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Original Manuscript

Enzymatic cross-linking of casein facilitates gel structure weakening induced by overacidification

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Abstract

Casein (α_{S1}, α_{S2}, β, κ) is the major protein fraction in milk and, together with heat denatured whey proteins, responsible for gel network formation induced by acidification. Rheological measurements during gelation typically reveal a maximum storage modulus (*G′*) at a pH close to the isoelectric point (pI) of casein (~4.6). With further decreasing pH gel stiffness decreases because of increased electrostatic repulsion, which is referred to as overacidification. In this study we investigated the effect of casein cross-linking with microbial transglutaminase on gel structure weakening induced by acidification to pH below the pI. Although enzymatic cross-linking increased the maximum stiffness (G'_{MAX}) of casein gels the reduction of *G′* during overacidification, expressed as ratio of the plateau value (G'_{FINAL}) to G'_{MAX}, was more pronounced. Almost no soluble protein was detected in the serum of gels from cross-linked casein, whereas considerable amounts of α_S- and κ-casein were released from reference gels below the pI. This suggests that covalent cross-linking of casein retains charged molecules within the gel network and therefore causes a higher reduction of protein-protein interactions because of higher electrostatic repulsion. Furthermore, higher amounts of uncross-linked β-casein, which was the only casein type not found in the serum, resulted in higher G'_{FINAL} to G'_{MAX} ratios, underlining the important contribution of β-casein to acid gel formation and prevention of gel structure weakening.

Keywords:

Casein; Gelation; Cross-linking; Transglutaminase; Rheology; Isoelectric point

1 Introduction

Casein is the major protein fraction in milk, where it is assembled to colloidal aggregates with a diameter from ~50 to 500 nm, the so called casein micelles. They consist of about 5,000 casein molecules and ~6 % colloidal calcium phosphate which contributes to micelle integrity through electrostatic interactions. Casein in milk is insoluble at pH 4.6, which is referred to as isoelectric precipitation, and the respective pH might therefore be called isoelectric point (pI) [1]. The pI is equivalent to the pH at zero net charge of a molecule, which is sometimes associated with a dramatic decrease of protein solubility. Progressive acidification of casein to pH 4.6, induced by either microorganisms or chemical acidulants such as glucono-δ-lactone (GDL), results in the reduction of casein net charge and, in case of casein micelles, in the loss of steric repulsion because of micelle disintegration so that attractive interactions can cause gelation. Overacidification, for example achieved through high concentrations of GDL, means that the net charge of casein molecules increases again. This leads to reduced protein-protein interactions within the gel network because of increased electrostatic repulsion [2]. When this process is monitored in time-based small strain oscillation rheometry, gel stiffness shows a maximum at a pH close to the pI of casein, which is followed by a decrease of storage and loss modulus *(G'* and *G")* with further decreasing pH [3, 4]. After homogenising gels in demineralised water, Braga et al. [5] detected that overacidification also causes dissociation of casein molecules into the serum. We previously reported similar findings for acid-induced soy protein gels by analysing the expelled liquid after centrifugation [6].

Besides heat-denatured whey proteins, four casein types with different hydrophobicity and charge are mainly involved in milk gel network formation, namely α_{S1}-, α_{S2}-, β-, and κ-casein. As regards the pI of casein it is important to note that pH at zero net charge of the individual caseins as estimated from their primary structure is higher than pH 4.6. However, because α_{S1}-, α_{S2}-, and β-casein are phosphorylated, experimentally observed pIs are ~4.4, ~4.9, and ~4.7, respectively [7]. However, for κ-casein a pI of ~5.6 – 5.8 has been reported [8].

Studies related to the contribution of individual caseins to acid-induced gel networks were already conducted. For example, Holland et al. [9] found no differences in the gelation behaviour of β-casein depleted and regular milk. On the other hand, O'Kennedy et al. [10] observed a higher stiffness of acid sodium caseinate gels enriched with α_{S1}- and β-casein. Furthermore, Portnaya et al. [11] and Moitzi et al. [12] reported that β-casein associates to disc-like micelles of approx. 6 – 8 monomers at pH 2.6, and McCarthy et al. [13] found that

β-casein is not completely soluble below its pI. This suggests that β-casein may positively affect the strength of casein gels through its relatively high hydrophobicity even at low pH. However, the effect of casein composition on gel properties during overacidification has not been investigated yet.

Microbial transglutaminase (mTGase; EC 2.3.2.13) catalyses the formation of cross-links between γ-carboxamide and ε-amino groups of protein bound glutamine and lysine residues, respectively, resulting in the polymerisation of proteins [14]. mTGase can be applied for enhancing texture and physical properties of acidified dairy products [15-17], and we previously suggested that casein cross-linking reduces structure weakening of acid gels during overacidification [18]. However, these gelation measurements were stopped before gel formation reached a steady state, so that this hypothesis was not completely ascertained.

With the current study we aim to comprehensively investigate how, during overacidification, casein gel structure is affected by both the presence of mTGase-induced cross-links and by the β-casein content, which was varied through addition of a β-casein rich fraction isolated from skim milk. Small strain oscillation rheometry was used to quantify structure weakening during overacidification. Special emphasis was placed on caseins released from the gel network because of electrostatic repulsion. The results provide fundamental insights into the interrelations between molecular rearrangements and casein gel stiffness below the pI.

2 Material and methods

2.1 Materials

Commercial acid casein (crude protein content 868 g/kg, Kjeldahl method, N x 6.38 [19]), and α_S-, β-, and κ-casein standards were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Low heat skim milk powder, crude protein content 340 g/kg, was obtained from Sachsenmilch Leppersdorf GmbH (Leppersdorf, Germany). mTGase (Activa MP from *Streptomyces mobaraensis*, 100 U per g powder) and glucono-δ-lactone (GDL) were received from Ajinomoto Foods Europe SAS (Paris, France) and Kampffmeyer Nachf. GmbH (Ratzeburg, Germany), respectively.

2.2 Preparation of β-casein poor and β-casein rich casein powder

Skim milk powder was reconstituted in demineralised water at 100 g/kg, and 0.3 g/kg sodium azide were added for preservation. The sample was kept in a water bath at 2.5 °C for at least 15 h to facilitate dissociation of β-casein from the casein micelles [20]. The reconstituted milk was then separated by cross-flow filtration [21, 22], 0.1 µm polyethersulfone membrane (Sartorius AG, Göttingen, Germany) with compensating the retentate concentration by adding one volume of refrigerated demineralised water. The retentate was kept at 2.5 °C and the process repeated on the next day to increase separation efficiency. After warming to room temperature, retentate and permeate were acidified to pH 4.6 with 6 mol/L HCl. The precipitated casein was then separated from the whey by filtration through cellulose filters (Rotilabo type 600P, Carl Roth GmbH+Co. KG, Karlsruhe, Germany), excessively washed with demineralised water, removed from the filter and freeze dried (Martin Christ GmbH, Osterode am Harz, Germany). Retentate and permeate powder had a crude protein content of 949 and 952 g/kg (Kjeldahl method, N x 6.38 [19]), and are further referred to as β-casein poor and β-casein rich fraction, respectively.

2.3 Preparation of casein solutions and treatment with mTGase

All acid casein powders were dispersed in 0.1 mol/L sodium phosphate buffer (pH 6.8), preserved with 0.3 g/kg sodium azide, and stirred over night at room temperature. The target protein concentration of all solutions was 27 g/kg.

Cross-linking in casein solutions was achieved by incubation with 3 U mTGase per g casein at 40 °C for 5 or 24 h. Subsequent enzyme inactivation was performed at 85 °C for 10 min, followed by cooling in ice water. Uncross-linked references were treated in the same way without enzyme addition and are referred to as 0 h.

Adjustment of the β-casein content was achieved by blending uncross-linked or cross-linked β-casein poor solution with uncross-linked β-casein rich solution in ratios of 70:30, 80:20, 90:10, or 100:0, with 90:10 roughly corresponding to the status of the original skim milk (this was estimated from the weight of the achieved powders). To obtain a similar number of cross-links in the samples, always 70 % of cross-linked β-casein poor casein solution was used in the respective mixtures and complemented with uncross-linked β-casein poor casein solution (Table 1). All mixtures were prepared separately for individual measurements.

2.4 Small strain oscillation rheometry

A strain controlled ARES RFS3 rheometer (TA Instruments, Eschborn, Germany) with a concentric cylinder geometry (d_i = 32 mm, d_o = 34 mm, h = 33.5 mm) was used for gelation monitoring. Temperature equilibrated casein solutions were blended with different amounts of GDL (30 – 120 mg per g casein solution), transferred into the rheometer geometry, and the sample surface covered with paraffin oil to prevent evaporation. The storage modulus G' (Pa) and the loss factor tan δ (-) were recorded every 60 s at constant strain amplitude (γ = 0.003) and frequency (ω = 1.0 rad/s), and temperature was maintained constant at 20, 30, or 40 °C. Measurements were stopped when G' decreased less than 0.5 % within 5 min. pH development during acidification was recorded in separate samples using a pH meter with memory function (InoLab, WTW GmbH, Weilheim, Germany). All results are mean values from duplicate experiments; the half deviation range was always < 5 % of the mean value.

2.5 Detection of soluble casein in the serum

Casein molecules, released from the gel network during overacidification, were measured according to Schuldt et al. [6] with slight modifications. Casein solutions were blended with 30 or 60 mg GDL per g and transferred into centrifugation tubes, which were kept in a water bath at 20, 30, or 40 °C until equilibrium pH was reached (~6 h). After cooling in ice water and centrifugation at 1000 x g for 20 min at 6 °C (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatants were carefully removed and filtered through a 0.45 µm cellulose syringe filter (Sartorius AG, Göttingen, Germany). The casein fractions in the serum were identified by gel electrophoresis and size exclusion chromatography.

2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a vertical equipment from C.B.S. Scientific Company Inc. (Del Mar, CA, USA). Separating and stacking gel contained 125 mg/mL and 40 mg/mL polyacrylamide, respectively, and were prepared according to Jaros et al. [23]. The casein samples were diluted in a 1:1 mixture of 8 mol/L urea and sample buffer (0.8 mol/L Tris, 4 mol/L glycerine, 20 mg/mL SDS, pH 8.0), and boiled for 5 min in the presence of dithiothreitol to cleave disulphide bonds (reducing SDS-PAGE). SDS-PAGE was run at 100 V, and bands were subsequently stained with Coomassie Brillant Blue. Electropherograms were semi-quantitatively evaluated regarding band intensities using TL100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine mass ratios between protein fractions.

2.7 Size exclusion chromatography (SEC)

SEC (AZURA Assistant ASM 2.1L, Knauer Wissenschaftliche Gerätebau GmbH, Berlin, Germany; Superdex 200 Increase 10/30 column, GE Healthcare, Uppsala, Sweden) was additionally used to detect monomeric and polymeric caseins in the serum separated by centrifugation. The samples were blended with elution buffer (6 mol/L urea, 0.1 mol/L NaCl, 0.1 mol/L Na_2HPO_4, 1 g/L CHAPS, pH 6.8) and treated with dithiothreitol to cleave disulphide bonds. Chromatographic separation was performed at room temperature with an isocratic flow rate (0.5 mL/min) and casein was detected at 280 nm.

2.8 Summary of experimental design

Two sets of gelation experiments were performed at similar conditions: acidification temperature was 20, 30, or 40 °C, and GDL concentration was 30 – 120 mg/g. In set 1, solutions of commercial casein, cross-linked for 0, 5 or 24 h, were used for obtaining base data. Set 2 comprised all the samples identified in Table 1.

3 Results and discussion

3.1 Acid-induced gelation of casein solutions

Fig. 1a illustrates the pH development in casein solutions after adding different amounts of GDL at 30 °C. mTGase-treatment does not affect the acidification profile [23, 24], ensuring the same pH course for all samples acidified at a particular GDL concentration and temperature. The equilibrium pH (pH_{EQ}) decreased from ~3.6 to ~2.7 (below the pI of casein) when increasing amounts of GDL were applied, but was not affected by gelation temperature (not shown).

Fig. 1b shows the gelation curves of uncross-linked and cross-linked casein solutions, acidified with 40 mg/g GDL at 30 °C. Gelation starts at a particular onset-pH and, subsequently, G' increases until a maximum in gel stiffness (G'_{MAX}) at pH ~4.0 – 4.2 is reached. Ongoing pH decrease results in a decrease of G' which then approaches a plateau. As previously reported, maximum gel stiffness increases after moderate casein cross-linking (e.g., 5 h incubation with mTGase), but decreases when cross-linking is excessive (e.g., for 24 h) [23, 25, 26]. This relation is neither affected by acidification temperature nor by GDL concentration although absolute values may differ [18, 27]. The present work, however, has

its focus on what happens to the gels when acidification is continued after maximum stiffness was exceeded.

Three key parameters were extracted from the gelation curves (Fig. 1b) and used for further characterisation of the samples: the maximum gel stiffness G'_{MAX}, the final gel stiffness G'_{FINAL}, and the corresponding pH at G'_{FINAL}. The ratio of G'_{FINAL} to G'_{MAX} serves as parameter to quantify structure weakening induced by overacidification. The storage modulus G' is related to the number and strength of bonds within the gel network [28], hence G'_{FINAL}/G'_{MAX} represents the fraction of bonds which remain, and $(1 - G'_{FINAL}/G'_{MAX})$ corresponds to the fraction of bonds that are disrupted during overacidification.

A peak in the tan δ profiles was observed at pH ~3.75 when samples underwent structure weakening during overacidification (Fig. 1c). In case of acid-induced milk gels, peaks in tan δ at the beginning of gelation (pH ~5.2) have been attributed to the solubilisation of colloidal calcium phosphate [29], or to a transition from a gel network dominated by denatured whey proteins to a network dominated by casein-casein-interactions [30]. However, both interpretations do not apply for the current study since we used acid casein, and the peak was observed at a lower pH. In general, higher tan δ values imply a more viscous character, indicating a relaxation of bonds and particle rearrangements [31]. The increase of tan δ at pH ~4.0 goes well with the decrease of G' as it underlines reduced protein-protein interactions because of increased electrostatic repulsion below the pI. However, tan δ decreased again while G' was also decreasing and, in contrast to G', did not reach a plateau value within the measurement period. As can be seen from the insert in Fig. 1c, the peak in tan δ coincided with a maximum in the first derivative of G' after pH, indicating that further increasing electrostatic repulsion at lower pH slows down structural changes and impedes particle rearrangements.

3.2 Effect of enzymatic cross-linking on gel structure weakening

As indicated by lower G'_{FINAL}/G'_{MAX} ratios of the gels (Fig. 2), structure weakening was more pronounced at higher GDL concentrations, certainly because of the lower pH_{EQ} and the associated higher electrostatic repulsion between casein molecules which impede protein-protein interactions [2]. In addition, structure weakening was more pronounced for samples with higher cross-linking intensity, and the reference (0 h) showed a distinct dependence on acidification temperature: some structure weakening was visible at 20 °C and 30 °C but, at 40 °C, G' did not reach a maximum during gelation. Gels from the sample incubated with mTGase for 24 h were also more weakened with decreasing acidification temperature,

whereas this could not be observed for the 5 h incubated casein solution. Upon acidification of the 24 h incubated casein with 120 mg/g GDL at 20 °C, *G′* decreased below 1 Pa at pH ~2.8, although the sample still exhibited a loss factor (tan *δ*) of approx. 0.2, indicating gel characteristics. Weaker hydrophobic interactions at lower temperature appear to be a key factor to structure weakening because of providing less resistance to electrostatic repulsion. However, this was not valid for the 5 h sample, indicating that moderate cross-linking may facilitate other interactions such as hydrogen bonds as a consequence of conformational changes. pH at G'_{FINAL} also decreased with the intensity of casein cross-linking. In case of the 24 h sample, pH at G'_{FINAL} was almost as low as pH_{EQ} at the corresponding GDL concentration. Temperature dependence of pH at G'_{FINAL} was generally less pronounced than that of G'_{FINAL}/G'_{MAX}. However, because no local maximum was found in gelation curves of the 0 h sample at 40 °C, the respective pH is not available. Likewise, the peak in tan *δ*, which was observed in all samples that exhibited structure weakening during overacidification, was shifted from pH ~4.1 to ~3.7 with increasing cross-linking intensity, but this pH was not affected by gelation temperature or GDL concentration (not shown), indicating a sample specific behaviour.

Braga et al. [5] observed dissociation of κ-casein from uncross-linked sodium caseinate gels after acidification below its pI (~5.6 – 5.8 [8]). From that it might be assumed that the pronounced structure weakening in overacidified gels from cross-linked casein is caused by the dissociation of high molecular weight casein polymers with larger polymers requiring a lower pH to get released into the serum. However, almost no soluble casein was found in the serum of gels formed from cross-linked casein by SDS-PAGE. In contrast, κ-casein became partly solubilised when the uncross-linked reference was acidified with 30 (pH_{EQ} ~3.6), and α_S-casein was additionally found after acidification with 60 mg/g GDL (pH_{EQ} ~3.1) (Fig. 3). As, however, some authors noted that highly polymerised casein cannot be detected by SDS-PAGE because it may be too large to enter the pores of the electrophoresis gel [32-34], the serum was also analysed by SEC. As can be seen from the chromatograms of the cross-linked casein samples, high molecular weight casein polymers are detected by this method, and analysis of the serum confirms that monomeric casein is more prone to be released from the gel network than cross-linked casein (Fig. 4). Consistent with SDS-PAGE, some dimers (elution at ~19 – 22 min) and degradation products (~26 – 30 min) were also found in the serum of the reference gels. This indicates that the structure weakening observed in the rheological experiments is not connected to the release of casein molecules from the gel network to the serum. The dissociation of charged casein molecules may rather

decrease the electrostatic repulsions in the network and enhance protein-protein interactions between the remaining molecules. Consequently, cross-linking of casein molecules would retain electrostatic repulsion within the network that counteracts to non-covalent attraction and leads to reduced protein-protein interactions as indicated by lower *G′*. It has been reported that covalent cross-links impede rearrangements of casein molecules [24, 35], suggesting that gels from mTGase-treated samples require more time to reach an equilibrium state, resulting in the shift of G'_{FINAL} to lower pH. However, the peak in tan *δ* (see Fig. 1c), which implies a temporary increase in rearrangements during overacidification, was only observed for gels that exhibited structure weakening and was therefore more specific for cross-linked casein.

3.3 Effect of uncross-linked β-casein on gel structure weakening

β-casein was not found in the serum phase of the reference gels (Fig. 3) although its pI (~4.8 – 5.0) is even higher than that of α_{S1}-casein (~4.4 – 4.8) [36]. This leads to the assumption that uncross-linked β-casein contributes most to gel structure because of its high hydrophobicity. Gelation experiments were therefore also performed with casein solutions with different β-casein content (sample set 2). The β-casein rich fraction had a purity of approx. 80 % with only minor impurities (Fig. 3), probably γ-caseins (products of enzymatic β-casein degradation) [13, 32]. Addition of higher amounts of uncross-linked β-casein rich solution to cross-linked β-casein poor solution resulted in considerably higher G'_{FINAL}/G'_{MAX} ratios of the gels at ≥ 60 mg/g GDL, whereas this was not observed at 40 mg/g GDL (Fig. 5). Samples comprised of only uncross-linked casein fractions differed only after gelation at 30 °C but not after gelation at 20 °C, meaning the ß-casein concentration was less important in this case. Independent of the β-casein concentration, no local *G′* maximum appeared during gelation at 40 °C. pH at G'_{FINAL} was hardly affected by the β-casein content, although slightly higher values at higher β-casein concentration might be identified. This is probably related to a shift towards the pI of β-casein.

Marchesseau et al. [38] determined a higher apparent hydrophobicity for β-casein than for κ-casein and α_S-casein between 10 and 25 °C. Although these findings are based on the adsorption of molecules to a hydrophobic surface without reflecting charge effects, this indicates that β-casein has a high potential to remain within a hydrophobic gel network. This is supported by results of McCarthy et al. [13] who showed that β-casein is not completely soluble below its pI. Other authors observed that β-casein exhibits amphiphilic properties even at pH 2.6 [11], which leads to temperature independent association below the pI [12].

These properties might prevent gel structure weakening during overacidification as indicated by increased G'_{FINAL}/G'_{MAX} ratios at higher β-casein contents. The conformation of associated β-casein differs between neutral and acidic pH [12], and such conformational changes during acidification might be crucial for gel structure. Cross-linking of β-casein, however, limits its reorganisation since molecules are fixed in a particular conformation. As a consequence, gels showed always a more pronounced structure weakening with increased casein cross-linking (Fig. 2, Fig. 5); the same tendency was also observed when the pure β-casein rich fraction was cross-linked and used for the same kind of experiments (data not shown).

3.4 Comparison of the sample sets

Comparing the two sets of experiments demonstrates that the results of the uncross-linked casein samples are in a similar range, whereas gels from cross-linked casein of the second sample set (Fig. 5) behaved like intermediates between the samples of the first set, cross-linked for either 5 or 24 h prior to acidification (Fig. 2). Since the β-casein poor fraction was also cross-linked for 24 h prior to blending with uncross-linked casein, these findings underline that adding uncross-linked casein in general, and uncross-linked β-casein in particular reduces gel structure weakening during overacidification. Incubation with mTGase for 24 h (at the given conditions) usually results in the polymerisation of approx. 98 % of the casein [18, 23], and also in an excessive internal cross-linking of casein polymers [25, 26]. The addition of 30 % uncross-linked casein (Table 1) decreases the amount of polymerised casein to approx. 70 %, which is considerably lower than the 87 % that we reported recently for casein cross-linked for 5 h [39]. However, gels from 5 h incubated samples (Fig. 2) exhibited higher G'_{FINAL}/G'_{MAX} ratios than gels from 24 h cross-linked samples with added uncross-linked casein (Fig. 5). This indicates that not only the polymerisation of casein, but especially the extent of cross-linking within casein polymers during further incubation suppresses the reorganisation of casein molecules, resulting in more pronounced structure weakening during overacidification.

4 Conclusion

High concentrations of GDL as acidulant were applied to investigate rheological properties of acid casein gels below the pI. Structure weakening induced by increased electrostatic repulsion at low pH was more pronounced for casein cross-linked by mTGase. Since dissociation of casein molecules into the serum was only observed for the uncross-linked

reference, we assume that cross-linking of casein molecules retains electrostatic repulsion within the gel network, causing a reduction of protein-protein interactions. Structure weakening was considerably reduced with higher contents of uncross-linked β-casein, highlighting the unique properties of β-casein below its pI. It is already known that cross-linking with mTGase improves many functional properties of casein, including gelation. However, this study shows that, below the pI, polymerisation negatively affects the structural mechanisms by which casein gels can preserve their maximum stiffness. These findings have to be considered in the development of applications for casein polymers which require low pH.

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Conflict of interests

The authors declare no conflict of interests.

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Tables

Table 1 Composition of 27 g/kg casein solutions with varied β-casein concentration.

Sample denotation	Cross-linked* β-casein poor solution [%]	Uncross-linked β-casein poor solution [%]	Uncross-linked β-casein rich solution [%]
Uncross-linked 70:30	0	70	30
Uncross-linked 80:20	0	80	20
Uncross-linked 90:10	0	90	10
Uncross-linked 100:0	0	100	0
Cross-linked 70:30	70	0	30
Cross-linked 80:20	70	10	20
Cross-linked 90:10	70	20	10
Cross-linked 100:0	70	30	0

* Cross-linking was performed with 3 U microbial transglutaminase per g casein at 40 °C for 24 h.

Figures

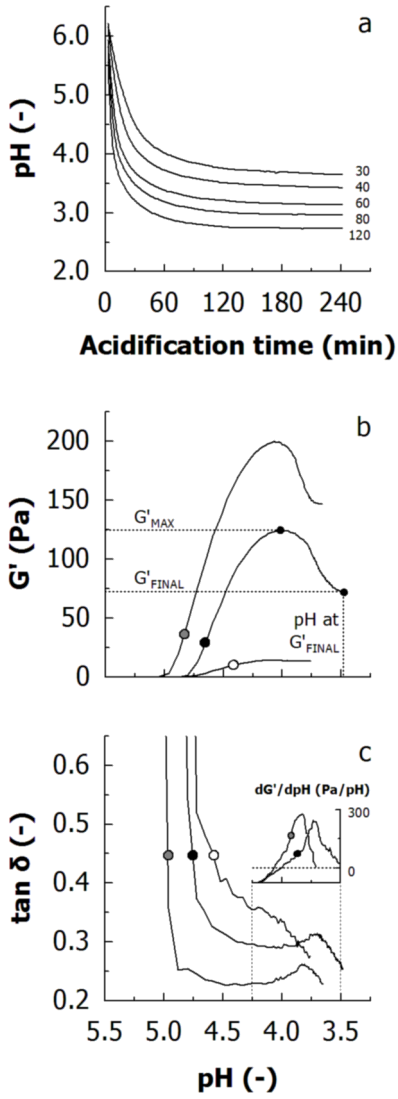

Fig. 1 (a) pH development in 27 g/kg casein solution during acidification with 30 – 120 mg/g glucono-δ-lactone (numbers) at 30 °C. (b) Gelation curves and (c) loss factor profiles of 27 g/kg casein solution, treated with 3 U microbial transglutaminase per g casein at 40 °C for 0 (white), 5 (grey), or 24 h (black), as a function of pH. Insert shows the development of the first derivative of the storage modulus (d*G'*/dpH) between pH 4.25 and 3.5. Gelation conditions: 30 °C, 40 mg/g glucono-δ-lactone. Key parameters G'_{MAX}, G'_{FINAL}, and pH at G'_{FINAL} (see text) were taken from all curves.

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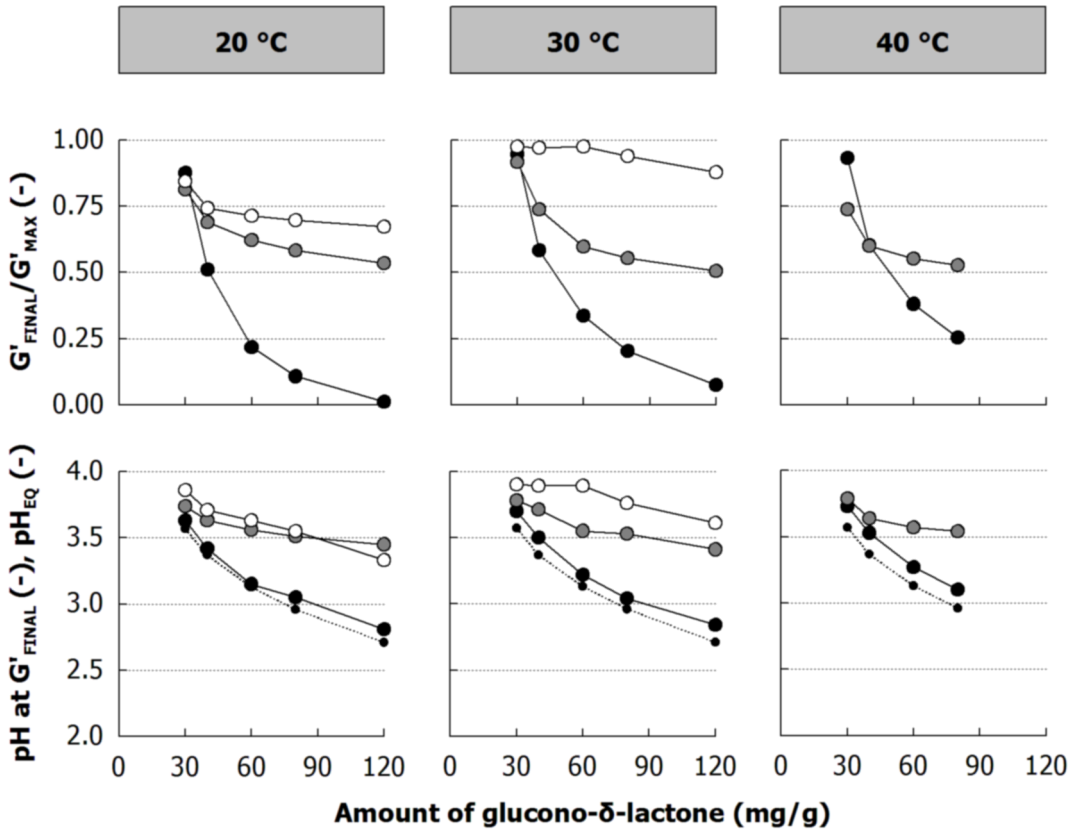

Fig. 2 Ratio of final gel stiffness to maximum gel stiffness (G'_{FINAL}/G'_{MAX}) (top) and pH at G'_{FINAL} (bottom) as affected by glucono-δ-lactone (GDL) concentration and gelation temperature. Solutions of commercial acid casein (27 g/kg) were cross-linked with 3 U microbial transglutaminase per g casein at 40 °C for 0 (white), 5 (grey), or 24 h (black) prior to acidification. Equilibrium pH (pH_{EQ}) at the particular GDL concentrations is given for comparison (dotted line).

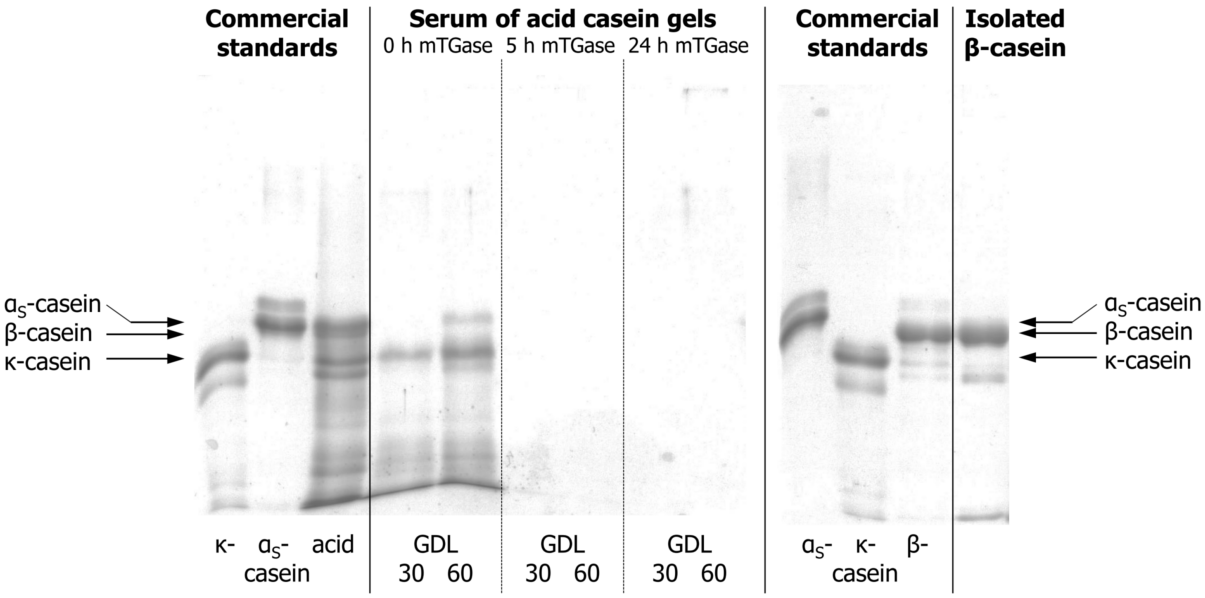

Fig. 3 Electropherograms of casein samples. Serum was obtained by centrifugation of 27 g/kg casein gels: casein was cross-linked with 3 U microbial transglutaminase (mTGase) per g casein at 40 °C for 0, 5, or 24 h, and acidified with 30 or 60 mg/g glucono-δ-lactone (GDL) at 30 °C; β-casein rich fraction was isolated from reconstituted skim milk.

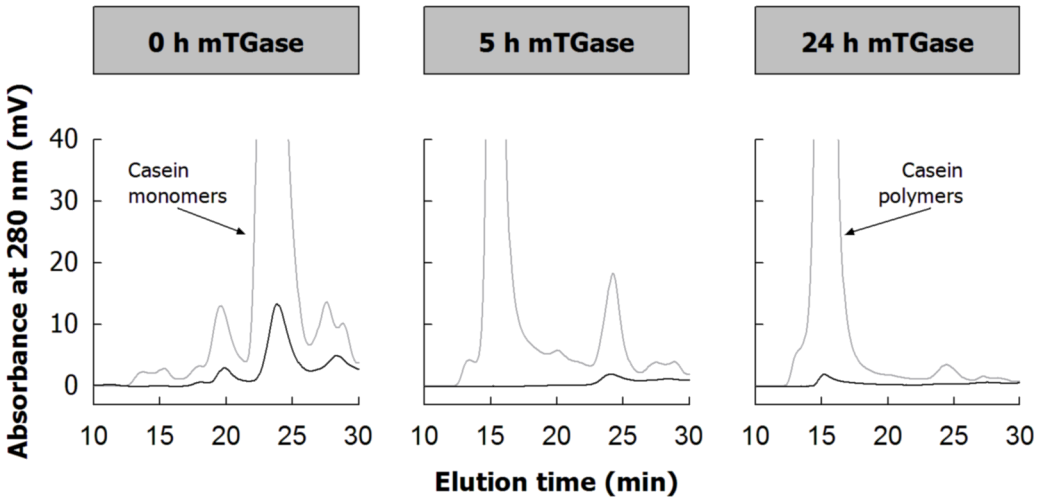

Fig. 4 Size exclusion chromatography of 27 g/kg casein solutions (grey lines) and serum phases (black lines) obtained by centrifugation of acid gels made thereof (60 mg/g glucono-δ-lactone, 30 °C). Casein was cross-linked with 3 U microbial transglutaminase (mTGase) per g casein at 40 °C for 0, 5, or 24 h.

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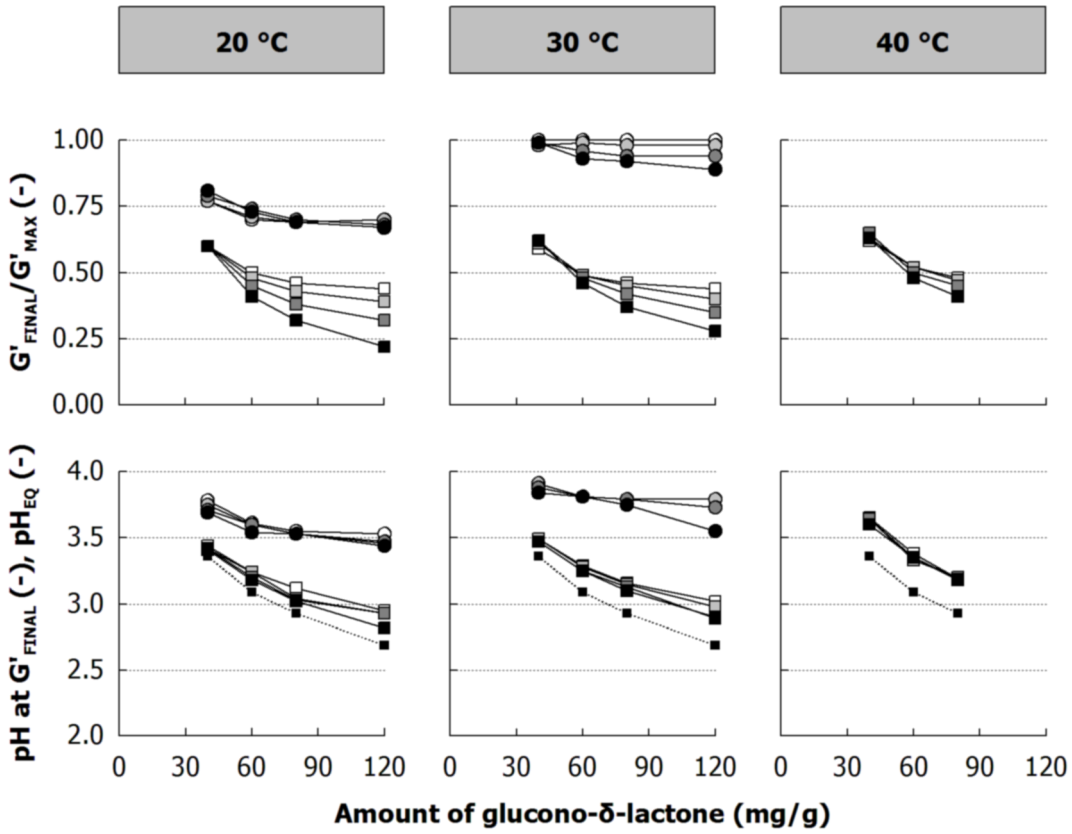

Fig. 5 Ratio of final gel stiffness to maximum gel stiffness (G'_{FINAL}/G'_{MAX}) (top) and pH at G'_{FINAL} (bottom) as affected by glucono-δ-lactone (GDL) concentration and gelation temperature. Samples were prepared by blending cross-linked (squares) or uncross-linked (circles) β-casein poor solutions (27 g/kg) with uncross-linked β-casein rich solutions (27 g/kg) in ratios of 70:30 (white), 80:20 (light grey), 90:10 (dark grey), and 100:0 (black). 70 % of cross-linked β-casein poor solution (3 U microbial transglutaminase per g casein, 40 °C, 24 h) was used in the respective mixtures and complemented with uncross-linked β-casein poor solution (see Table 1). Equilibrium pH (pH_{EQ}) at the particular GDL concentrations is given for comparison (dotted line).