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Heat induced gelation of acid milk: balance between weak and covalent bonds

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Gelation of acidified milk at pH ⩾ 5, after heat treatments is a well known phenomenon, due to the precipitation of whey proteins, and especially β-lactoglobulin onto κ-casein (Sawyer, 1969). High heat treatments cause denaturation of whey proteins which associate with κ-casein through disulphide interchange reactions (Hill, 1989). Since their charge is reduced, the denatured proteins associated with casein micelles become susceptible to aggregation when milk is then acidified, which promotes enhanced protein–protein interactions (Lucey et al. 1997). The gelation phenomenon involves disulphide bonds (Hashizume & Sato, 1988; Goddard, 1996) which are responsible for the gel firmness (Goddard, 1996). However, other interactions between proteins can occur, such as hydrogen and hydrophobic bonds, especially at the initial stage of interactions (Haque et al. 1987; Haque & Kinsella, 1988; Jang & Swaisgood, 1990). It is therefore relevant to investigate a possible contribution of weak linkages to the gel structure and firmness.

In the present study, the balance between disulphide bonds and other linkages has been studied. Different reagents (urea or SDS) have been added to skim milk in order to affect weak linkages in heat induced gels of acid milk (i.e. hydrogen and hydrophobic bonds). 2-mercaptoethanol was added to milk to increase the gel firmness (Goddard, 1996) through the promotion of interchange between disulphide bonds. The different reagents have been added alone to measure their influence on the gel firmness compared with untreated controls. The reagents have also been added in combination to measure the balance between the influence of disulphide and weak linkages on the gel firmness. Finally, dynamic oscillatory trials were carried out, with or without urea, to investigate parameters of the kinetics during thermal gelation.

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Materials and Methods

Milk preparation

Skim milk powder was obtained from Lovelait (St Martin de Belleroche, France); $CaCl_2$, urea, and SDS were obtained from Prolabo (Paris, France) and 2-mercaptoethanol, (MeSH) from Sigma (Saint Quentin Fallavier, France).

The skim milk powder was dissolved at a final concentration of 150 g/l into deionised water with sodium azide (1 g/l) as preservative. $CaCl_2$ was added (30 mM), the pH was adjusted with 1 M-HCl to 5·0, 5·5, or 5·9, and the final solution was kept overnight at 4 °C. Samples were then placed in 150 ml beakers, and the pH was verified and readjusted if necessary. As required, 5 mM-MeSH, 22 or 44 mM-SDS (which corresponded to 5 or 10 g/l) or 1 or 4 M-urea (which are low concentrations compared with classical 6 M-urea used for denaturation of micelles in gel electrophoresis) were added, alone or in combination (5 mM-MeSH/1 M-urea; 5 mM-MeSH/22 mM-SDS).

Physical analysis

Compression test. Gelation of milk was performed in an incubator at 100 °C for 1 h. Skim milk gels were then cooled in a water bath at 20 °C for 1 h before physical measurements. Compression tests were performed with a Lloyd TA500 apparatus (Lloyd, 78180 Montigny le Bretonneux, France) fitted with a 10 N sensor and a cone of 45° made of Plexiglass. The procedure involved a penetration step of 17 mm into the sample at a speed of 0·75 mm/s, and a holding time of 40 s in the sample. A typical evolution of strength versus time was a first sharp increase followed by a slow decrease. The data monitored were the maximum force (F*max,* N), and the work of the compression step (W, mJ).

Dynamic oscillation. Gelation was followed by dynamic rheological measurements on an AR1000 Texture Analyser (TA Instrument, F78056, Saint-Quentin-en-Yvelynes) with

a cone-plate geometry plate 92-mm diameter; cone 60-mm diameter, a = 0·069 rd). The liquid milk sample was placed in the geometry, covered with paraffin oil and stored 10 s at 25 °C. It was heated in the geometry from 25 to 100 °C at 5 deg C/min (duration 15 min), Then the sample was immediately cooled to 25 °C at 20 deg C/min (duration 4 min) and stored at 25 °C for 2 min. Measurements at 1 Hz at 0·1% strain were performed during the gelation process in duplicate or triplicate with different preparations of milk during heating and cooling steps. The temperatures are those measured at the surface of the plate and are not far from the temperature of the 5 ml liquid. Gelation was defined as the point when elastic modulus, G′, had reached 1 Pa (van Marle & Zoon, 1995). Values of G′ at 25 °C at the end of the cooling step were also reported.

Statistics

Statistics were performed with SPSS software (Microsoft); ANOVA was performed using the value of the residual variance as the actual variance between data. Then the LSD test was applied at 5% signification level.

Results and Discussion

Influence of the pH on gel firmness

Table 1 presents the data obtained for the physical analysis of gels. The F*max*, work, W and G′ at 25 °C, decreased as the pH increased excepted at 5·0. These results were in agreement with those obtained by Goddard & Augustin (1995). Gels obtained at pH 5·0 did not exhibit the same behaviour, which was also reported by Goddard & Augustin (1995) at pH 5·1. This pH is close to the pI which leads to an aggregated weak and excudative gel. Dynamic shear oscillations showed a much lower gelation temperature of 45 °C at pH 5·0, instead of 60 or 65 °C at pH 5·5 and 5·9 respectively (Fig. 1). Similar differences between pH 5·0 and other pH were also noticeable for tan δ at 25 °C which was significantly higher at pH 5·0 ($P<0·05$; Table 1) which suggested a modification of the gel structure.

During heat treatment of milk, the diameter of casein micelles increases as does the amount of colloidal calcium phosphate (Singh & Creamer, 1991). Some precipitation of insoluble calcium phosphate can occur (Walstra & Jenness, 1984), but nevertheless, the release of calcium phosphate from the micelles is still believed to be controlled by the pH (Augustin & Clarke, 1991). The difference in gelation temperature is therefore mainly due to the decrease of electrostatic repulsions between proteins which is modified by the previous aggregation of whey proteins (Lucey et al. 1997). The decrease of the gel strength at pH 5·0 was probably due to the type of acidification used in the study, which was not progressive and did not promote a micelle disruption that occurs at pH 5·2 (Le Graët & Brulé, 1993) or below. At pH 5·0 the low gelation temperature was reached rapidly (Fig. 1) and the gel was very excudative and difficult to measure. Furthermore Hashizume & Sato (1988) have also noticed a difference in the behaviour of GDL gels or acid precipitates (both at pH 4·5) regarding their solubility, especially in urea. This fact underlines the influence acidification method. In fact, in our case, we presume the use of HCl at this pH enhanced protein–protein interactions which makes the gelling phenomenon possible, but lowered protein–water interaction which gave an excudative structure.

Table 1. Compression test parameter [maximum force (F*max*), work done during 17 mm compression (W)], and dynamic oscillation parameter (elastic modulus, G′ and tan δ) values of heat-set gels prepared at various pH

Values are means ± SD for $n=3$

	F*max* (N)	W (mJ)	G′ (Pa)†	Tan δ†
pH 5·0	$0·96 \pm 0·04^{a}$	$2·17 \pm 0·06^{a}$	118 ± 56^{a}	$0·2839 \pm 0·0012^{a}$
pH 5·5	$1·93 \pm 0·13^{b}$	$4·07 \pm 1·2^{b}$	689 ± 81^{b}	$0·2729 \pm 0·0027^{b}$
pH 5·9	$1·33 \pm 0·09^{c}$	$3·19 \pm 0·77^{c}$	510 ± 28^{c}	$0·2714 \pm 0·0029^{b}$

† Elastic modulus, G′ and Tan δ measured the end of the cooling step at 25 °C

[a,b,c] Values within the same column with different superscripts were significantly different, $P<0·05$

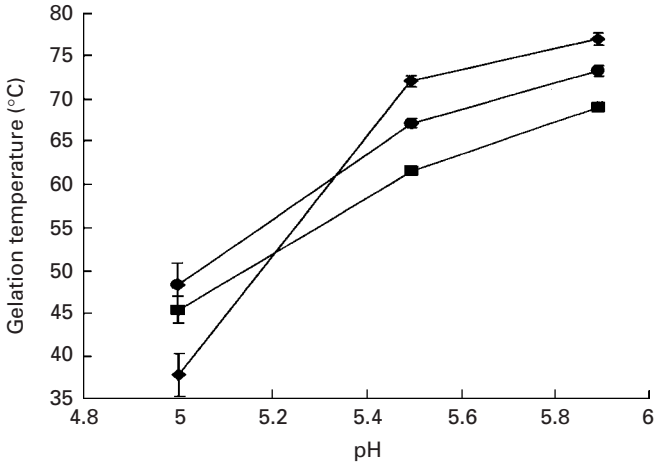

Fig. 1. Changes in the gelation temperature versus pH ■, without urea addition (control); ●, with 0·5 M-urea and ◆, 1 M-urea. The gelation temperature was defined as the point where G′ = 1 Pa.

Thermotropic gelification of reconstituated skim milk at 150 g/l. Heat treatment: 25 to 100 °C at 5 degC/min (15 min duration), then cooling step to 25 °C at 20 degC/min (4 min duration) and storage at 25 °C for 2 min. Measurements at 1 Hz at 0·1% with a cone plate rheometer. Mean of 3 experiments, bars represent standard deviation.

Influence of chemical additives

Variance analysis exhibited significant effects of both pH and reagents. What was more interesting, was the

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Table 2. Effect of addition of 2-Mercaptoethanol (MeSH), urea, or SDS to skim milk on the texture (maximum force in compression, F*max*) of heat-set gels prepared from milk adjusted to various pH

Values are means ± SD for $n=3$

	pH 5·0	pH 5·5	pH 5·9
Control	$0{\cdot}93 \pm 0{\cdot}22^{a}$	$1{\cdot}93 \pm 0{\cdot}13^{a}$	$1{\cdot}33 \pm 0{\cdot}09^{a}$
MeSH	$1{\cdot}74 \pm 0{\cdot}02^{b}$	$2{\cdot}19 \pm 0{\cdot}30^{b}$	$1{\cdot}53 \pm 0{\cdot}23^{b}$
1 M-urea	$0{\cdot}95 \pm 0{\cdot}21^{a}$	$0{\cdot}46 \pm 0{\cdot}17^{c}$	$0{\cdot}39 \pm 0{\cdot}08^{c}$
4 M-urea	$0{\cdot}05 \pm 0{\cdot}005^{c}$	$0{\cdot}05 \pm 0{\cdot}005^{d}$	$0{\cdot}07 \pm 0{\cdot}02^{c}$
22 M-SDS	$0{\cdot}55 \pm 0{\cdot}05^{d}$	$0{\cdot}12 \pm 0{\cdot}08^{d}$	$0{\cdot}047 \pm 0{\cdot}01^{d}$
44 M-SDS	$0{\cdot}36 \pm 0{\cdot}17^{d}$	$0{\cdot}06 \pm 0{\cdot}01^{d}$	$0{\cdot}046 \pm 0{\cdot}01^{d}$
MeSH/1 M-urea	$0{\cdot}85 \pm 0{\cdot}08^{a}$	$0{\cdot}64 \pm 0{\cdot}025^{c}$	$0{\cdot}99 \pm 0{\cdot}03^{c}$
MeSH/22 M-SDS	$0{\cdot}497 \pm 0{\cdot}07^{d}$	$0{\cdot}42 \pm 0{\cdot}08^{c}$	$0{\cdot}50 \pm 0{\cdot}045^{e}$

[a,b,c,d,e] Values within the same column with different superscripts were significantly different, $P<0{\cdot}05$

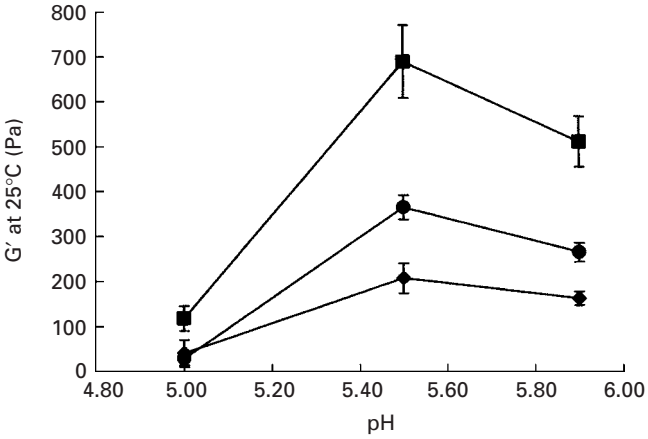

Fig. 2. Elastic modulus, G′ at 25 °C at the end of the cooling step versus pH ■, without urea addition (control); ●, with 0·5 M-urea and ◆, 1 M-urea.

Thermotropic gelification of reconstituated skim milk at 150 g/l. Heat treatment: 25 to 100 °C at 5 degC/min (15 min duration), then cooling step to 25 °C at 20 degC/min (4 min duration) and storage at 25 °C for 2 min. Measurements at 1 Hz at 0·1% with a cone plate rheometer. Mean of 3 experiments, bars represent standard deviation.

significance of the interaction, which suggested a combined effect of pH and reagents on the gelation phenomenon. This could be linked to the well known modification of micelle structure with pH.

In agreement with Goddard (1996), F*max* (and W) increased with the addition of MeSH at pH 5·9. Nevertheless the magnitude of the increase was lower (1·12 compared with 2·6; Goddard, 1996), because of a difference in the preparation of milk powder and in the measurement of gel strength. On the contrary, we observed a decrease of F*max* (and W) after addition of urea or SDS at any concentration. Results are presented in Table 2. The amount of urea and SDS (1 M and 22 mM respectively) needed to obtain a decrease in the physical characteristics of the gels was drastically higher than the amount of NEM (N-Ethylmaleimide) needed to obtain the same phenomenon. Goddard (1996) has shown that at 0·2 mM-NEM there is a plateau of a minimum gel strength. He used high-heat milk powder at 200 g/l. According to Guingamp et al. (1993) this may contain, 0·05–0·39-mM free sulphydryl groups; 0·05-mM for high temperature treated milk (probably the minimum after a very strong treatment) 0·39-mM for raw milk (the maximum level in the absence of heat treatment). The samples tested by Goddard (1996) may have had an intermediate level of free sulphydryl groups, since the concentration of the blocking agent is supposed to be sufficient to mask all free sulphydryl groups. Nevertheless gel formation still occurs, even if there are no interchange of disulphide bonds. He concludes that other interactions between proteins i.e. hydrogen and hydrophobic bonding must be involved in the formation of the gels. This is consistent with our results, since urea significantly decreased gel firmness and SDS did not allow gel formation at pH 5·9 and 5·5. However it was necessary to use rather high SDS concentration, which suggested that formation of a large number of bonds had to be overcome. It was possible to partially prevent loss of gel firmness by adding MeSH to the milk with either urea or SDS. However, the resulting gel exhibited a lower firmness than the control (Table 2).

Since urea is known to have an effect both on hydrogen and hydrophobic interactions (Lefebvre et al. 1998), only this reagent was used in dynamic oscillation studies. The results confirmed compression tests, since in most cases urea addition to milk led to an increase of the gelation temperature (Fig. 1), which suggests that more energy was needed to obtain a gel if weak interactions between proteins were reduced. The elastic modulus G′ at the end of the cooling step (Fig. 2) exhibited the same tendency; its value was lower when urea was added. These results also suggested that weak interactions were involved in the creation of the gels.

The behaviour of gels at pH 5·0 was still different with urea since the gelation temperature did not increase with this reagent. This was probably also due to the importance of electrostatic phenomena, when the pH decrease towards 4·5. In fact at pH 5·0 the micelles have probably principally started to disrupt as submicelles, and 1 M-urea was responsible for the disruption of the residual micellar structure. Thus enhanced protein–protein interactions led to the onset of a very weak gel.

During the onset of acid milk gellation, interchange of disulphide bonds occurs and control basically the firmness of the heat-set acid-milk gels. In fact this has been identified quite easily by other authors since the amount of interchange is basically dependent on the intensity of heat treatments. Furthermore, the energy of this kind of linkages (330–380 KJ/mol) contributes to the stability of the gels and a small quantity of linkages broken can induce a drastic decrease of the gel firmness. On the contrary it

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is necessary to disrupt large quantities of weak bonds (of which energy is low 4–40 KJ/mol) to significantly affect the gel structure. This could suggest probably the contribution of weak bonds inside the micelle structure (such as suggested by Lefebvre-Cases et al. 1988), whereas disulphide interchange could be rather involved in the skeleton of the gels. Thus, if the micelle structure is affected, it is no more possible to create a solid skeleton in the gel.

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