Kinetic aspects of casein micelle cross-linking by transglutamininase at different volume fractions

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1. Introduction

Caseins are major dairy proteins, and not only a recognised source of nutrients such as essential amino acids, calcium and phosphorus, but also widespread techno-functional ingredients in food manufacturing. In recent years, the concentration and fractionation of milk proteins by membrane filtration have become increasingly relevant (Carter, Cheng, Kapoor, Meletharayil, & Drake, 2021; Corredig, Krishnakutti Nair, Li, Eshpari & Zhao, 2019). In such processes, skimmed milk is passed over a semi-permeable membrane, allowing for the partial transmission of certain components into the permeate fraction while increasing the ratio of protein to dry matter in the retentate. Depending on the molecular weight cut off (MWCO) of the membrane, whey proteins are either fully retained (ultrafiltration (UF); typically ~10 kDa MWCO) or partially depleted (microfiltration (MF); typically ~0.1–0.2 μm pore size), resulting in either milk protein concentrate (MPC) or micellar casein concentrate (MCC), respectively (Raak & Corredig, 2022). The total protein content (MPC) or casein content (MCC) of the products can be increased further by diafiltration, i.e., the addition of water or other media to the concentrate during filtration, before they are typically spray-dried and sold as powders for reconstitution.

Post-translational modifications of casein proteins in ingredients such as caseinates, MPCs or skim milk powders have been studied as means to tailor their properties and create added value. One example is the use of enzymatic cross-linking via microbial transglutamininase (mTGase; EC 2.3.2.13); this enzyme catalyses the formation of covalent isopeptide bonds between glutamine and lysine residues (Buchert et al., 2010). Many studies have been conducted to demonstrate how cross-linking of caseins may improve the technological properties of dairy products, such as yoghurt, cheese or ice cream (Gharibzahedi & Chronakis, 2018; Gharibzahedi et al., 2018; Raak, Rohm, & Jaros, 2021; Romeih & Walker, 2017). Until recently, however, less attention has been given to the potential of casein ingredients modifications. It has been shown for instance, that sodium caseinate cross-linked by mTGase, when added to skim milk, increases the stiffness of the resulting acid gels (Raak, Rohm, & Jaros, 2020). Moreover, the addition of a powder derived from MPC (~70% protein) cross-linked by mTGase to enrich protein in yoghurt, results in a higher gel strength compared to the same treatment but without cross-linked MPC (Chen et al., 2018; Li et al., 2021).

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Only intra-micellar cross-linking with limited changes in casein micelle size has been reported so far for casein micelles suspensions when treated at their native volume fraction (Huppertz & de Kruif, 2008; Mounsey, O’Kennedy, & Kelly, 2005). Furthermore, only few studies have addressed the mTGase cross-linking reaction for casein micelles in concentrated suspensions, meaning volume fractions higher than those of native milk. For example, Huppertz (2014) used reconstituted skim milk powder at 180 g/kg dry matter (~2x concentration) and observed that low levels of cross-linking improves heat stability, as shown by an increased heat coagulation time when treated at pH > 6.8. Lam et al. (2017) treated reconstituted skim milk at 276 g/kg dry matter (~3x) with mTGase. When these samples were diluted back to 1x with different concentrations of EDTA, an increased stability of casein micelles against disruption induced by Ca²⁺-chelation was observed. Moreover, Er, Sert, and Mercan (2019) produced powders from mTGase-treated skim milk, concentrated by evaporation to 450 g/kg dry matter (~4x), and observed lower powder particle sizes and decreased caking with cross-linking. Fewer studies are available on casein micelles suspensions such as MPC. Power, Fenelon, O’Mahony, and McCarthy (2020), treated a casein micelles suspension from MPC reconstituted to 100 g/kg protein (~3x), and showed that cross-linking could stabilise the protein particles against the disruption induced by the addition of sodium hexametaphosphate.

To date, no systematic investigation has been reported of the mechanism of casein cross-linking by mTGase in concentrated casein micelles suspensions such as fresh milk protein concentrates obtained by membrane filtration. As in these systems the inter-particle distance between casein micelles is considerably reduced down to lengths much smaller than the casein micelle diameter (Karlsson, Ipsen, Schrader, & Ardo, 2005), it might be hypothesised that in milk concentrates the enzyme activity is accelerated and cross-linking may occur not only intra- but also inter-micellarly. The aim of this study was to investigate the kinetics of casein micelle cross-linking by mTGase in 4x MPC compared to unconcentrated milk. Fresh casein micelles suspensions were prepared from skim milk using UF (MPC), and treated with different levels of enzyme and for different periods of time to fully explore the kinetic details of the reaction. The cross-linking extent was evaluated using denaturing size exclusion chromatography, and dynamic light scattering was applied to assess potential changes in the casein micelle structure. This research will lead to a better understanding of the mode of action of mTGase on concentrated milk protein suspensions, and contribute knowledge to the development of casein ingredients of improved functionality.

2. Materials and methods

2.1. Materials

Pasteurised skim milk (72 °C, 15 s) was provided by Arla Foods Amba (Viby, Denmark). The enzyme mTGase “Activa MP” from Streptomyces mobaraensis was a gift from Ajinomoto Foods Europe SAS (Mesnil-Saint-Nicaise, France) and had a declared activity 100 U/g. Standards of αs1, β- and κ-casein, α-lactalbumin and β-lactoglobulin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and had declared purities of ≥70%, ≥98%, ≥70%, ≥85% and ≥90%, respectively. Bovine serum albumin (BSA) was obtained from VWR Life Sciences (Søborg, Denmark) and had a declared purity of ≥98%. All other chemicals were of analytical grade.

2.2. Sample preparation

2.2.1. Manufacture of milk protein concentrate

Skim milk was treated with 2 g/kg NaN₃ for preservation and concentrated using a Vibro Lab3500 system equipped with a PESH plate-and-frame UF membrane unit (30 kDa MWCO; 0.35 m² membrane area) and connected to a 9 L stainless steel pressurised container as feed tank (Sani Membranes ApS, Allerød, Denmark). The container was kept in an ice bucket during the filtration to maintain a temperature of approx. 10 °C. The filtration was driven by pressurised air at 0.2 MPa, and the retentate flow was regulated manually via the valve opening. The concentrate passing through the membrane chamber was continuously circulated by an external pump (RLFP122202, Sani Membranes ApS). The system was operated in vibration mode to minimise fouling and thus facilitate the separation. The filtration was ended when the retentate reached a 4x concentration factor, based on volume of permeate removed.

2.2.2. Cross-linking by microbial transglutaminase

Skim milk and 4x MPC were equilibrated in a water bath at 40 °C for 1 h before mTGase was added at concentrations of 0.125, 0.5 and 2.0 mg/g (skim milk) or 0.5, 2.0 and 8.0 mg/g (4x MPC), corresponding to similar enzyme:protein ratios for the two substrates. The samples were incubated at 40 °C for 20, 80, 320 or 1280 min, and the enzymatic reaction was stopped by adding 8 mmol/L N-ethylmaleimide (NEM). Enzyme levels and incubation times were based on previous published work on skim milk (Jaros, Jacob, Otto, & Rohm, 2010). Reference samples referred to as 0 min incubation time corresponded to skim milk and MPC with 8 mmol/L NEM but without addition of mTGase. Additional reference samples were prepared by adding 8 mmol/L NEM before incubating skim milk and 4x MPC with 2.0 and 8.0 mg/g mTGase at 40 °C for 1280 min, respectively. All samples were prepared and analysed within one week.

For a more comprehensive interpretation of the results, the theoretical concentration of cross-links nₜ (µmol/g) that could have been formed, assuming infinite availability and susceptibility of glutamine and lysine residues was calculated:

\[
nₜ = \frac{I_{\text{inc}} \cdot c_{E} \cdot A_{P}}{1000 \cdot \text{CF}}
\]

(1)

Here, \(I_{\text{inc}}\) is the incubation time (min), \(c_{E}\) is the enzyme concentration (mgm/mTGase/g), \(CF\) is the concentration factor (i.e., 1 for skim milk and 4 for MPC), and 1000 is a conversion factor. \(A_{P}\) corresponds to the declared enzyme activity of 100 U/mTGase, where 1 U = 1 µmol reaction product per min (Jin et al., 2016). Although \(nₜ\) may not be an accurate predictor of the actual concentration of cross-links, it is a useful parameter for normalisation when comparing samples treated with different enzyme concentrations (Raak et al., 2019).

2.2.3. Separation of colloidal and serum phases

Skim milk and 4x MPC samples were separated into serum and colloidal phase by ultracentrifugation (Optima L-80 XP, Beckman Coulter, Inc., Brea USA; rotor type Ti70; 23 mL tube volume) at 100,000 × g and 21 °C for 1 h. The supernatant was carefully removed, and the casein micelle pellet was discarded.

2.2.4. Preparation of casein and whey protein reference samples

Standard caseins (αs1-, β- and κ-casein) standards were dissolved in demineralised water at a concentration of 6 mg/g, and α-lactalbumin, β-lactoglobulin and BSA were dissolved in demineralised water at a concentration of 3 mg/g. All reference samples were analysed in the same way as the skim milk samples.

2.3. Characterisation of sample composition

The total nitrogen contents of skim milk, 4x MPC, their supernatants, and permeate from ultrafiltration were determined using a Gerhardt Dumastherm (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The protein contents were calculated after subtracting the nitrogen content of the ultrafiltration permeate (non-protein nitrogen) and using a conversion factor of N × 6.38.
2.4. Evaluation of the cross-linking extent

2.4.1. Size exclusion chromatography

A Superose 6 Increase 10/300 GL column (GE Healthcare, Uppsala, Sweden) was integrated into an AKTA purifier system comprising a P-900 pump, an IN-907 injection valve, and a UV-900 UV detector (GE Healthcare). The elution buffer consisted of 6 mol/L urea, 0.1 mol/L NaCl, 0.1 mol/L Na₂HPO₄·2H₂O and 0.2 g/L NaN₃, and was adjusted to pH 6.8 using 6 mol/L HCl. Skim milk samples as well as their supernatants were diluted 1:10 (v/v) with elution buffer containing 10 mg/mL dithioerythritol, whereas 4x MPCs and the corresponding supernatants were first diluted 1:4 (v/v) with UF permeate before diluting them 1:10 (v/v) with the buffer system. The samples were incubated at room temperature for at least 1 h to cleave disulphide bonds, and subsequently injected using a 100 µL sample loop. The separation was carried out at ambient temperature (approx. 21 °C) and an isocratic flow rate of 0.75 mL/min, and the eluting protein was detected at 280 nm. The data was collected and processed using the UNICORN software (v5.31; GE Healthcare). As whey proteins co-elute with the caseins (Raak, Abbate, Lederer, Rohm, & Jaros, 2018), the chromatograms of the supernatants were subtracted from those of the corresponding skim milk and 4x MPC samples to analyse specifically the cross-linking extent of the casein micelles. The area (A) under the curve corresponding to polymerised caseins (eluting at 7–15.5 mL) and monomeric caseins (15.5–17.5 mL) were determined, and the polymerisation degree PD (%) was calculated as follows:

\[
PD = \frac{A_{\text{polymerised}}}{(A_{\text{monomeric}} + A_{\text{polymerised}})}\times 100\%
\]

Standards of αS-, β- and κ-casein, α-lactalbumin, β-lactoglobulin and BSA were run as well to determine their elution volumes.

2.4.2. Gel electrophoresis

In order to obtain additional insights on the cross-linking of the different casein types (αS, β, κ) in skim milk and MPC, denaturing and reducing gel electrophoresis was carried out using an Invitrogen™ system and the corresponding supplies (ThermoFisher Scientific, Waltham, MA, USA). 4x MPC samples as well as their supernatants were first diluted 1:4 (v/v) with UF permeate. Afterwards, all samples were diluted 1:15 (v/v) with demineralised water and subsequently mixed with NuPAGE™ LDS sample buffer and 1 mol/L dithioerythritol in a ratio of 6.5:2:5:1 (v/v). The mixtures were boiled at 95 °C for 5 min in a thermosthaker (IKA-Werke GmbH&Co.KG, Staufen, Germany). Aliquots (10 µL) were injected to NuPAGE™ precast gradient gels (4–12% polyacrylamide), and the experiment was run at 200 V for 40 min using an XCell SureLock™ Mini-Cell filled with NuPAGE™ MES SDS running buffer. The gels were stained overnight in SimplyBlue™ SafeStain solution, rinsed in demineralised water for 24 h, and subsequently digitalised using a ChemiDoc XRS + gel imaging system (Bio Rad Laboratories, Inc., Hercules, CA, USA). The protein bands were analysed semi-quantitatively using Image Lab (v6.0.1; Bio Rad Laboratories, Inc.), and the band intensities of the different casein fractions in mTGase-treated samples were divided by those of the uncross-linked reference to evaluate the accessibility of the different casein types for the enzyme. A molar mass standard (Precision Plus Protein™, Bio Rad Laboratories) and standards of αS-, β- and κ-casein, α-lactalbumin, β-lactoglobulin and BSA were run on a separate gel together with uncross-linked skim milk and 2.0 mg/g mTGase for band identification.

2.5. Dynamic light scattering

The hydrodynamic diameter \(D_h\) of the protein particles was estimated using a Zetasizer Lab (Malvern Panalytical Ltd., Malvern, UK) in side scattering mode (\(θ = 90°\)). Skim milk and 4x MPC samples were diluted 1:1000 and 1:4000 (v/v) with filtered (0.45 µm nylon) UF permeate, respectively, and the measurements were carried out at 30 °C after 120 s equilibration time. Correlation functions were acquired and processed using ZS Xplorer (v2.0.1.1; Malvern Panalytical Ltd.). The refractive index for solvent was set to 1.341 for UF permeate (Beliciu, Sauer, & Moraru, 2012), and solvent viscosity was 1.029 mPa s (DHR 20, TA Instruments, New Castle, DE, USA; \(γ = 10/s\)). For each biological replicate, three subsequent measurements of two technical replicates were performed and averaged. Results shown are z-averages of \(D_h\).

![Fig. 1. Typical size exclusion chromatograms of skim milk samples without microbial transglutaminase (dotted lines) and treated with 0.5 mg/g microbial transglutaminase for 1280 min (full lines). (A) Total skim milk samples, (B) centrifugal supernatants, (C) difference between (A) and (B). Note the difference in y-axis scaling for (B).](image-url)
2.48 mg/g, which is in good agreement with the results obtained by enzyme concentrations.

Individual milk batches from different weeks were used. In each week, supernatants (Fig. 1b) the majority of the protein eluted between 15.5 gal supernatants. In untreated milk (Fig. 1a, dotted line) and the membrane. The total protein contents of the skim milk and 4x MPC were that neither whey proteins nor non-micellar caseins passed the 30 kDa membrane. The BSA standard eluted at about 13.7 mL (not shown), but this protein was not detected in these samples.

3. Results and discussion

3.1. Sample composition

The ultrafiltration permeate was analysed with SEC and gel electrophoresis without detecting proteins (data not shown), demonstrating that neither whey proteins nor non-micellar caseins passed the 30 kDa membrane. The total protein contents of the skim milk and 4x MPC were 35.9 ± 0.52 mg/g and 127.4 ± 9.54 mg/g, respectively, and the corresponding soluble protein contents were 7.59 ± 0.45 mg/g and 40.88 ± 2.48 mg/g, which is in good agreement with the results obtained by Renhe and Corredig (2018).

3.2. General polymerisation kinetics

Fig. 1 shows representative size exclusion chromatograms of untreated and mTGase-treated skim milk and the corresponding centrifugal supernatants. In untreated milk (Fig. 1a, dotted line) and the supernatants (Fig. 1b) the majority of the protein eluted between 15.5 and 18.5 mL. These fractions contained mostly monomeric proteins, as demonstrated from elution of isolated fractions of αs1-, β- and κ-casein, eluting at 15.9, 16.3 and 16.6 mL, respectively (data not shown). Caseins were not resolved from the whey proteins, α-lactalbumin (eluting at 17.4 mL) and β-lactoglobulin (16.7 mL). These results were fully consistent with previous reports (Raak et al., 2018). The centrifugal supernatants showed three unresolved peaks eluting at 15.8, 16.7 and 17.4 mL (Fig. 1b), identifiable as soluble β-casein, β-lactoglobulin, and α-lactalbumin. The BSA standard eluted at about 13.7 mL (not shown), but this protein was not detected in these samples.

Fig. 1a compares the elution of untreated skim milk and the same sample incubated with mTGase for 1280 min. The enzyme treatment clearly decreased the intensity of the peak corresponding to the monomeric proteins (eluting between 15.5 and 18.5 mL) and resulted in the appearance of a new peak at ~15.5 mL, corresponding to a fraction of polymerised caseins with molecular weights larger than 4·10^4 kDa (based on the exclusion volume of the column). The size exclusion chromatograms of the two supernatants (Fig. 1b) showed overlapping elution behaviour, suggesting similar concentrations of residual monomeric proteins. The area of this peak was not significantly different throughout incubation, as it contained mainly residual serum proteins, which, under these conditions, are barely cross-linked by mTGase.

Native whey proteins are known to be a very poor substrate for mTGase due to the low accessibility of their globular structure (Buchert et al., 2010). Using gel electrophoresis, previous authors (Chen et al., 2016) observed a slight decrease in free α-lactalbumin and β-lactoglobulin after mTGase-treatment of reconstituted MPC powder; however, in this case, process-induced changes may have resulted in a partially denatured whey protein fraction, with an increased susceptibility to the cross-linking enzyme.

Due to the co-elution of soluble caseins and whey proteins, differential chromatograms were generated by subtracting the supernatants (Fig. 1b) from the corresponding samples’ chromatograms (Fig. 1a). From this differential elution pattern (Fig. 1c) it was possible to estimate the extent of casein micelle cross-linking by the amount of uncross-linked caseins in the samples (see Eq. (2)). Fig. 1c clearly indicates that, while in untreated skim milk (dotted line) a large peak corresponding to the uncross-linked, dissociated caseins, without any aggregate peak eluting earlier in the chromatogram was present, after

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Polymerisation Degree (%)</th>
<th>4x Milk Protein Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skim Milk</td>
<td>0.5 mg/g mTGase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 mg/g mTGase</td>
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<tr>
<td></td>
<td></td>
<td>4.0 mg/g mTGase</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>1.7 ± 1.5 A</td>
</tr>
<tr>
<td>20</td>
<td>4.3 ± 1.4 A</td>
<td>1.7 ± 1.5 A</td>
</tr>
<tr>
<td>80</td>
<td>8.2 ± 0.6 A</td>
<td>1.7 ± 1.5 A</td>
</tr>
<tr>
<td>320</td>
<td>25.5 ± 3.0 A</td>
<td>7.0 ± 3.0 A</td>
</tr>
<tr>
<td>1280</td>
<td>43.8 ± 2.2 A</td>
<td>35.1 ± 3.5 A</td>
</tr>
</tbody>
</table>

Table 1 Polymisation degree of skim milk and 4x milk protein concentrate treated with different levels microbial transglutaminase (mTGase) for different incubation times at 40 °C. Incubation time of 0 min corresponds to samples with no enzyme added. Letters correspond to significant differences (p < 0.05) within the same row (small case letter) and within the same column (capital letter). Values represent average of two replicates and their standard deviation.

2.6. Statistical analyses

All samples were produced in two biological replicates, and three individual milk batches from different weeks were used. In each week, both skim milk and MPC were treated with mTGase at two different enzyme concentrations.

Data were analysed using SigmaPlot (v14.0.3.192; Systat Software Inc., San Jose, CA, USA). A Student’s t-test was used to identify significant effects of incubation time and sample type (i.e., concentration factor + enzyme level). Spearman’s rank order was performed for correlation analyses. The statistical acceptance level was p < 0.05.

Fig. 2. Polymisation degree of skim milk (closed symbols) and 4x milk protein concentrate (open symbols) that were treated with 0.125 (circles), 0.5 (squares), 2.0 (diamonds), or 8.0 (triangles) mg/g microbial transglutaminase at 40 °C for different incubation time (0–1280 min) as a function of the theoretical amount of formed reaction product in the sample (nθ; see Eq. (1)). Values represent averages of two replicates and their standard deviation.
generally similar for skim milk and 4x MPC, as within the same level of increased significantly with increasing incubation time and higher the presence of NEM for 1280 min (Table 1) significantly different from the endogenous reactions occurring in the milk (i.e., non-enzymatic cross-linking mainly caused by the presence of an aggregate peak resulting from mTGase). The control samples (0 min) showed minimal levels of polymerisation, mainly caused by the presence of an aggregate peak resulting from endogenous reactions occurring in the milk (i.e., non-enzymatic cross-linking during storage or pasteurisation). These values were also not significantly different from the PDs of skim milk and MPC incubated in the presence of NEM for 1280 min (p < 0.05) (data not shown). The PD increased significantly with increasing incubation time and higher enzyme levels. The polymerisation kinetics of casein micelles were generally similar for skim milk and 4x MPC, as within the same level of enzyme, there were no significant differences in the PD values between skim milk and 4x MPC, apart from one treatment (medium mTGase, 320 min incubation), where the PD was lower in the concentrate than in the skim milk.

In Fig. 2, the development of the polymerisation degree is illustrated as a function of the theoretical amount of product formed (nₘ, see Eq. (11)). It is clear that the PD can be scaled by normalising the incubation time to enzyme level and concentration factor (see Eq. (11)). A positive correlation was found between the PD and nₘ with a Spearman correlation coefficient of 0.967 (p < 0.05), demonstrating the proportionality between these three factors in the polymerisation kinetics of skim milk and 4x MPC. It was therefore possible to conclude that the overall polymerisation by mTGase of casein proteins is not affected by the proximity of the casein micelles, as there were no differences with volume fraction.

The highest PD values reached in this study ranged from ~81% for skim milk and ~87% for 4x MPC (Table 1), indicating an almost complete cross-linking of the casein micelles with less than 20% monomeric casein remaining. Other authors reported maximum PD values of, e.g., ~80% (Jaros et al., 2010) and ~67% (Duerasch et al., 2020; Duerasch et al., 2018) for casein micelles in skim milk. However, comparison to literature values is difficult as, e.g., unidentified deviations between actual and declared enzyme activity or missing information on the enzyme activity make the comparison of the incubation conditions a challenge. For instance, Huppertz, Smidty, and de Kruiff (2007) stated to have reached complete cross-linking of casein micelles after 24 h as monomeric caseins were absent in gel electrophoresis, but did not provide information on the enzyme activity of their mTGase.

### 3.3. Cross-linking kinetics of the individual casein types

Gel electrophoresis was used to determine possible differences in the reactivity of the different casein types (αₛ, αₛ₂, β, κ) in skim milk and 4x MPC to mTGase. Fig. 3a shows a representative migration pattern of skim milk compared to that of the various caseins and whey protein standards. A 2.0 mg/g mTGase solution was too diluted to appear as an additional band under the analytical conditions used in this study, demonstrating that mTGase is not visible in the casein samples. The skim milk sample showed three major bands for uncross-linked casein. The un-cross-linked caseins migrated to positions that were not in agreement with their molar masses (~28–31 kDa instead of ~19–25 kDa), as was also reported in other studies (Creamer & Richardson, 1984; Liu & Damodaran, 1999; Raak et al., 2018). Therefore, the standard was not further used to characterise the polymerised casein.

Fig. 3b shows typical electrophoretic migration patterns of mTGase treated casein. In agreement with the size exclusion chromatography analysis, the band intensity of monomeric caseins decreased and new high molecular weight fractions appeared with ongoing incubation. The band intensities of the whey proteins (α-La, β-lactoglobulin, BSA) in the milk samples and in their centrifugal supernatant did not vary with incubation time, confirming what was shown in Fig. 1b.

As shown by the casein standards, β- and κ-casein could not be distinguished, contradictory to previous studies (e.g., Bogahawaththa, Trajkovska, Markoska, & Vasiljevic, 2021; Liyanaarachchi & Vasiljevic, 2018). This lack of separation efficiency could be due to the use of gradient gels, where the resolution decreases with decreasing molecule size. NuPAGE™ gradient gels were not applied in many other studies. Only the work by Fuentes-Lemus, Jiang, Häglund, and Davies (2021), who investigated isolated αₛ, β, and κ-caseins on separate gels, indicates a similar behavior. The other two upper bands refer to αₛ₂-casein, respectively (Macierzanka et al., 2011). The intensity of the band referring to αₛ₂-casein appears to be high in relation to the expected concentration of this fraction in the sample, which was most likely due to differences in the binding of the dye in the staining solution between the different casein types.

β- and κ-casein were therefore integrated together, and compared to the total area of αₛ-caseins, as β-, and especially κ-casein, have been
reported to be most susceptible to mTGase in skim milk due to their location at the outer regions of the casein micelle (Duerasch et al., 2018; Hinz, Huppertz, & Kelly, 2012). It was hypothesised that a higher susceptibility of αS-caseins to mTGase in 4x MPC compared to skim milk might indicate severe structural changes of the casein micelle upon concentration that increased the accessibility of its inner regions. For instance, it was demonstrated by Duerasch et al. (2018) that αS-caseins are more accessible to mTGase after alkaline treatment of the casein micelle at pH 7.9, whereas κ-casein is less cross-linked than at the native pH 6.8, suggesting an opening and/or rearrangement of the internal structure of the micelle with alkalinisation.

Fig. 4 a and b illustrate the relative decrease of uncross-linked αS-caseins (Fig. 4 a) and β- and κ-casein (Fig. 4 b) in skim milk and 4x MPC as obtained from gel electrophoresis band intensities as a function of incubation of with mTGase. Regardless of the concentration factor, the band intensity of each casein fraction decreased in the same way with no obvious differences between skim milk and 4x MPC. At the highest cross-linking extent, the band intensity of αS-caseins was ~30% of the original value, and that of the mixed β- and κ-casein fraction was ~10% of the original value. This means that the latter were more accessible to mTGase than αS-caseins. To illustrate this more clearly, the intensity ratios of uncross-linked αS-to uncross-linked β- and κ-casein are shown in Fig. 4c. The increasing trend of the curve demonstrates clearly that β- and κ-casein are more rapidly cross-linked, resulting in a higher share of αS-caseins in the fraction of uncross-linked proteins. These results fully agree with previous research showing a susceptibility of the different caseins to mTGase in the order κ > β > αS1 > αS2 for casein micelles in skim milk (Duerasch et al., 2018). No statistically significant differences between skim milk and 4x MPC were found for each combination of incubation time and enzyme level, and Spearman’s rank order suggested significant negative correlations between the relative concentrations of αS-caseins and the β- and κ-casein mixed fraction with n_th, with correlation coefficients of −0.843 and −0.915, respectively (p < 0.05).
It is worth noting that the decrease in band intensity of all casein fractions together was also in good agreement with the PD values obtained from SEC. For instance, the highest enzyme dosage and longest incubation time resulted in a relative concentration of residual monomeric caseins of ~20% (data not shown), meaning that ~80% of the casein must have been polymerised. This is comparable to the observations made by SEC (see Table 1). Spearman’s rank order suggested a significant negative correlation of the PD and the relative concentration of all residual monomeric caseins with a correlation coefficient of −0.915 (p < 0.05).

It was concluded that concentrating casein micelles by ultrafiltration to 4x did not change the kinetics of polymerisation, and also did not change the accessibility of the enzyme to the various casein proteins.

**3.4. Effect of cross-linking on casein micelle size**

Fig. 5 illustrates the apparent diameter of the casein micelles in skim milk and 4x MPC as a function of the cross-linking extent. During the initial stages of cross-linking (i.e., n_{th} < 4 μmol/g and PD ≤ 25%), the casein micelle diameter was unchanged, with a value of about 160 nm. With increasing extent of cross-linking, the apparent diameter D_{th} deviated from the initial value, with skim milk and 4x MPC showing opposite trends. Casein micelles in skim milk showed a significant decrease to ~150 nm (p < 0.05), corresponding to a radius decrease of about 5 nm, indicating a potential collapse of the polyelectrolyte layer due to enhanced crosslinking of the surface of the casein micelles. Furthermore, it has been shown previously for sodium casein particles that extensive cross-linking can cause an increase in their density (Abbate et al., 2019).

On the other hand, the diameter in 4x MPC showed a significant increase to ~170 nm at the highest cross-linking extent (p < 0.05). This increase in size was not great enough to indicate inter-micellar cross-linking, as the polydispersity index (PDI) was not significantly affected by neither the sample type nor the incubation time (PDI = 0.116 ± 0.020). It is also important to point out that there was no statistically significant difference between D_{th} of casein micelles in uncross-linked skim milk and 4x MPC, as previously reported (e.g., Coskun, Wiking, Rahimi Yazdi, & Corredig, 2022; Liu, Weeks, Dunstan, & Martin, 2014).

It can be hypothesised that the mTGase-induced changes in D_{th} are a direct result of the closer interactions between micelles in the concentrated suspensions, with an increased surface interaction between β- and κ-caseins. However, it cannot be excluded that the enzymatic cross-linking may have resulted in an increased stability of the micelles upon dilution for the light scattering analysis, and that the increase in apparent diameter was caused by the rearrangements occurring during concentration and not during enzymatic cross-linking.

Previous studies at 1x concentration factor suggested no change in the size of casein micelles during cross-linking by mTGase (e.g., Duerasch et al., 2018; Lam et al., 2017; Nogueira et al., 2019), including Huppertz and de Kruif (2008), who stated to have reached complete concentration of all residual monomeric caseins with a correlation coefficient of 75%, meaning that ~80% of the micelle surface. To gain complete understanding of the structural changes that casein micelles might undergo as a consequence of concentration and enzymatic cross-linking, further studies on the undiluted systems are necessary.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author statement**

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