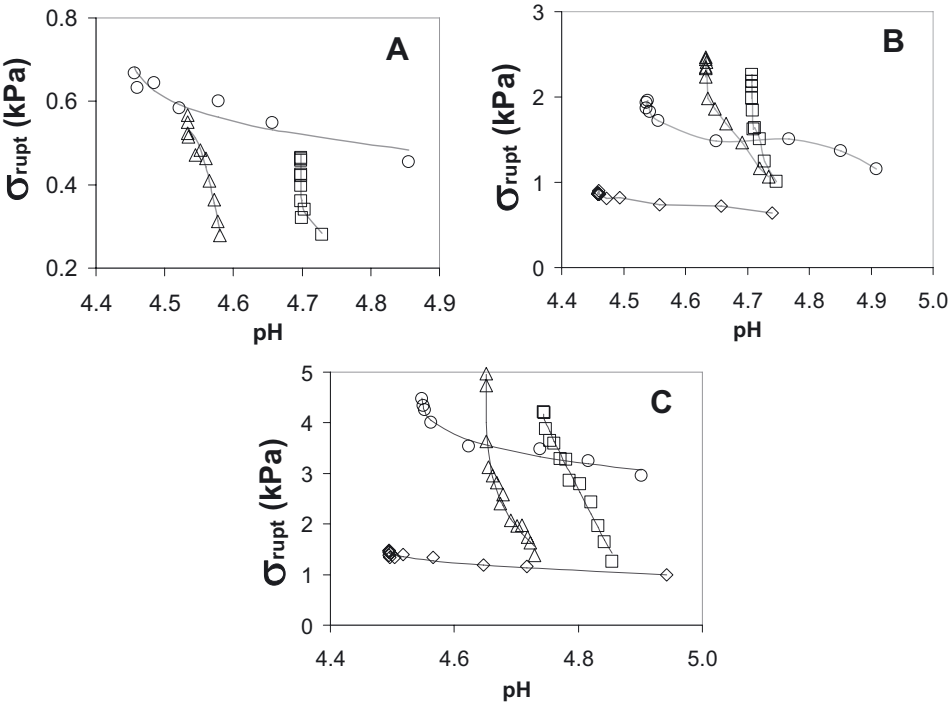

Figure 3. Effect of pH on the stress at rupture of sodium caseinate gels acidified with GDL at incubation temperatures (T) of 4 (□), 10 (△), 25 (○) and 40 °C (◊). The sodium caseinate concentrations were 2% (A), 4% (B) and 6% (w/w) (C).

a gradual neutralization of the negative charge of the protein molecules, diminishing electrostatic repulsion and promoting extensive protein-protein interactions. At higher protein concentrations, the gels formed at lower temperatures (lower acidification rates), principally at 4 and 10 °C, showed a sharper increase in stress at rupture at a determined pH value (Fig. 3). This increase was attributed to strengthening of the gel network due to the slower changes to reach final pH value, permitting more effective protein-protein interactions due to the lower electrostatic repulsion.

Such an increase in stress at rupture, more abrupt at lower temperatures and more gradual at higher temperatures, was initiated at different pH values, depending on the CS concentration and incubation temperature. Gel network reorganization or rearrangement of the sodium caseinate particles, and even cluster formation in the limited pH range, could improve the strength and interconnectivity of the network [29].

In addition to the incubation temperature, gel network strengthening was also influenced by a combination of factors such as the small difference between the initial and final pH values (lower electrostatic repulsion forces) during network reinforcement and the time scale to evaluate the reorganization process, which was indirectly associated with the acidification rates. Increasing the incubation temperature caused a decrease in final pH, which could affect the balance of the interaction forces during the network reorganization

Table II. Values for pH, stress at rupture and time at the start and end of the network reorganization process of GDL-induced sodium caseinate gels (2–6% w/w) formed at different incubation temperatures (4, 10, 25 and 40 ˚C).

Temperature	CS (% w/w)	pH		Stress at rupture (kPa)		Time (kmin)	
		initial	final	initial	final	initial	final
T = 4 ˚C	**2**	4.73	4.70	0.28	0.40	2.73	5.69
	4	4.75	4.71	1.01	2.13	2.47	5.52
	6	4.85	4.74	1.25	4.22	1.54	5.43
T = 10 ˚C	**2**	4.58	4.53	0.28	0.57	1.84	5.46
	4	4.73	4.63	1.06	2.47	1.17	5.73
	6	4.73	4.65	1.38	4.97	1.16	5.49
T = 25 ˚C	**2**	4.52	4.51	0.67	0.79	1.59	2.79
	4	4.56	4.54	1.72	1.96	1.35	2.78
	6	4.56	4.55	4.00	4.47	1.35	2.76
T = 40 ˚C	**2**	4.46	4.45	–	–	–	–
	4	4.46	4.46	0.86	0.87	1.20	3.44
	6	4.50	4.50	1.34	1.46	0.87	3.43

process. Indeed, it was observed that the difference between the initial and final stress at rupture (during the reorganization process) was strongly influenced by the temperature and pH at the beginning of network rearrangement (Tab. II).

Gels analyzed before reorganization showed the strongest network when incubated at 25 ˚C. However, after gel reorganization the highest values for stress at rupture were observed at 10 ˚C, probably because of the lower acidification rate giving more time for rearrangement at pH values close to 4.6. Thus, as a general trend, the highest values for hardness of the gel network were observed at the maximum values of reorganization time (difference between final and initial time) and in the environment with the lowest repulsion forces of an electrostatic nature.

The gels formed at incubation temperatures of 4, 10 and 25 ˚C showed similar stress at rupture values but different final pH values at fixed sodium caseinate concentration, showing the effect of temperature on the balance of the interaction forces during a long ageing time.

Increasing temperatures lead to increasing hydrophobic interactions (entropy-driven interaction) and electrostatic repulsion, but decreasing hydrogen bonding (attractive interactions) [5, 31]. Thus, the balance in the interaction forces would be shifted more towards repulsion and a weakening of the matrix [5], which is ultimately observed in gels formed at 40 ˚C.

Incubation temperature did not have the same effect on strain at rupture (Fig. 4) as occurred for stress at rupture (Fig. 2). A trend of decreasing strain at rupture during acidification was observed up to a steady state value, which was similar to the time taken to reach final pH. Such behavior was more pronounced at higher CS concentrations.

Gels containing 2% (w/w) CS and formed at temperatures of 4, 10 and 25 ˚C showed smaller strain at rupture values (about 0.1 to 0.3) and were weaker than those containing 4 (results not shown) and 6% (w/w) (strain at rupture in the range from 0.4 to 0.8, for all temperatures). Thus, gel deformability was a function of network density due to macromolecule concentration and final pH, in contrast to hardness, which also depended on the acidification rate and time for particle rearrangement.

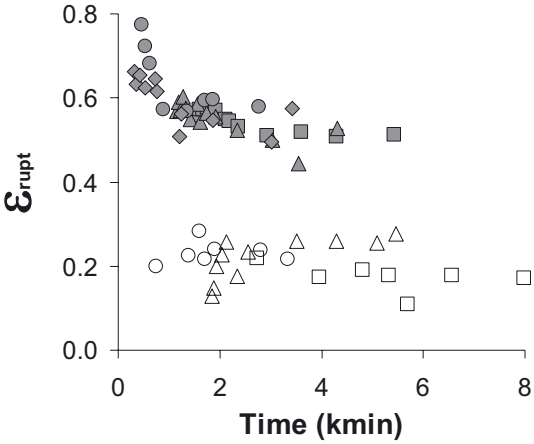

Figure 4. Effect of acidification time on the strain at rupture (ε_{rupt}) of sodium caseinate gels acidified with GDL at incubation temperatures of 4 (□), 10 (△), 25 (○) and 40 ˚C (◊). The open and solid symbols are CS at 2% and 6% (w/w), respectively.

3.2. Gel properties at the steady state of pH: microstructure and syneresis

Gels containing 6% (w/w) sodium caseinate and formed at different incubation temperatures were chosen to evaluate the microstructure at the steady state of pH, as they were firmer and showed the same trend as the 2 and 4% gels. Figure 5 shows the micrographs of the 6% (w/w) sodium caseinate gels. There are striking differences between the microstructures, showing the effect of increasing the temperature on the organization and strand formation in the casein particle gel networks (Figs. 5C and 5D). The acidified casein gels consisted of a coarse particulate network of casein particles linked together in clusters, chains and strands, as reported by Kalab et al. [16].

At 4 ˚C, the gel network appeared to be fairly homogeneous, with smaller pores than the other gels formed at higher temperatures, but it showed a similar rigidity to the gels formed at 10 and 25 ˚C. As a general trend, above 10 ˚C, a more particulate structure was observed, and increasing temperatures resulted in an increase in the pore size in the gel network. In addition, the formation of a gel network at 4 ˚C can be clearly observed in Figure 5A, unlike the reports of Roefs et al. [25], and de Kruif and Roefs [8]. These authors showed an inhibition of gelation of the casein micelles at temperatures below 10 ˚C and at pH values near the pI (4.6), as a consequence of decreased aggregation reactions due to a marked reduction in hydrophobic interactions under such conditions [24].

The gelation of sodium caseinate particles observed at 4 ˚C was probably the result of the aggregation of casein fractions released into the serum, mainly β- and κ-casein, promoted by an intense reorganization process due to a slow acidification rate [8, 13, 32]. At lower temperatures, the hydrophilic negatively charged region of the β-casein protrudes from the micelle surface due to reduced hydrophobic interaction [32], increasing the density of the hairy surface layer. When the pH value is reduced towards pI, neutralization of the negative charge causes a decrease in electrostatic repulsion [32], promoting more intensive protein-protein interactions and leading to a gel network formation [24, 25].

As a general trend, high values for syneresis, from 35 to 82% (w/w), were observed (Fig. 6). Gels containing 2% (w/w) sodium caseinate exhibited the highest values for syneresis and the influence of incubation temperature was not

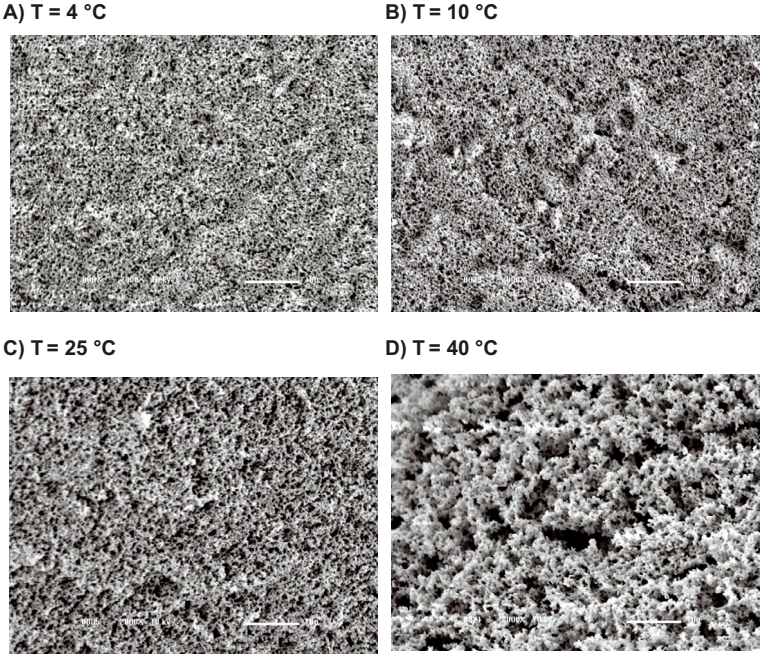

Figure 5. Scanning electron micrographs of acidified sodium caseinate gels (6% (w/w)) formed at incubation temperatures of A) 4 ˚C, B) 10 ˚C, C) 25 ˚C and D) 40 ˚C. Magnification is 2000× and the scale bar = 10 μm for all the micrographs.

observed (Fig. 6). Gels containing higher sodium caseinate concentrations showed lower syneresis values (Fig. 6), probably due to the greater amount of hydrophilic sites able to bind water molecules and more interconnected capillary pores.

At higher sodium caseinate concentrations (4 and 6% (w/w)) an increase in the syneresis values was observed with an increase in incubation temperature from 4 to 25 ˚C, when stronger gels were also observed (Figs. 6 and 7). However, a further increase in temperature to 40 ˚C led to a decrease in syneresis (Fig. 6), in spite of the more fragile gel network (Fig. 7).

Lower syneresis values were observed at lower incubation temperatures, mainly at 4 and 10 ˚C (Fig. 6), which could be related to the higher amount of dissociated β- and κ-casein from sub-micelles released into the serum [3, 7] during the acidification process prior to reaching the final pH value. The κ-casein molecule has a strongly hydrophilic portion, favoring hydrogen bonding between water-protein [28]. Interestingly, at 25 ˚C the effect of sodium caseinate concentration on lowering spontaneous syneresis was not observed, probably due to the influence of the temperature on the protein-water interactions (Fig. 6) despite the differences observed in the gel network hardness at steady state (Fig. 7).

A correlation between the syneresis values and stress at rupture (σ_{ss}) did not show a clear relation between the hardness of the gel network and its ability to retain water in the matrix and avoid spontaneous syneresis. One would expect a stronger gel network, which is mainly maintained by dense strands formed by macromolecular interactions, to show a greater amount of available

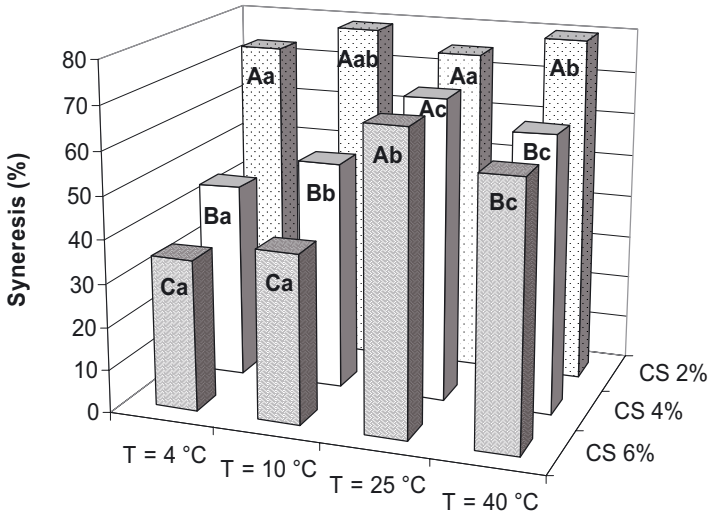

Figure 6. The influence of incubation temperatures (T: 4, 10, 25 and 40 ˚C) and sodium caseinate concentrations (CS: 2, 4 and 6% (w/w)) on gel syneresis. Capital letters: differences ($P < 0.05$) among the CS concentrations at the same incubation temperature and small letters: differences ($P < 0.05$) among the incubation temperatures at the same CS concentration.

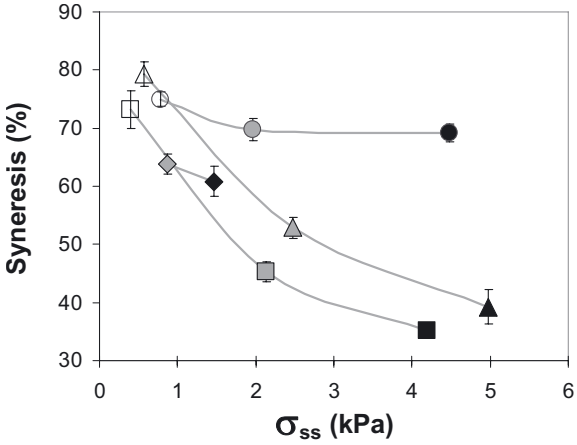

Figure 7. Relationship between the stress at rupture and syneresis of sodium caseinate gels acidified with GDL at the steady state of pH. The incubation temperatures are 4 (□), 10 (△), 25 (○) and 40 ˚C (◊) and the sodium caseinate concentrations are represented by colors: white, gray and black, representing 2, 4 and 6% (w/w), respectively.

sites for holding water than a weaker gel network, which was indeed the case for gels formed at 4 and 10 ˚C. Possibly, the weaker structured gels could only support a small stress, probably due to their low strand density, which would be more ineffective at entrapping the free water. However, the results observed for gels formed at 25 ˚C showed that their more porous structure was an indication of lower water-holding capacity in spite of their harder structure. Thus, the syneresis properties were probably a complex result of the effect of incubation temperature on the balance between the attractive and repulsive interactions between protein-protein and

protein-water. In addition, the effect of the final pH at steady state of the gels could also influence the kind of protein-protein interaction and water-protein affinity.

4. CONCLUSIONS

The results of this study showed that the acid-induced gelation of sodium caseinate induced by GDL was affected by the concentration, and by the incubation temperature because of the changing acidification rate. Increasing sodium caseinate concentrations caused higher pH values at steady state, higher rates of acidification and gel network formation, higher stress at rupture at steady state and less syneresis. On the other hand, increasing incubation temperatures led to higher rates of acidification and gel network formation, but greater syneresis and hardness of the gel network, due to a more porous network. Stronger gels and greater rearrangement of the network were observed for final pH values close to 4.6 and with lower acidification rates, which caused a longer ageing time. The most important interactions between the proteins involved in gel microstructure stabilization were mainly electrostatic, followed by hydrophobic and hydrogen bonds, which influenced the protein-protein and protein-water interactions. Gels formed at 10 °C showed high hardness with less syneresis for the same sodium caseinate concentration, due to the balance between the attractive and repulsive forces, promoted by the long time for rearrangement at pH close to 4.6. Thus, sodium caseinate acidification at concentrations of 4 and 6% (w/w) could be considered the most optimal for practical (industrial) processes, because of their particular texture and water-holding capacity.

Acknowledgements: This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil (FAPESP, Grant No. 04/08517-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

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