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Acid-induced gelation of enzymatically cross-linked caseinate in different ionic milieus

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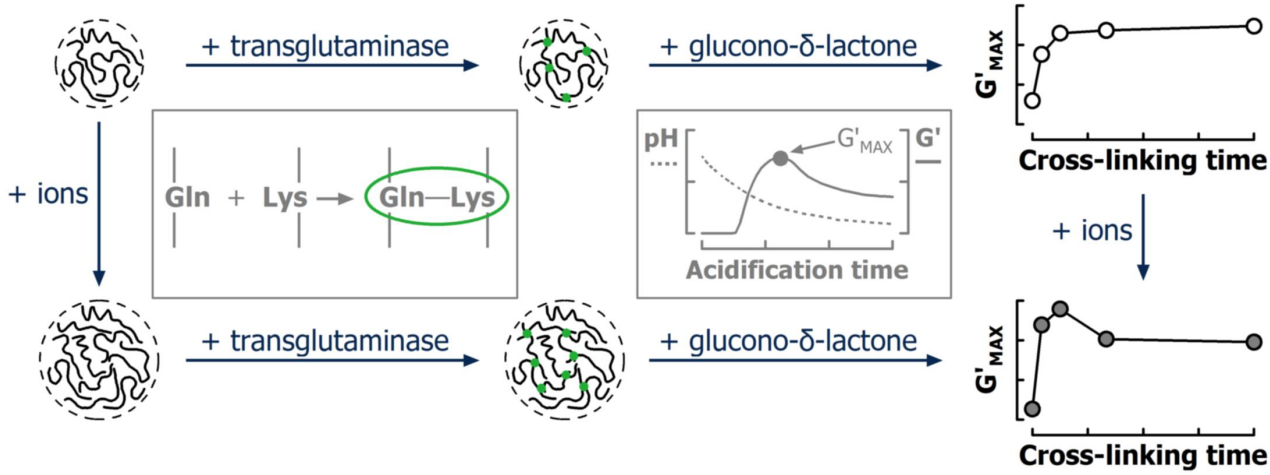
Abstract

Acid casein powder was used to prepare caseinate solutions (27 g/kg) with different ionic milieus: sodium caseinate (NaCN, *I* ~0.015 mol/L) and calcium caseinate (CaCN, *I* ~0.03 mol/L) were obtained by neutralisation with NaOH and $Ca(OH)_2$, respectively, and dissolving in phosphate buffer resulted in a high ionic strength caseinate solution (CN-PB, *I* ~0.16 mol/L). Treatment with microbial transglutaminase (mTGase) for defined incubation times lead to different extents of casein cross-linking, which were characterised by size exclusion chromatography and the N-ε-(γ-glutamyl)-lysine isopeptide content (IC). Maximum polymer size was reached at ~90 % casein polymerisation, and increased in the order NaCN < CN-PB < CaCN. Further enzyme treatment, however, increased the IC, pointing to cross-links within existing polymers. We suggest that the maximum polymer size is determined by the size of casein particles resulting from self-assembly in solution and that mTGase preferably acts on molecules within the same particle. Enhanced association at higher ionic strength (CN-PB) or in the presence of bivalent cations (CaCN) may therefore result in larger covalently cross-linked casein polymers. Furthermore, oscillation rheometry revealed that the relationship between casein cross-linking and stiffness of gels acidified with glucono-δ-lactone depends on the ionic milieu. While for NaCN G'_{MAX} increased with the time of cross-linking, the presence of ions resulted in the highest G'_{MAX} at moderate cross-linking intensities. This was also observed when NaCl was added to cross-linked NaCN. The results suggest that electrostatic attraction during gel formation are interfered by ions and cannot be compensated by rearrangements in case of extensively cross-linked casein particles.

Keywords: Casein, Transglutaminase, Cross-linking, Gelation, Ionic strength

Graphical abstract



Highlights

- Size of casein polymers formed by transglutaminase depends on the ionic milieu
- Polymers are internally cross-linked during prolonged transglutaminase treatment
- Addition of ions changes the relationship between incubation time and gel stiffness

1. Introduction

Casein is the major protein fraction in milk. As a consequence of weak non-covalent interactions between caseins and strong electrostatic interactions with colloidal calcium phosphate, the molecules are associated to so called casein micelles (de Kruif, Huppertz, Urban, & Petukhov, 2012). These are disintegrated by acidification as charge neutralisation causes the dissociation of colloidal calcium phosphate, and the loss of electrostatic repulsion results in precipitation at pH ~4.6 (O'Regan & Mulvihill, 2011).

Precipitated casein can be redissolved in water by neutralisation with alkali, using, e.g., NaOH that results in sodium caseinate (NaCN), or $Ca(OH)_2$ that results in calcium caseinate (CaCN) (O'Regan & Mulvihill, 2011). In NaCN solutions, molecules were shown to self-assemble to small particles (Chu, Zhou, Wu, & Farrell, 1995), and this association was more pronounced at increasing temperature, decreasing pH, and increasing ionic strength (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008). Huppertz et al. (2017) recently suggested that particles in NaCN solution are similar to the primary casein particles which casein micelles consist of. Self-association was also observed in solutions of pure β- or κ-casein (Cragnell et al., 2017; Dauphas et al., 2005; Moitzi, Portnaya, Glatter, Ramon, & Danino, 2008; O'Connell, Grinberg, & de Kruif, 2003a; Ossowski et al., 2012), and this had substantial consequences for the enzymatic cross-linking of casein. Microbial transglutaminase (mTGase; EC 2.3.2.13), which catalyses the formation of isopeptide bonds between protein-bound glutamine and lysine residues (Buchert et al., 2010; Jaros, Partschefeld, Henle, & Rohm, 2006; Romeih & Walker, 2017), was found to act mainly on casein molecules within the same β- or κ-casein particle. Thus, intramolecular isopeptide bonds were formed when β-casein existed as individual molecules at low temperature (around 0 °C), whereas polymerisation occurred when either β- or κ-casein was associated to particles at elevated temperature (35 – 40 °C) (de Kruif, Tuinier, Holt, Timmens, & Rollema, 2002; O'Connell & de Kruif, 2003b).

We suggest that this behaviour applies also for caseinate solutions which comprise of α_{S1}-, α_{S2}-, β-, and κ-casein in a ratio of approx. 3.8 : 1 : 3.5 : 1.5 (O'Mahony & Fox, 2013). This would mean that there is a maximum polymer size with respect to the number of molecules within an individual casein particle. Furthermore, this maximum polymer size might be adjustable via changes in temperature, pH, and ionic strength (HadjSadok et al., 2008).

In previous studies we dissolved acid casein powder in 0.1 mol/L sodium phosphate buffer (pH 6.8), resulting in a system with a relatively high ionic strength. Cross-linking with 3 U mTGase per g protein at 40 °C lead to almost complete polymerisation of casein during incubation for 24 h as was indicated by a disappearing monomer peak in size exclusion chromatography (SEC) (Jaros, Jacob, Otto, & Rohm, 2010; Rohm, Ullrich, Schmidt, Löbner, & Jaros, 2014).

A considerable amount of isopeptide bonds was, however, formed even after ~95 % of casein was polymerised, but almost no changes in the polymer fraction were observed in SEC (Jaros et al., 2014a, b). This underlines the idea of a maximum polymer size. Interestingly, acidification of the cross-linked casein with glucono-δ-lactone (GDL) resulted in gels with the highest stiffness after 3 h incubation (~80 % of casein was polymerised), and further cross-linking lead to lower gel stiffness (Jaros et al., 2010, 2014a; Rohm et al., 2014).

In other studies NaCN was used without addition of ions, however, cross-linking of casein with laccase (EC 1.10.3.2), tyrosinase (EC 1.14.18.1), or mTGase was less extensive and considerable amounts of monomers remained (Ercili Cura et al., 2009, 2010; Myllärinen, Buchert, & Autio, 2007). Therefore, comparability to our gelation experiments is limited.

The aim of this study was to compare different systems reported in literature (i.e., acid casein in phosphate buffer and NaCN solution) to examine the impact of ionic strength on casein polymerisation by mTGase, and on acid-induced gelation of cross-linked casein. Furthermore, CaCN was included as substrate where Ca^{2+} ions cause aggregation of mainly α_{S1}- and α_{S2}-casein and partly β-casein to particles with diameters of up to 430 nm (Cuomo, Ceglie, & Lopez, 2011; Pitkowski, Nicolai, & Durand, 2009; Smialowska, Matia-Merino, Ingham, & Carr, 2017; Thomar, Gonzalez-Jordan, Dittmer, & Nicolai, 2017). To the best of our knowledge, CaCN was not used before for this kind of cross-linking and gelation experiments.

2. Materials and methods

2.1. Materials

Fresh raw milk was obtained from a local farmer (Pulsnitz, Germany), and standards of α_S-, β- and κ-casein were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Microbial transglutaminase (Activa MP from *Streptomyces mobarensis*) received from Ajinomoto Foods Europe SAS (Hamburg, Germany) had an enzyme activity of 92 U/g (determined by the hydroxamate method; Folk & Cole, 1966). Glucono-δ-lactone was obtained from Kampffmeyer Nachf. GmbH (Ratzeburg, Germany).

2.2. Sample preparation

Raw milk was skimmed by centrifugation (3000 g, 6 °C, 20 min; Heraeus BioFuge Stratos, Thermo Electron Corporation, Waltham, MA, USA) and adjusted to pH 4.6 with 6 mol/L HCl at room temperature for casein precipitation. The precipitate was separated from whey by filtration through cellulose filter (Rotilabo type 600P, Carl Roth GmbH & Co. KG, Karlsruhe, Germany),

washed with 0.02 mol/L sodium acetate buffer (pH 4.6), and freeze dried (Martin Christ GmbH, Osterode am Harz, Germany). This resulted in acid casein powder with a crude protein content of 889 g/kg as determined by Kjeldahl method (N x 6.38; IDF, 1979).

For preparation of NaCN and CaCN solutions, acid casein powder was dispersed in demineralised water and dissolved at room temperature by neutralising (pH 6.8) with 1 mol/L NaOH or 0.02 mol/L $Ca(OH)_2$, respectively. To obtain a system with high ionic strength, the powder was dissolved in 0.1 mol/L phosphate buffer (pH 6.8) (CN-PB) (see previous studies; Jaros et al., 2010; Raak, Gehrisch, Rohm, & Jaros, 2015). Target protein concentration was 27 g/kg, and 0.3 g/kg sodium azide was added for preservation. Based on ion concentrations required for the preparation step, ionic strengths of ~0.015 mol/L, ~0.16 mol/L, and ~0.03 mol/L were calculated for the (protein-free) serum phases of NaCN, CN-PB, and CaCN, respectively.

Enzymatic cross-linking of casein was achieved by incubation with 3 U mTGase per g casein at 40 °C. The enzyme was inactivated by heat treatment (85 °C, 10 min) after 1, 3, 8, or 24 h, and reference samples (0 h) were treated in the same way without enzyme addition. In an additional sample set, NaCl was added to fresh NaCN solutions after enzyme treatment in concentrations of 0, 0.1, or 0.2 mol/L, and $CaCl_2$ was added in a concentration of 0.015 mol/L, the highest amount not causing casein precipitation, followed by pH readjustment to 6.8 using 1 mol/L NaOH.

2.3. Evaluation of casein cross-linking

The amount of cross-links expressed as isopeptide content (IC, mg N-ε-(γ-glutamyl)-lysine per g casein) was determined by amino acid analysis according to Lauber, Henle, & Klostermeyer (2000) after a three-step enzymatic hydrolysis of the samples according to Henle, Walter, & Klostermeyer (1991). Casein polymerisation was investigated using SEC with an urea containing buffer to suppress non-covalent interactions and after treating the samples with dithiothreitol to cleave disulphide bonds. The polymerisation degree (PD) was calculated from the chromatograms by relating the peak area of polymerised casein to the entire area (Lauber et al., 2000). Details on equipment, buffers, and procedures are given in a previous paper (Raak, Rohm, & Jaros, 2017a). All determinations were carried out as two individual experiments.

2.4. Monitoring of acidification

pH development of the samples during acidification with GDL was monitored using a six channel data logger for pH and temperature (MCC-SYSti-6b, EA Instruments Ltd., Wembley, UK). Temperature equilibrated samples were blended with GDL, transferred into a water bath, and pH was recorded at 30 °C every 60 s using the instrument-associated software (MCC-MON-6c, Version 2.3.1). After preliminary tests, we selected GDL concentrations of 35 mg/g for NaCN and CaCN and 40 mg/g for CN-PB to obtain similar acidification behaviour of the different casein

systems. GDL concentrations were adapted from previous studies (Jaros et al., 2010, 2014; Raak et al., 2015, 2017a; Rohm et al., 2014) and resulted in rather fast acidification to minimise the measurement times. All measurements were conducted as duplicate experiments.

2.5. Rheological measurements

Small strain oscillation rheometry using a strain controlled ARES RFS3 rheometer (TA Instruments, Eschborn, Germany) with a cup and bob geometry (d_i = 32 mm, d_o = 34 mm, h = 33.5 mm) was conducted to record gelation curves. Temperature equilibrated samples were blended with GDL (35 mg/g for NaCN and CaCN, 40 mg/g for CN-PB), transferred into the rheometer geometry immediately after mixing, and the sample surface was covered with paraffin oil to prevent evaporation. Temperature was controlled at the outer cylinder by a computer controlled circulator, and storage modulus G' (Pa) and loss factor (tan δ) were recorded every 60 s at 30 °C, ω = 1 rad/s, and γ = 0.003, which is within the linear viscoelastic region (Rohm et al., 2014). Measurements were stopped after 60 min, and maximum storage modulus (G'_{MAX}) and tan δ at G'_{MAX} were taken as evaluation measures. All results shown are mean values from duplicate experiments.

3. Results and discussion

3.1. Enzymatic cross-linking of caseinate in different ionic milieus

Fig. 1 shows the progress of enzymatic cross-linking in the different ionic milieus. For all samples, polymerisation was almost complete after 24 h of incubation (i.e., PD ~100 %), although PDs observed for NaCN were slightly lower at all incubation times. IC of NaCN and CN-PB were, however, in good agreement, suggesting that the activity of mTGase was hardly affected by ionic strength. In contrast, CaCN showed a lower IC after 24 h of mTGase treatment. Kütemeyer, Froeck, Werlein, & Watkinson (2005) reported that mTGase activity at 50 °C was considerably decreased by $CaCl_2$ concentrations of 0.45 – 1.80 mol/L. Both temperature and Ca^{2+} concentration were lower in the current study (40 °C, 0.013 mol/L), but may be responsible for a partial inactivation of mTGase during incubation for 24 h. Since the progress of polymerisation was similar for CaCN and CN-PB, we assume that the susceptibility of casein monomers to mTGase cross-linking was not dramatically reduced by Ca^{2+}-induced aggregation, although other authors observed dense casein domains comparable to micro-phase separation (Thomar, Nicolai, Benyahia, & Durand, 2013).

The respective chromatograms from SEC (Fig. 2) depict that individual casein types show slightly different elution times when injected separately, whereas they eluted as a single peak when caseinate samples were analysed. Nevertheless, single casein standards indicate the position of α_S-, β-, and κ-casein within the monomer peak and allow drawing conclusions on the polymerisation velocity of the different casein types. Independent of the ionic milieu, β-casein was completely polymerised after 1 h of mTGase treatment since no signal was obtained at ~22 – 23 min of elution. In NaCN and CN-PB, a bump at ~24 – 25 min implies that κ-casein remained longest in the monomeric state, which is consistent with previous results obtained from gel electrophoresis (Jaros et al., 2010; Macierzanka et al., 2011; Partanen et al., 2008). In CaCN, however, κ-casein was polymerised faster than α_S-casein. It has been reported for casein micelles that κ-casein is first cross-linked because of its good accessibility at the micelle surface (Hinz, Huppertz, & Kelly, 2012; Huppertz & de Kruif, 2007; Jaros et al., 2010). This suggests that κ-casein in CaCN is also a preferred substrate as a consequence of Ca^{2+}-induced aggregation of α_S-caseins.

Casein polymers in CN-PB eluted earlier (~14 – 17 min) than polymers in NaCN (~15 – 20 min), indicating that polymers in CN-PB are larger (see Fig. 2). Furthermore, polymer peaks of NaCN and CN-PB differed only marginally between 3 and 24 h incubation although IC increased by a factor of ~2 (Fig. 1). This implies that isopeptide bonds were mainly formed within already existing casein polymers, and suggests that this maximum polymer size increases with increasing ionic strength as a consequence of self-association into casein particles with a higher monomer number (HadjSadok et al., 2008). Cross-linked CaCN contained the largest casein polymers (elution at ~13 – 16 min), but in contrast to NaCN and CN-PB, the shape of the polymer peak changed with ongoing cross-linking: a peak of lower intensity was obtained after 1 h of cross-linking (~13 – 15 min), which became sharper and was shifted to higher elution times with further mTGase treatment (~14 – 16 min). This suggests a change in polymer conformation from loose and open-structured macromolecules to dense and compact particles with decreased hydrodynamic volume as a result of internal isopeptide bonds and monomer incorporation. A shoulder emerging at ~13 – 14 min after cross-linking for ≥3 h indicates an additional small fraction of very large polymers.

3.2. Acid-induced gelation of cross-linked caseinate

Fig. 3 shows the maximum stiffness (G'_{MAX}) of acid-induced caseinate gels formed at 30 °C as a function of mTGase incubation time. Consistent with previous results (Jaros et al., 2010, 2014a; Rohm et al., 2014), G'_{MAX} of gels from CN-PB increased with mTGase treatment for up to 3 h, but decreased with prolonged incubation. In contrast, G'_{MAX} of NaCN gels increased progressively with increasing cross-linking, and CaCN showed highest G'_{MAX} after 1 h of incubation. Since G'_{MAX} of the

uncross-linked references (0 h) were not identical despite of similar acidification profiles (not shown), the ionic milieu must have had a direct impact on the gelation process. We therefore prepared a second batch of NaCN and added NaCl after mTGase treatment to evaluate the effect of ions on the gelation behaviour of cross-linked casein (Fig. 4). In general, addition of NaCl decreased the gelation onset pH, delayed the evolution of G' and shifted G'_{MAX} to lower pH, which is consistent with previous results on acid-induced sodium caseinate gels (Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997) as well as soy protein gels (Bi, Li, Wang, & Adhikari, 2013; Schuldt, Raak, Jaros, & Rohm, 2014). Since the GDL concentration was rather high, G' of NaCN gels without NaCl decreased considerably with continued acidification after G'_{MAX} was reached at pH ~4.2 because of increased electrostatic repulsion below the isoelectric point. This decrease was more pronounced at higher cross-linking intensity and was previously attributed to the fact that covalent cross-linking retains increasingly charged molecules within the gel network so that protein-protein interactions are reduced (Raak, Rohm, & Jaros, 2017b). This structure weakening, however, was prevented by the addition of NaCl, likely because repulsive charges were screened by the ions. Consequently, no clear maximum was reached within the measurement period (60 min) for NaCN gels with 0.2 mol/L NaCl.

Addition of NaCl also shifted highest G'_{MAX} to the 3 h incubated NaCN sample (see Fig. 4), resulting in a relationship between cross-linking intensity and G'_{MAX} similar to CN-PB (see Fig. 3). G'_{MAX} was generally lower after salt addition, however, gels from 1 and 3 h incubated CN-PB had considerably higher stiffness than the respective NaCN samples. Given that IC and PD were independent from ionic strength (see Fig. 1), this indicates that the smaller polymers in NaCN (see Fig. 2) contributed less to a proper gel formation. This is consistent with data from a recent study where we observed lower gel stiffness at a particular IC in case smaller polymers were present in CN-PB (Raak et al., 2017a).

Previously, we reported an inverse linear correlation between G'_{MAX} and the corresponding loss factor (tan δ) of acid gels from cross-linked CN-PB (Jaros et al., 2010; Rohm et al., 2014). The present results, however, demonstrate that this is not a universal relationship as the lowest tan δ at G'_{MAX} was always found for the 3 h incubated samples (IC ~14.5 mg/g, PD ~90 %; see Fig. 1) independent of the ionic milieu and thus independent of G'_{MAX} (see Fig. 3). In general, rather loosely cross-linked polymers were formed during the first 3 h of incubation: an IC of 14.5 mg/g corresponds to 1.26 mol isopeptides per mol casein (m_M of casein ~24,000 g/mol), which is relatively close to the minimum amount of cross-links required for a polymer (0.9 mol/mol; Lauber et al., 2000). A decrease in tan δ therefore points to increased elastic properties of the gels with moderately increasing cross-linking. In case of peroxidase-cross-linked α-lactalbumin the density of protein particles decreased with increasing polymerisation because of a loose and open structure (Dhayal, Gruppen, de Vries, & Wierenga, 2014). For concentrated solutions

(50 – 100 g/L) of these nanoparticles, Saricay, Wierenga, & de Vries (2016) reported a gel-like behaviour with tan δ (ω = 1 rad/s) decreasing from ~0.65 to ~0.20 with increasing particle size (R_h = 25 – 100 nm). They assumed that the higher elastic response results from enhanced contact and stronger short-range interactions between large jammed particles with a low density. Acidification reduces electrostatic repulsion and thus enables contact between casein particles at much lower concentrations, resulting in a network with physical interactions between the particles within casein clusters. Therefore, the findings of Saricay et al. (2016) principally correlate with acid gels formed from loosely cross-linked casein polymers. On the other hand, continued mTGase treatment resulted in the formation of cross-links within existing polymers: 2.53 and 2.85 mol isopeptides per mol casein were found in CN-PB and NaCN incubated for 24 h, respectively, which is approximately three times higher than the minimum amount of cross-links in a polymer (0.9 mol/mol) and likely increased the density of the casein polymers again. With regard to gel networks, a higher tan δ is usually associated with a more pronounced dissipation of the applied shear energy when the disruption of weak physical bonds allows particle movements (Belyakova et al., 2003). This means that the mobility of loosely cross-linked, open-structured casein polymers in the gel network is probably impaired for steric reasons (e. g., size, entanglements) and/or because of stronger interactions. In contrast, particle movements might be facilitated again after extensive cross-linking because of an increased compactness of the polymers.

Fig. 3 also shows the effect of the ionic milieu on tan δ at G'_{MAX}. NaCN gels exhibited the highest and CaCN gels the lowest values, except for the uncross-linked reference. We suggest that NaCN contained the most compact and CaCN the loosest casein polymers (see section 3.1). The observed tan δ are therefore in good agreement with the argumentation of Saricay et al. (2016) that denser protein particles show a lower elastic response. Interestingly, gels from uncross-linked NaCN and CaCN had relatively similar tan δ (~0.38), whereas the respective CN-PB gel showed a considerably lower value (~0.3). This indicates that the favoured self-association at high ionic strength results in casein particles with lower density and thus in gels with a higher elastic response. Inversely, Belyakova et al. (2003) observed higher tan δ for acid gels after NaCN particles dissociated in the presence of sucrose. In contrast, Ca^{2+}-induced casein aggregation might be easily revoked by acidification as a consequence of neutralisation of negatively charged amino acid residues. This is in accordance with Matia-Merino, Lau, & Dickinson (2004) who observed a release of Ca^{2+} ions from casein particles with decreasing pH of caseinate gels. In tan δ profiles of acidified skim milk a peak at pH ≥5.0 indicates the dissociation of colloidal calcium phosphate (Anema, 2009). Such a peak was not observed during gelation of CaCN (not shown), suggesting that the majority of Ca^{2+} ions were already released prior to the gelation onset (pH ~4.9).

The addition of 0.1 mol/L NaCl to uncross-linked NaCN decreased tan δ at G'_{MAX} from ~0.36 to ~0.29 (see Fig. 4). This effect was much smaller after 24 h incubation (~0.34 vs. ~0.32) although a considerably lower tan δ was observed when ions were already present during cross-linking of CN-PB (~0.28; see Fig. 3). This suggests that extensive cross-linking of casein particles fixates their conformation and impedes molecular reorganisation induced by ions. On the other hand, the profiles clearly show that tan δ was not constant during acidification: a minimum was reached at G'_{MAX} but, with further acidification, tan δ increased up to a local maximum before it dropped again. This peak coincides with a maximum in the first derivative of G' after pH, and the decrease in tan δ indicates that structural changes are slowed down at low pH (Raak et al., 2017b). After adding NaCl, this peak was less pronounced and, consistent with previous results on acid-induced soy protein gels (Schuldt et al., 2014), higher tan δ were observed at the end of the measurement period. This confirms that screening of repulsive charges by ions counteracts structure weakening below the isoelectric point of casein by facilitating particle movements in the gel network.

The final question that remains is why ions change the relationship between casein cross-linking and maximum gel stiffness. Lucey et al. (1997) elaborated that attractive electrostatic interactions between casein particles are important for network formation, and that screening of charges by ions weakens these interactions; this resulted in lower gel stiffness. It is, however, obvious that this effect is more pronounced when casein particles are extensively cross-linked. In pure NaCN, mainly positively charged ions (~0.025 mol/L Na^+) are present to interfere with electrostatic interactions by screening negative charges on the surface of the casein particles. Hydrophobic interactions as well as additional covalent cross-links in the interior contribute to particle stiffness, leading to increasing G'_{MAX} of acid gels with ongoing mTGase treatment. At higher ionic strength, introduced either prior to (CN-PB) or after cross-linking (NaCN with NaCl), positive charges are additionally screened by high concentrations of negatively charged ions (i. e., phosphate, chloride), and attractive electrostatic interactions during gelation are reduced. Loosely cross-linked casein molecules (incubation for ≤3 h) may be able to reorganise during acidification so that especially hydrophobic interactions between casein particles are facilitated and covalent cross-links contribute additionally to gel stiffness. Extensively cross-linked particles, however, are fixated in their distinct conformation and such rearrangements are therefore impeded, resulting in lower G'_{MAX}.

As concerns the gelation of CaCN, considerably lower G'_{MAX} were reached compared to the respective NaCN samples (see Fig. 3). This is consistent with results from Matia-Merino et al. (2004), who observed a decreasing stiffness of caseinate gels with increasing $CaCl_2$ concentration (0 – 4.0 mol Ca^{2+} per mol casein) and attributed this to a decreased number of interacting casein particles and reduced attractive forces because of interference with electrostatic and hydrophobic interactions. Even though the concentration of Ca^{2+} ions was much lower compared to NaCN with

added NaCl, the decrease in G'_{MAX} was much more pronounced. This indicates a very strong effect of bivalent Ca^{2+} ions, which might also have caused this dramatic change in the relationship between incubation time and gel stiffness, meaning that Ca^{2+} impeded the reorganisation of casein particles even in samples cross-linked for only 3 h, therefore showing the highest G'_{MAX} after 1 h incubation. This assumption could be confirmed to some extent by adding 0.015 mol/L $CaCl_2$ to cross-linked NaCN prior to gelation: G'_{MAX} of 1 and 3 h incubated NaCN were very similar in this case (Fig. 5), i. e., the behaviour was intermediate between NaCN and CaCN. Ca^{2+} concentrations of CaCN and NaCN with added $CaCl_2$ were 0.013 and 0.015 mol/L, respectively, but it might be possible that the effective Ca^{2+} content of the latter was lower because of incomplete $CaCl_2$ dissociation and/or lower Ca^{2+} sensitivity of casein after cross-linking. In fact, solutions were much less turbid after $CaCl_2$ addition when NaCN was cross-linked (not shown), pointing to reduced interactions with Ca^{2+} ions. Furthermore, tan δ at G'_{MAX} of these gels were very close to those of the pure NaCN gels (see Fig. 4), underlining that the low values of CaCN gels are connected to the polymer size rather than the ionic milieu.

4. Conclusions

The current study strongly points to the importance of considering the ionic milieu for enzymatic cross-linking and acid-induced gelation of caseinate. Larger casein polymers were formed by mTGase at higher ionic strength and in the presence of bivalent cations, and polymers underwent extensive internal cross-linking during prolonged incubation, indicating that maximum polymer sizes depend on the self-association of casein molecules in different ionic milieus. Additionally, the presence of ions affected the relationship between casein cross-linking and gel stiffness. G'_{MAX} of NaCN without ions increased progressively with increasing cross-linking intensity, probably because electrostatic interactions were not disturbed and isopeptide bonds contributed to stiffness of the casein particles. In the presence of ions, introduced either prior to or after cross-linking, a reorganisation of casein molecules might facilitate other attractive interactions, e.g. hydrophobic interactions. Such rearrangements were, however, impeded in case of extensively cross-linked casein particles and the loss in electrostatic attraction could not be compensated; this resulted in a shift of the highest G'_{MAX} to shorter incubation times. The current findings underline that knowledge on interactions between cross-linked caseinate and food constituents such as salts is crucial for the application as a techno-functional ingredient in food design.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

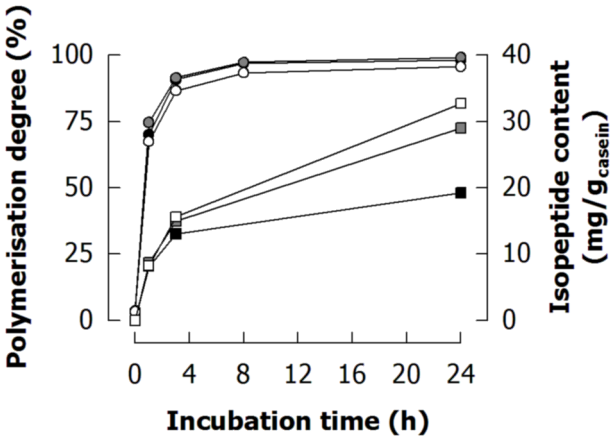

Fig. 1: Polymerisation degree (circles) and isopeptide content (squares) of sodium caseinate (white), casein in 0.1 mol/L phosphate buffer (grey), and calcium caseinate (black) after incubation with 3 U microbial transglutaminase per g casein at 40 °C.

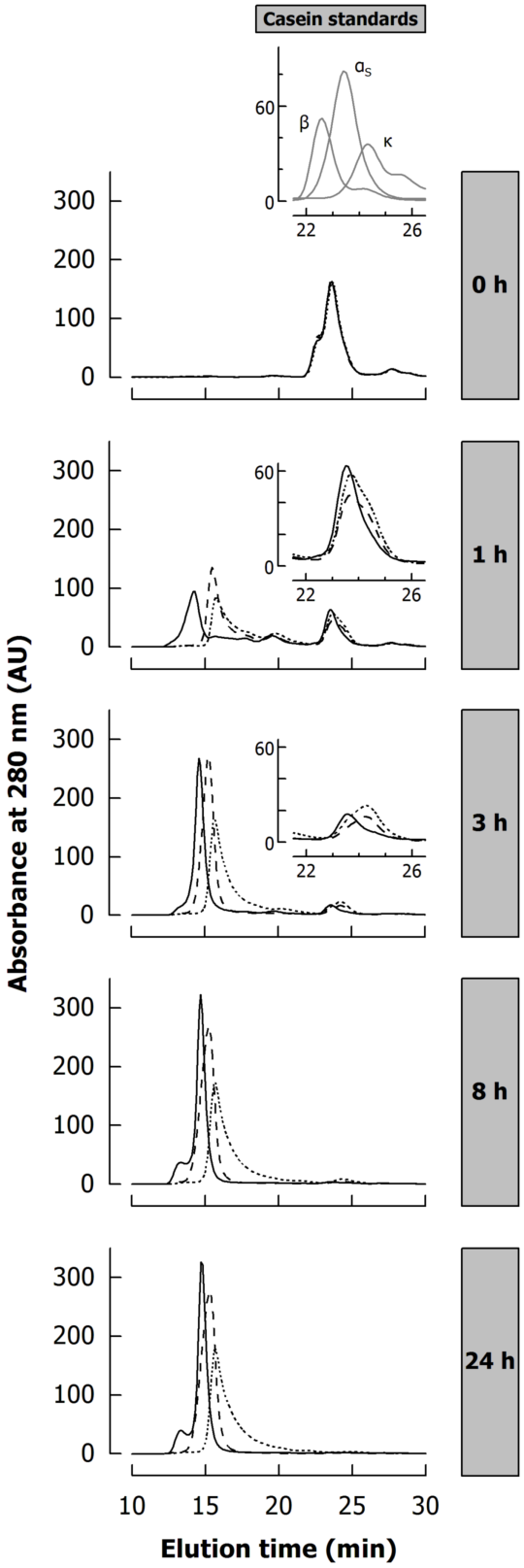

Fig. 2: Size exclusion chromatography of casein standards and sodium caseinate (dotted lines), casein in 0.1 mol/L phosphate buffer (dashed lines), and calcium caseinate (full lines) cross-linked with 3 U microbial transglutaminase per g casein at 40 °C for the specified periods of time. Inserts depict an enlargement of the monomer peaks.

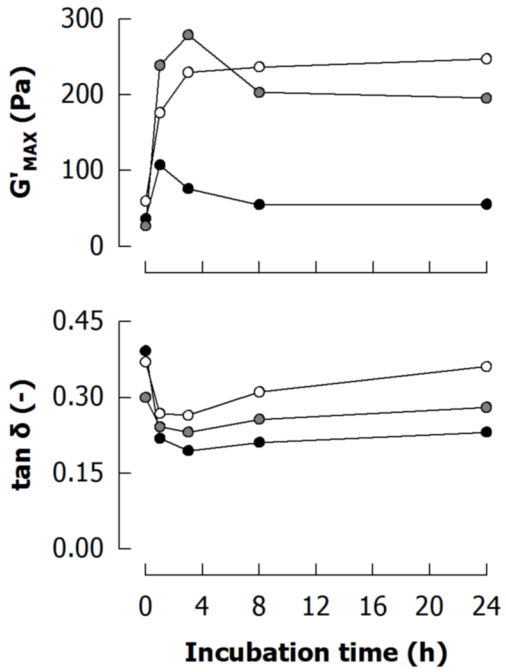

Fig. 3: Maximum stiffness G'_{MAX} (top) and tan δ at G'_{MAX} (bottom) of acid-induced gels (T = 30 °C) from sodium caseinate (white, 35 mg/g glucono-δ-lactone), casein in 0.1 mol/L phosphate buffer (grey, 40 mg/g glucono-δ-lactone), and calcium caseinate (black, 35 mg/g glucono-δ-lactone) cross-linked with 3 U microbial transglutaminase per g casein at 40 °C. Acidulant concentrations were adjusted to result in similar acidification profiles for the samples.

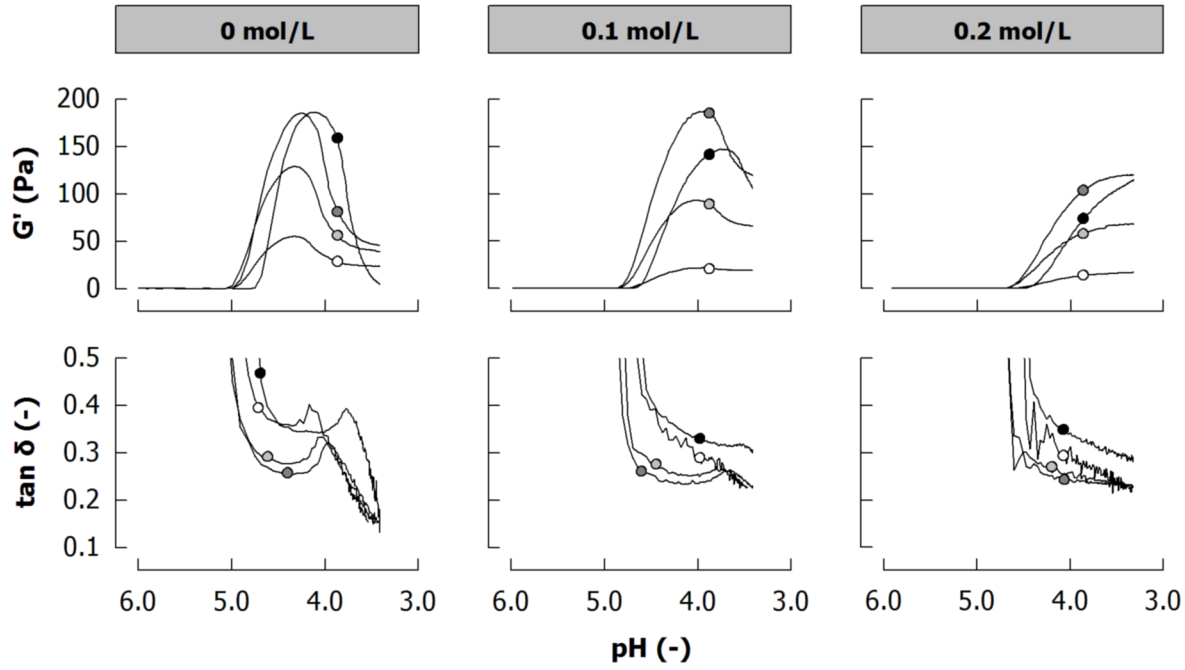

Fig. 4: Storage modulus G' (top) and loss factor tan δ (bottom) as a function of pH during gelation (35 mg/g glucono-δ-lactone, 30 °C) of sodium caseinate cross-linked for 0 (white), 1 (light grey), 3 (dark grey), and 24 h (black) with 3 U microbial transglutaminase per g casein at 40 °C. NaCl was added in the specified concentrations after incubation and had no effect on the acidification profile.

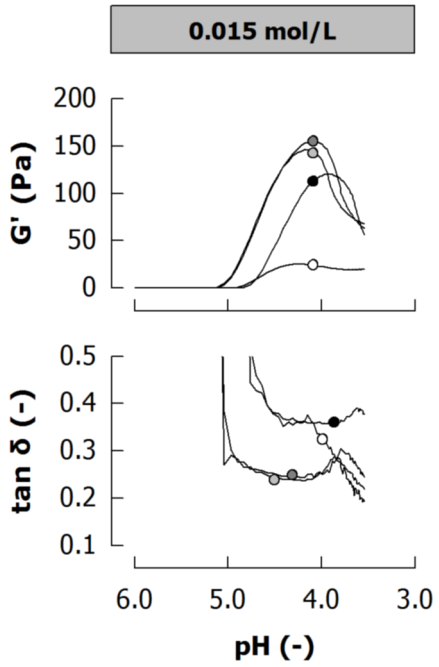

Fig. 5: Storage modulus G' (top) and loss factor tan δ (bottom) as a function of pH during gelation (35 mg/g glucono-δ-lactone, 30 °C) of sodium caseinate cross-linked for 0 (white), 1 (light grey), 3 (dark grey), and 24 h (black) with 3 U microbial transglutaminase per g casein at 40 °C. $CaCl_2$ was added in a concentration of 0.015 mol/L after incubation.