Cross-linking with microbial transglutaminase: Isopeptide bonds and polymer size as drivers for acid casein gel stiffness

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Abstract

Acid casein in phosphate buffer was cross-linked with microbial transglutaminase for different periods of time (1 – 8 h) and used for preparing mixtures with the uncross-linked reference to obtain samples with an identical number of cross-links (expressed as N-ε-(γ-glutamyl)-lysine isopeptide content, IC). For mixtures from longer incubated casein solutions, where cross-links were also formed within and between already present polymers, this procedure resulted in a lower polymerisation degree because a higher proportion of the reference was necessary to adjust the target IC. The experiments indicated that for a similar gel stiffness at the same isopeptide content a particular size of the polymers is crucial; a high content of dimers resulted in lower gel stiffness at a given IC. In contrast, gels with high stiffness were formed at high amounts of larger polymers. Furthermore, gelation temperature had a great impact: gel stiffness at 30 °C was lower at identical IC when casein solutions cross-linked for more than 5 h were used for blending, but this was not observed for gelation at 20 or 40 °C. Additionally, large deformation properties of the casein gels were clearly affected by cross-linking.
1. Introduction

Cross-linking of casein, the main building block in acidified dairy products, offers a large potential for the enhancement of physical and sensory properties of, e.g., yoghurt (Buchert et al., 2010; Jaros, Heidig, & Rohm, 2007; Loveday, Sarkar, & Singh, 2013), and for the stabilisation of the structure of the respective products during post-fermentation treatment (Mokoonlall, Nöbel, & Hinrichs, 2016a). The cross-linking enzyme laccase (EC 1.10.3.2; oxidoreductase) has an acidic pH optimum and could therefore be added to stirred yoghurt after fermentation, but was reported to also cause protein degradation and thus weaker gel structure, impairing a controlled application in the industry (Ercili Cura et al., 2009; Mokoonlall et al., 2016b). In contrast, microbial transglutaminase (mTGase; EC 2.3.2.13; acyl transferase) catalyses acyl transfer reactions between glutamine and lysine residues at neutral pH that result in covalent protein cross-links (Buchert et al., 2010; Jaros, Partschefeld, Henle, & Rohm, 2006a). Cross-linked casein micelles showed an improved stability against ethanol, urea, as well as chelating agents (e.g., Heber, Paasch, Partschefeld, Henle, & Brunner, 2012; Huppertz & de Kruif, 2007a; Huppertz, Smiddy, & de Kruif, 2007b; Partschefeld, Schwarzenbolz, Richter, & Henle, 2007), and increased milk gel stiffness after acidification (Ercili-Cura et al., 2013). On the other hand, the formation of weak and coarse gels was also reported and assumed to be related to the presence of large polymers or effects on the physical interaction potential between the casein subunits because of the cross-linking (Bönisch, Huss, Lauber, & Kulozik, 2007a; Jaros, Pätzold, Schwarzenbolz, & Rohm, 2006b).

The extent of cross-linking is often determined by separation techniques such as gel electrophoresis (e.g., Moon, Hong, Huppertz, Fox, & Kelly, 2009; Partanen et al., 2008) or size exclusion chromatography (SEC; e.g., Bönisch et al., 2007a; Ercili Cura et al., 2009) and may be expressed as polymerisation degree (PD, relative amount of cross-linked protein molecules). The PD can be varied by the enzyme/substrate ratio (Bönisch, Lauber, & Kulozik, 2007b; Jaros et al., 2006b) and/or incubation time (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Jaros, Jacob, Otto, & Rohm, 2010; Lauber, Henle, & Klostermeyer, 2000). However, this parameter does not provide information on the actual polymer size, which progressively increases during enzyme treatment (Jaros et al., 2010; Macierzanka et al., 2011; Partanen et al., 2008). Until yet it is not known how to control the polymer size. In a previous study, casein solutions that were cross-linked for different times were therefore blended with an uncross-linked reference to obtain samples with similar PD, but the respective mixtures did not exhibit the same gel stiffness upon acidification (Jaros et al. 2014a; Jaros et al., 2014b). This indicates that the size of the casein polymers must play an important role for their gelation behaviour.
When plotting the number of ε-(γ-glutamyl)-lysine (Glu-Lys) isopeptide (IC) bonds induced by mTGase against gel stiffness, mixtures from casein solutions cross-linked up to a critical incubation time were almost identical. It was concluded that a critical polymer size must exist, because mixtures with the largest polymers (as indicated from SEC) showed always lower gel stiffness at a given IC. These experiments, however, were conducted at only one particular acidification condition, without any further rheological characterisation of the gels. In previous studies temperature and acidification rate affected gelation properties of caseins dependent on the extent of cross-linking: for instance, stiffness of uncross-linked casein gels was higher at 20 °C compared to 30 °C, but the contrary was observed for cross-linked casein gels (Myllärinen, Buchert, & Autio, 2007; Rohm, Ullrich, Schmidt, Löbner, & Jaros, 2014). It was assumed that shrinkage of the casein particles, which reduces contact areas at higher temperatures, is prevented after cross-linking. Furthermore, strain sweeps performed subsequently to gelation revealed strain hardening, which was less pronounced after longer casein cross-linking (Rohm et al., 2014).

This study expands on a previous work (Jaros et al. 2014a; Jaros et al., 2014b; Rohm et al. 2014) by considering additional experiments in which temperature and acidulant concentration were systematically varied. For that, samples with a similar IC were created by blending differently cross-linked casein with an uncross-linked reference, and rheological assessment at small and large deformation was performed on the gels made thereof. The aim was to examine whether the gelation of casein could be tailored by the IC alone, or if this is possible only for particular acidification rates and gelation temperatures.

2. Materials and methods

2.1. Preparation of polymerised casein samples

Acid casein with a crude protein content of 868 g·kg\(^{-1}\) (Kjeldahl method, N x 6.38; IDF, 1979) was obtained from Sigma-Aldrich GmbH (Steinheim, Germany). The powder was dispersed in 0.1 mol·L\(^{-1}\) phosphate buffer (pH 6.8) at a protein concentration of 27 g·kg\(^{-1}\) and stirred overnight at room temperature to ensure complete hydration. Sodium azide was added in a concentration of 0.3 g·kg\(^{-1}\) to prevent microbial growth.

mTGase powder (Activa MP from Streptomyces mobaraensis) with a specified enzyme activity of 100 U·g\(^{-1}\) (Ajinomoto Foods Europe SAS, Paris, France) was added to the casein solution in a concentration of 3 U per g casein. Incubation was carried out in a water bath at 40 °C, and the enzyme was inactivated in hourly intervals between 1 and 8 h by heat-treatment at 85 °C for
10 min, followed by cooling in ice water. A reference was treated in the same way without enzyme addition and is referred to as 0 h.

2.2. Determination of the isopeptide content

The casein samples were enzymatically hydrolysed in three steps using pepsin and pronase E (Merck KGaA, Darmstadt, Germany), and leucine aminopeptidase with prolidase (Sigma-Aldrich GmbH, Steinheim, Germany) according to Henle, Walter, & Klostermeyer (1991), and the hydrolysates were subjected to a cation exchange chromatography based amino acid analysis (S4300, Sykam GmbH, Eresing, Germany) for determination of the N-ε-(γ-L-glutamyl)-L-lysine (Glu-Lys) content according to Lauber et al. (2000). By considering the molar mass of Gly-Lys (275 g·mol⁻¹) the isopeptide content (IC) was further expressed as g Glu-Lys per 100 g casein.

2.3. Size exclusion chromatography

Size exclusion chromatography (SEC) was conducted using a liquid chromatography system (AZURA Assistant ASM 2.1L, Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) coupled with a Superdex 200 Increase 10/30 column (GE Healthcare, Uppsala, Sweden). Casein solutions were diluted with the elution buffer (6 mol·L⁻¹ urea, 0.1 mol·L⁻¹ NaCl, 0.1 mol·L⁻¹ Na₂HPO₄, 1 g·L⁻¹ CHAPS, pH 6.8) to a concentration of 2 g·L⁻¹ and treated with DTT to cleave disulphide bonds. The separation was carried out at ambient temperature at an isocratic flow rate of 0.5 mL·min⁻¹. Casein was detected at 280 nm, and ClarityChrom v.3.0.7 (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) was used for data acquisition. The relative amount of monomer, dimer, and polymer fractions (%) was estimated from the corresponding peak areas in the chromatograms related to the entire sample area, and the polymerisation degree (PD) was defined as 100 % – monomer content (Lauber et al., 2000).

2.4. Preparation of casein mixtures with identical isopeptide content

Casein solutions, which were treated with mTGase for different periods of time, were blended with the uncross-linked reference sample (0 h) in ratios necessary to reach the corresponding target ICs. PD and the relative amount of the polymeric fractions were calculated from the respective numbers of the basis samples and the mixing ratio. All mixtures were prepared separately for individual measurements.

2.5. Rheological measurements

Small amplitude oscillatory shear rheology was conducted in a concentric cylinder system (dᵢ = 32 mm, dₒ = 34 mm, h = 33.5 mm) of a strain-controlled ARES RFS3 rheometer (TA
Instruments, Eschborn, Germany) to monitor gelation. Temperature equilibrated casein solutions were blended with 35, 40, or 45 g glucono-δ-lactone (GDL) per kg casein solution for acidification and transferred into the rheometer immediately after mixing. Sample surface was covered with paraffin oil to prevent evaporation, and the storage modulus G’ (Pa) was recorded every 60 s at a frequency of 1 rad·s\(^{-1}\) and a strain amplitude of 0.003 with a circulator regulating the temperature at the outer cylinder to 20, 30, or 40 °C. At pH 4.0, time sweeps were stopped, and the strain amplitude was subsequently increased from 0.003 to 3.0 (10 points per decade) at 1 rad·s\(^{-1}\) to investigate the large deformation properties of the gels. Additionally, pH development during acidification was recorded in a separate sample using a pH meter with memory function (InoLab, WTW GmbH, Weilheim, Germany).

All rheological measurements were performed in duplicate; half deviation range was always smaller than 5 % of the mean value.

3. Results and discussion

3.1. Composition of casein mixtures

Table 1 summarises IC, monomer, dimer and polymer contents, and the PD of the reference (0 h) and the casein solutions after cross-linking with mTGase (incubation time serves as sample coding). IC, polymer content and PD continuously increased with incubation time, monomer content decreased, and dimer content showed highest values after 1 and 2 h of incubation. Although different lots of casein and mTGase were used, both IC and PD fit well to the data published previously (Jaros et al., 2014b; Fig. S1). The data in Table 1 illustrates the idea behind the sample set-up. IC after 1 h incubation was 0.46 g\(\cdot\)100 g\(^{-1}\), and the same IC was obtained by mixing the 4 h and the 0 h samples in a ratio of 4 : 6, or by mixing the 8 h and the 0 h samples in a ratio of 3 : 7 (see also Fig. 1). The PD of the respective samples was however different, namely 49.4, 39.3 and 34.0 %, respectively.

Fig. 1. shows the monomer, dimer, and polymer contents of casein mixtures adjusted to different target ICs. The right-hand end point of each line corresponds to PD and IC given in Table 1 for the respective incubation times. Because the dimer content reached a maximum after 1 h of incubation, this sample and its mixtures had higher dimer but lower polymer contents than the other samples at an identical IC. Jaros et al. (2010) identified also a trimer fraction which also showed its highest concentration after the same mTGase treatment for ~1 h. It is therefore likely that polymers in the 1 h incubated sample and its mixtures were generally smaller. The PD showed
3.2. Gel formation of casein mixtures

Fig. S2 shows typical curves of pH and G' during acidification of casein with GDL. The pH development is not affected by mTGase treatment (Ercili-Cura et al., 2013; Jaros et al., 2010), ensuring the same acidification rate for all samples. The maximum storage modulus (G'_MAX) was observed at pH ~4.3 and taken as a measure of gel stiffness. G' of acid-induced protein gels typically decreases with pH below the isoelectric point of proteins, which has been ascribed to recreated electrostatic repulsions and to the release of particular protein fractions from the gel network into the serum phase (Braga, Menossi, & Cunha, 2006; Dickinson & Matia Merino, 2002; Schuldt, Raak, Jaros, & Rohm, 2014).

Fig. 2 displays G'_MAX of the gels obtained at different acidification conditions. Mixtures from 3 – 8 h incubated casein solutions with the reference showed similar G'_MAX at the same IC after gelation at 20 and 40 °C (only performed with 40 g·kg⁻¹ GDL), whereas the 1 and 2 h incubated samples had lower G'_MAX independent from temperature and GDL concentration. A similar trend was reported previously (Jaros et al., 2014a; Jaros et al., 2014b), however, this difference was considered to be negligible. At a gelation temperature of 30 °C, mixtures from 3, 4, and 5 h samples with the reference can be clearly distinguished from mixtures of 7 and 8 h with 0 h incubated casein solutions, and this difference was not affected by the acidification rate as the same tendency was also obtained at 35 and 45 g·kg⁻¹ GDL. This implies that differences between mixtures with the same IC are rather connected to other temperature driven effects than its impact on the acidification rate.

It has been reported that non-micellar casein molecules associate to small particles in solution as a consequence of hydrophobic interactions (Chu, Zhou, Wu, & Farrell, 1995; Farrer & Lips, 1999), with the particle conformation (size, density, monomer number) depending on temperature, pH, and ionic strength (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008). mTGase-treatment of purified β-casein and κ-casein suggested that cross-linking takes place mainly within the casein particles (de Kruif, Tuinier, Holt, Timmins, & Rollema, 2002; O'Connel & de Kruif, 2003). For gelation conducted at 40 °C (equal to the enzyme incubation temperature), no temperature induced change in particle conformation was expected. The cross-linked casein could
then directly contribute to gel stiffness, resulting in an increase of $G'_{\text{MAX}}$ with IC. It was previously suggested that especially the casein dimers and trimers act as gelation nuclei, which facilitate a proper arrangement of casein monomers during gelation (Jacob, Nöbel, Jaros, & Rohm, 2011; Jaros et al., 2010). This is, however, not valid for mixtures with ICs >0.7 g\cdot100 g^{-1}, which had the highest $G'_{\text{MAX}}$ and consisted mainly of larger polymers (Fig. 1), and for the 1 and 2 h incubated casein solutions with more dimers (and probably trimers) but lower $G'_{\text{MAX}}$ at a respective IC. The results of this systematic approach therefore imply that a particular amount of polymers (~85 – 90 %) and also a specific polymer size (>trimer) is needed for the formation of a network with high $G'_{\text{MAX}}$ (Tab. 1, Fig. 2).

HadjSadok et al. (2011) reported that the number of casein monomers per particle decreased with lowering temperature from 50 to 10 °C as a consequence of reduced hydrophobic attraction. Hence, casein particles would be more prone to conformational changes when gelation is conducted at 20 or 30 °C. Highest $G'_{\text{MAX}}$ was shifted from IC ~1.6 g\cdot100 g^{-1} at 40 °C to IC ~1.2 g\cdot100 g^{-1} at 30 °C (independent from GDL concentration), and to IC ~1.1 g\cdot100 g^{-1} at 20 °C (Fig. 2). This shift might result from the dissociation of monomers, dimers, and maybe trimers from the cross-linked casein particles, which could then support gel formation by their more flexible integration into the network. Furthermore, mixtures from 3, 4, or 5 h incubated samples with the reference showed higher $G'_{\text{MAX}}$ at 30 °C than mixtures of the 8 h and 0 h sample at the same IC. This is in good agreement with a previous study (Jaros et al., 2014a; Jaros et al., 2014b), where it was concluded that gel formation was hindered by the presence of polymers above a critical size, which were formed during prolonged incubation. However, since this was not the case at 40 °C, and 1 and 2 h incubated casein had also lower $G'_{\text{MAX}}$ at the same IC, this hypothesis must be expanded. A decrease from an incubation temperature of 40 °C to a gelation temperature of 30 °C reduces hydrophobic attraction between casein molecules within distinct particles (de Kruif, Anema, Zhu, Havea, & Coker, 2015; Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997), which might cause a loosening or partly dissociation from the casein particles. As long as these conformational changes are not considerably restricted because of excessive cross-linking, this would result in enlarged contact area and an enhanced interaction potential of the particles. The current results suggest that the loosening of casein particles was similar in 3, 4, and 5 h incubated casein solutions and mixtures thereof, resulting in a similar interaction potential and similar $G'_{\text{MAX}}$ at identical IC. In contrast, conformational changes were probably restricted after incubation for 8 h, therefore decreasing the interaction potential and $G'_{\text{MAX}}$. At 20 °C, hydrophobic interactions are weaker, but the strength of hydrogen bonds is greater (Ringgenberg, Alexander, & Corredig, 2013). When electrostatic repulsions are diminished by acidification, hydrogen bonds would facilitate interactions between the hydrophilic molecule parts located at the surface of the casein particles.
Hence, conformational changes of cross-linked casein particles would be less important, resulting in similar $G'_{\text{MAX}}$ of mixtures of 3 – 8 h with 0 h incubated casein solutions at identical IC. Additionally, the differences between the reference and cross-linked samples were much lower at 20 °C ($\Delta G'_{\text{MAX}} \sim$100 Pa) compared to 30 °C ($\Delta G'_{\text{MAX}} \sim$185 Pa at 40 g·kg$^{-1}$ GDL) and 40 °C ($\Delta G'_{\text{MAX}} \sim$150 Pa), underlining the peculiar effects of uncross-linked casein in the mixtures. At 20 °C, dissociation of casein is easiest for the uncross-linked particles. Hence, when present in a specific amount, the gel network is supported by the more flexible integration of the molecules. For instance, at an IC $\sim$0.46 g·100 g$^{-1}$, the 1 h incubated casein solution had a lower monomer content and a lower $G'_{\text{MAX}}$ than mixtures from longer incubated casein solutions with the reference at the same IC. Likewise, the 8 h incubated casein solution had an IC $\sim$1.6 g·100 g$^{-1}$ and the lowest monomer content, and $G'_{\text{MAX}}$ was lower than samples and mixtures with IC between 0.9 – 1.3 g·100 g$^{-1}$, which contained a higher amount of uncross-linked casein. A similar tendency was observed previously for gelation at 30 °C (Jaros et al., 2014a; Jaros et al., 2014b). The conformational changes of casein particles at temperature changes and during gelation are still not fully explored. However, it seems evident that the particles do not maintain their shape because a simple interrelation between $G'_{\text{MAX}}$ and IC could not be found in the present gelation experiments.

3.3. Large deformation properties

Fig. S2 shows a typical strain sweep experiment conducted immediately after monitoring gelation at small deformation. As reported previously (Rohm et al., 2014), casein gels showed pronounced strain hardening, which was manifested by an increase of $G'$ with increasing strain amplitude between the linear viscoelastic region and fracture. For comparison of the samples, the stress overshoot factor was calculated as the ratio of peak value ($G'_{\text{OS}}$) to plateau value ($G'_{\text{p}}$) of the curves (Gisler, Ball, & Weitz, 1999). Although casein mixtures with similar $G'_{\text{MAX}}$ showed also similar gelation profiles in small strain measurements (Fig. S3), they differed clearly in their large deformation properties.

Fig. 3 shows the stress overshoot factor of the gels (from pure and mixed casein solutions) as a function of their IC. With some limitations, the pure cross-linked casein samples (last point of each curve) showed a decreasing stress overshoot factor with increased cross-linking, which is consistent with previous results (Rohm et al., 2014). In contrast, Ercili-Cura et al. (2013) observed strain hardening only for acid-induced gels from mTGase treated skim milk but not for the uncross-linked reference. Casein gels in the current study exhibited a similar behaviour at 40 °C, where the stress overshoot factor was higher for all samples with IC $\leq$0.5 g·100 g$^{-1}$ compared to the reference. Nevertheless, mixtures with the same IC revealed a tendency to a lower stress overshoot factor for longer incubated casein solutions at all acidification conditions, although some
discrepancies were noticeable especially at lower ICs. Consistent with previous research (Rohm et al., 2014), decreasing stress overshoot factors were also accompanied by slightly lower fracture strains.

According to Gisler et al. (1999), a particle gel network exhibits a curved backbone, and an increasing rigidity with increasing strain amplitude is the result of stretching out the backbone until it breaks. Ercili-Cura et al. (2013) described this as the intrinsic strength of the particles, which form the backbone of the gel. For the samples of the present study this behaviour could mean that either the backbones are a priori less curved in gels containing larger polymers, or a lower flexibility of larger polymers simply results in fracture at lower strain amplitudes. At least for mixtures with a similar gelation behaviour and similar $G'_\text{MAX}$ in small deformation measurements (section 3.2, Fig. 2, Fig. S3), a similar network formation might be expected. Therefore, a lower stress overshoot factor would be rather a consequence of less flexibility of the polymers. Gels from the reference and 1 and 2 h incubated casein solutions, however, may have had a more curved backbone and thus a higher intrinsic strength, which would be exposed only at increased strain amplitudes. This would result in lower stiffness in small deformation measurements, but in a high stress overshoot factor in large deformation measurements.

4. Conclusions

Mixing uncross-linked casein with casein that was treated with mTGase for different times resulted in samples with identical IC but different PD. The gelation of these samples showed that the number of cross-links (expressed as IC) is an important but not the only driver for stiffness of casein gels. Although for a certain range of incubation times gel stiffness was directly dependent on the IC of the different mixtures, a high amount of small polymers (especially dimers) was shown to result in lower gel stiffness compared to casein mixtures, which had the same IC but larger polymers. On the other hand, casein solution incubated for longer time (8 h) and mixtures prepared thereof had the lowest gel stiffness at a given IC when gelation was conducted at 30 °C instead of 40 °C (equal to temperature during mTGase treatment). The stiffest gels were always formed at PD ~85 – 90 %, suggesting that amount and size of casein polymers are probably not the only reasons for weaker gels. Instead, a decreased interaction potential because of restricted temperature driven changes in casein conformation after excessive cross-linking was assumed to negatively affect gel stiffness. The results point on the importance of a deeper knowledge on the interactions between specific casein polymer fractions to understand their gelation behaviour.
Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References


Tab. 1: Effect of incubation time on isopeptide formation and enzymatic polymerisation of 27 g·kg⁻¹ casein solutions treated with microbial transglutaminase (3 U per g casein, 40 °C).

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Figures

**Fig. 1:** Monomer (top), dimer (middle), and polymer contents (bottom) relative to total protein as a function of isopeptide contents of 27 g·kg⁻¹ casein solutions which were prepared by blending the uncross-linked reference with casein solutions which were mTGase-treated (3 U per g casein, 40 °C) for 1 h (white squares), 3 h (white circles), 4 h (grey circles), 5 h (black circles), and 8 h (black squares). The last point in each curve represents the pure cross-linked casein (see also Table 1). Casein cross-linked for 2 and 7 h and mixtures thereof were left out for the clearness of the graphs.
Fig. 2: Stiffness ($G'_{\text{MAX}}$) of acid-induced gels prepared from 27 g·kg$^{-1}$ casein solutions as a function of their isopeptide content. Samples were obtained by blending the uncross-linked reference with casein solutions which were mTGase-treated (3 U per g casein, 40 °C) for 1 h (white squares), 3 h (white circles), 4 h (grey circles), 5 h (black circles), and 8 h (black squares). The last point in each curve represents the pure cross-linked casein. Gelation temperature and glucono-δ-lactone concentration were varied as indicated by the horizontal and vertical boxes, respectively. Casein cross-linked for 2 and 7 h and mixtures thereof were left out for the clearness of the graphs.
Fig. 3: Stress overshoot factors of acid-induced gels prepared from 27 g·kg⁻¹ casein solutions as a function of their isopeptide content. Samples were obtained by blending the uncross-linked reference with casein solutions which were mTGase-treated (3 U per g casein, 40 °C) for 1 h (white squares), 3 h (white circles), 4 h (grey circles), 5 h (black circles), and 8 h (black squares). The last point in each curve represents the pure cross-linked casein. Gelation temperature and glucono-δ-lactone concentration were varied as indicated by the horizontal and vertical boxes, respectively. Casein cross-linked for 2 and 7 h and mixtures thereof were left out for the clearness of the graphs.
Fig. S1: Polymerisation degree (circles) and isopeptide content (squares) of 27 g·kg⁻¹ casein solution as a function of incubation time with microbial transglutaminase (3 U per g casein, 40 °C). Data of the current study (closed symbols) are compared with previous results (open symbols; Jaros et al. (2014) Int. Dairy J., 39, 345-347).
**Fig. S2:** Typical curves of pH (dotted line) and $G'$ (full line) during acidification of casein solutions with glucono-δ-lactone (left), and typical strain sweep experiment conducted subsequently to gelation measurements (right). Sample was a 27 g·kg$^{-1}$ casein solution incubated for 3 h with 3 U mTGase per g casein at 40 °C and acidified with 40 g·kg$^{-1}$ glucono-δ-lactone at 30 °C. Highlighted parameters $G'_{\text{MAX}}$, $G'_0$, and $G'_{\text{OS}}$ were taken for further sample characterisation.
Fig. S3: Gelation profiles of 27 g·kg⁻¹ casein solutions as a function of pH. Samples were obtained by blending the uncross-linked reference with casein solutions which were treated with microbial transglutaminase (3 U per g casein, 40 °C) for 1 h (white squares), 3 h (white circles), 4 h (grey circles), 5 h (black circles), and 8 h (black squares). Numbers within the graphs refer to the isopeptide content (IC, g·100 g⁻¹ casein) of the corresponding mixture. Gelation temperature and glucono-δ-lactone concentration were varied as indicated by the horizontal and vertical boxes, respectively. Samples prepared from the 1 and 8 h incubated casein solutions are shown in dotted lines to simplify distinguishing.