Identification of the initial reactive sites of micellar and non-micellar casein exposed to microbial transglutaminase

Anja Duerasch · Maja Konieczny · Thomas Henle

Received: 17 February 2022 / Revised: 2 May 2022 / Accepted: 28 May 2022 / Published online: 18 July 2022
© The Author(s) 2022

Abstract

To investigate the influence of the internal micellar structure on the course of enzymatic cross-linking especially in the initial phase of the reaction, casein micelles isolated from raw milk via ultracentrifugation were incubated with microbial transglutaminase (mTG) in comparison with non-micellar sodium caseinate. Reactive lysine and glutamine residues were identified using a label-free approach, based on the identification of isopeptides within tryptic hydrolysates by targeted HRMS as well as manual monitoring of fragmentation spectra. Identified reactive sites were furthermore weighted by tracking the formation of isopeptides over an incubation time of 15, 30, 45 and 60 min, respectively. Fifteen isopeptides formed in the early stage of mTG cross-linking of caseins were identified and further specified concerning the position of lysine and glutamine residues involved in the reaction. The results revealed lysine K176 and glutamine Q175 of β-casein as the most reactive residues, which might be located in a highly flexible region of the molecule based on different possible reaction partners identified in this study. Except for the isopeptide αs1 K34–αs2 Q101 in sodium caseinate (SC), all reactive sites were detected in micellar and in non-micellar casein, indicating that the initial phase of enzymatic cross-linking is not affected by micellar aggregation of caseins.

Graphical abstract
Keywords  Casein · Microbial transglutaminase · Proteomics · De novo sequencing · Time-of-flight mass spectrometry

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AJS ESI | Agilent Jet Stream Electrospray Ionization |
| DTT | Dithiothreitol |
| EC | Extra-micellar casein |
| MC | Micellar casein |
| mTG | Microbial transglutaminase |
| RP-HPLC-HRMS | Reversed-phase high-performance liquid chromatography with high-resolution mass spectrometry |
| SC | Sodium caseinate |
| SEC | Size exclusion chromatography |
| SMUF | Simulated milk ultrafiltrate |
| TG | Transglutaminase |

Introduction

Microbial transglutaminase (mTG) is an enzyme which catalyzes an acyl transfer from a glutamine moiety to a primary amine. When lysine residues are available, the reaction results in a three-dimensional cross-linking of proteins. This is widely used in the food industry to modify peptides/proteins and thus optimize their functionalities [1–4]. For instance, enzymatic cross-linking of milk proteins increases viscosity as well as stability of acid-induced dairy gels and thus decreases syneresis of yogurt gels [5–7]. Furthermore, the structure of casein micelles, naturally formed nanocarriers in milk, shows higher stability against dissociating agents or pressure after enzymatic cross-linking by mTG [8, 9].

On a molecular level, α S-caseins are expected to be better substrates for mTG than β-casein and κ-casein due to a higher number of glutamine (α S1/α S2/β/κ = 14/16/20/15) and lysine (α S1/α S2/β/κ = 14/24/11/9) residues in their amino acid sequence. However, in casein micelles, α S-caseins are considered to be predominantly located in the interior of the micellar structure, resulting in less accessibility for mTG and an inverted reactivity in micellar casein compared to non-micellar casein [10, 11]. Several scientific groups have already evaluated the sites of individual caseins which are reactive for enzymatic cross-linking [12–14]. Usually, labeling substances such as [14 C]-putrescine, [14 C]-monodansylcadaverine and N-(glucose-glucose-glucitol-1)-cadaverine participating in the enzymatic reaction as acyl donor or acceptor were used. Afterward, the hydrolyzed protein samples were screened for correspondingly labeled peptides to identify reactive sites [12–14]. This approach would evoke several problems when applied for complex protein associates like casein micelles by influencing the intermolecular protein network. Hence, to the best of our knowledge, reactive sites for enzymatic cross-linking by mTG in caseins were only evaluated in individual caseins or in sodium caseinate, but not in micellar casein (MC).

The primary aim of our study was to use a label-free approach to investigate reactive sites within casein micelles while maintaining their natural structure. To ensure the maintaining of the natural micelle structure, the procedure for isolation casein micelles from raw milk standardized and optimized in our working group was used as previously reported and evaluated [10, 15, 16]. Furthermore, direct identification of formed isopeptides should deliver new insights into the enzymatic cross-linking as well as the casein micelle structure by identification of both (i.e., lysine and glutamine) moieties involved in the cross-link.

To restrict the results of this study to the most reactive ("hottest") sites of casein, we focused on the initial phase of the protein cross-linking using mild reaction conditions. Because reactivity of individual caseins in casein micelles had been investigated particularly at a high degree of cross-linking in previous studies [10, 17, 18], our approach furthermore delivers information about the influence of micellar structure on the course of the enzymatic cross-linking at the very beginning of the reaction.

Materials and methods

Materials

Bovine raw milk was obtained from a local organic farmer. Microbial transglutaminase ACTIVA MP (EC 2.3.2.13) was from Ajinomoto (Hamburg, Germany). Sodium azide was purchased from Acros (Geel, Belgium). Tris(hydroxymethyl)aminomethane was obtained from AppliChem (Darmstadt, Germany), tri-potassium citrate monohydrate from GPR Rectapure (Leuven, Belgium) and potassium chloride from Carl Roth (Karlsruhe, Germany). Formic acid, sodium hydrogen phosphate dehydrate, calcium chloride dihydrate, magnesium chloride hexahydrate, sodium hydroxide and sodium acetate were all purchased from Grüssing (Filsum, Germany). Hydrochloric acid, monopotassium phosphate, tri-sodium citrate dehydrate and potassium sulfate were obtained from Merck (Darmstadt, Germany). 3-[(3-Cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) was bought from Molekula (Dorset, UK). Trypsin from bovine pancreas for sequencing was obtained from Sigma-Aldrich (Steinheim, Germany). Dithiothreitol (DTT), acetonitrile, urea, sodium chloride, acetic acid glacial as well as ethanol were all purchased from VWR (Darmstadt, Germany). Double-distilled water, prepared by Bi-Distillation...
Preparation of casein suspension

Caseins were isolated from the same batch of raw milk to exclude genetic variations between the samples. In the first step, raw milk was defatted by centrifugation at 2000×g for 30 min at 4 °C and subsequently divided into two parts: one for production of sodium caseinate and the other for isolation of casein micelles.

Sodium caseinate was prepared according to Recio and Olieman [19]. Briefly, caseins were precipitated by acidification of skim milk with 1 M sodium acetate buffer (pH 4.3), separated from whey by centrifugation (2000×g, 20 min, 4 °C) and again dissolved in deionized water under pH adjustment to pH 7.0 by adding 1 M sodium hydroxide. To completely remove whey proteins, this procedure was performed three times. An additional defatting step was performed by washing precipitated casein with 1 L acetone followed by 2×1 L of ethanol, followed by drying under a nitrogen stream overnight. To convert the dried acid caseinate into sodium caseinate, the precipitate was dissolved in deionized water, pH was adjusted to pH 7.0 with 1 M sodium hydroxide, and the solution was lyophilized for obtaining a storable powder.

Isolation of casein micelles was performed as reported in our previous study [10]. Defatted raw milk was lyophilized to obtain storable skim milk powder, which could be then reconstituted in portions by adding bi-distilled water. MC was obtained as a pellet and separated from whey using an Optima XE-90 ultracentrifuge with a SW41-Ti rotor (both obtained from Beckman Coulter, Krefeld, Germany) at 100,000×g for 1 h at 20 °C. To remove remaining whey proteins, the pellets were washed two times with simulated milk ultrafiltrate (SMUF), which was prepared according to Jenness and Koops [20] with additional preservation using 0.02% (w/v) sodium azide. Each washing step was followed by ultracentrifugation as above. For the experiments, pellets of MC were suspended in SMUF utilizing an ultrasonic homogenizer HD2070 combined with sonotrode MS72 (Bandelin electronics, Berlin, Germany) for 2×2 min at 75% power and afterward stirred overnight at 6 °C.

All experiments were performed with suspensions of MC or sodium caseinate standardized to 1% (w/v) protein concentration by protein determination via UV signal at 280 nm using commercial sodium caseinate for calibration.

Enzymatic cross-linking

Identification of the most important (“hottest”) reactive sites of caseins was performed in an early stage of enzymatic cross-linking, indicated by the predominant formation of only a small amount of dimeric proteins and as few as possible trimeric or higher cross-linked species. Therefore, mild reaction conditions were chosen with an enzyme–substrate ratio of 1:80 (1 U/g casein) and an incubation time of 1 h. Cross-linking was carried out at the temperature optimum of the enzyme at 40 °C and the pH optimum of casein micelles at pH 6.8. For the preservation of the natural casein micelle structure, all samples were solubilized in SMUF. The enzyme reaction was terminated by heating samples at 85 °C for 7 min. As a blank, SMUF instead of mTG solution was added to casein samples and incubated equally.

After incubation, sodium caseinate was dialyzed (molecular weight cutoff 14,000 Da) and subsequently freeze-dried. Cross-linked MC was ultracentrifuged as described above. The supernatant, containing extra- MC, was removed, dialyzed and lyophilized. The MC pellet was suspended in 2 mL bi-distilled water and subsequently lyophilized. All freeze-dried samples were further subjected to size exclusion chromatography (SEC) as described below.

For weighting the importance of the different reactive sites, casein samples were also incubated for 15, 30, 45 and 60 min under the same conditions as described above but without further purification via SEC.

Semipreparative purification of dimeric casein species by size exclusion chromatography (SEC)

Semi-preparative SEC was used for the separation of dimeric from monomeric proteins after mTG treatment based on a previously reported analytical method [21]. For that, cross-linked and freeze-dried samples were solubilized to 0.2% protein in elution buffer containing 6 M urea, 0.1 M sodium chloride, 0.1 M sodium hydrogen phosphate dihydrate, 1.6 mM CHAPS with additional 1% DTT. For complete reduction of disulfide bonds, samples were kept overnight at 8 °C. After membrane filtration (1.2 µm), aliquots of 8 mL were injected in the protein purifying chromatography system NGC Quest 100 consisting of two eluent pumps, one sample pump, conductivity as well as UV detector and the fraction collector bioFrac, all from Bio-Rad Laboratories GmbH (Munich, Germany). Separation of cross-linked and monomeric proteins was carried out by using the column Superdex 200 HiLoad 26/60 prep grade (GE Healthcare Life Sciences, Buckinghamshire, UK) and an isocratic elution at 2 mL/min for 320 mL by using the above defined elution buffer. Proteins were automatically collected in 7 mL fractions, when UV signal rose above 10 mAU at 280 nm. Fractions containing dimeric proteins were pooled, subsequently dialyzed utilizing a dialysis tubing cellulose membrane with a weight cutoff of 14,000 Da (Sigma Aldrich, Steinheim, Germany) and freeze-dried for following tryptic digestion.
The degree of oligomerization was calculated using the ChromLab Software (Bio-Rad Laboratories GmbH, Munich, Germany) by relating the peak area of cross-linked species to the whole area under the curve like previously reported [22].

Enzymatic hydrolysis

Freeze-dried samples were solubilized in TRIS buffer (0.1 M tris(hydroxymethyl)aminomethane, pH 7.8) to a protein concentration of 1 mg/mL. Aliquots of sample solution were added to 0.02 mM trypsin, which was previously solubilized in 1 mM hydrochloric acid according to the instructions of the distributor Sigma-Aldrich (Steinheim, Germany), to reach an enzyme–substrate ratio of 1:100. Enzymatic hydrolysis was then carried out at 37 °C for 16 h and stopped by freezing the samples followed by lyophilization.

Peptide sequencing via RP-HPLC-HRMS

For identification of tryptic peptides and isopeptides, reversed-phase high-performance liquid chromatography combined with high-resolution mass spectrometry (RP-HPLC-HRMS) was performed. Lyophilized hydrolysates were dissolved in eluent A (0.1% formic acid in bi-distilled water) containing 0.1% DTT and membrane filtered before analysis. Peptides were separated via RP-HPLC equipped with the column AdvanceBio Peptide Plus (2.1 × 150 mm; 2.7 µm) and the detector Q-TOF 6545, all from Agilent (Waldbronn, Germany). For elution, a gradient system as summarized in Table 1 with 0.1% formic acid in bi-distilled water as eluent A and 0.1% formic acid in acetonitrile as eluent B was used, starting with an isocratic step of 98% A for 2 min, followed by a gradient to 90% A for 5 min, then to 80% A in 23 min, then to 60% A in 10 min, then to 5% A in 1 min, followed by an isocratic step at 5% A for 8 min and a gradient back to 98% A in 4 min, followed by a post-run time of 18 min. The oven temperature was set to 35 °C, and the flow rate was 0.2 mL/min. Peptides were ionized positively by the ionization source Dual AJS ESI (Agilent Jet Stream Electrospray Ionization). The mass spectrometer was tuned every day in the mass range up to 3200 m/z at 4 GHz and in high-resolution mode. The fragmentor was set to 200 V, skimmer to 65 V, Oct1 RF Vpp to 750 V, nebulizer to 50 psig, VCap to 4000, nozzle voltage to 1000 V. The drying gas was used at a temperature of 300 °C and a gas flow of 12 L/min, whereas sheath gas temperature was 350 °C and gas flow 11 L/min.

For simultaneous identification of as many peptides as possible, Auto-MSMS mode was used. Accordingly, precursor selection was restricted to the three most abundant precursors per scan and selected precursors were excluded after three spectra and released after 0.5 min, while highly charged peptides were preferred. All precursors were fragmented by collision energy of 20 eV. Detection was made with 2 spectra/s and in a mass range of 100–3200 in MS modus or 50–3200 in MS/MS modus, respectively.

Peptides were identified by the software PEAKS Studio 8.5 (Bioinformatics Solutions Inc., Waterloo, Canada) using the UniprotKB/Swiss-Prot database (https://www.uniprot.org/). Each identified peptide was then defined in the software as marker for other peptides to search automatically for isopeptides formed by enzymatic cross-linking. Respective marker masses were calculated by subtracting the monoisotopic mass of ammonia (17.0264 Da) from the original peptide mass because the formation of one isopeptide bond altered the peptide mass because the formation of one isopeptide bond and mTG results in the release of one mol of ammonia. All defined markers originating from α s1-, αs2-, β- and κ-casein as well as up to five missed cleavages, so that a decrease in hydrolysis efficiency because of cross-linked protein was taken into account. Results from the software analysis were afterward verified by searching for the isopeptide masses in the cross-linked samples manually and proving the absence of these masses in the blank samples (non-cross-linked casein as well as monomeric fraction of semi-preparative SEC). Furthermore, fragmentation spectra of each isopeptide were improved by a targeted analysis to ensure correct peptide sequencing. For that, the identified peptide masses were used as precursors and the collision energy was varied between 10 and 40 eV. The observed fragmentation pattern was then compared to the theoretically formed y- and b-ions.

For evaluating the importance of identified isopeptides, caseins were incubated with mTG for different times (15, 30, 45 min) and undergone tryptic digestion without previous

### Table 1

| Time (min) | Eluent A (%) | Eluent B (%) |
|------------|--------------|--------------|
| 0          | 98           | 2            |
| 2          | 98           | 2            |
| 7          | 90           | 10           |
| 30         | 80           | 20           |
| 40         | 60           | 40           |
| 41         | 5            | 95           |
| 49         | 5            | 95           |
| 53         | 98           | 2            |
| +18 (post-run) | 98     | 2            |
purification via SEC. Subsequently, mass spectrometry was carried out in scan modus using the same MS parameters as in Auto-MSMS modus. The previously identified masses of isopeptides were searched in the scan, and their peak areas were related to the peak area of the peptide αs1 F145-R151 (FYPELFR, m/z 486.2568; z = 2), which could not be cross-linked by mTG because of the absence of glutamine and lysine in its amino acid sequence.

Statistical treatment

Incubation of caseins for 15, 30, 45 and 60 min was performed in duplicates.

Results and discussion

This study examines the influence of the micellar structure on the course of the early stage of enzymatic cross-linking of caseins by mTG. To pursue this objective, MC and sodium caseinate (SC) were incubated with a low activity of mTG, so that only the most reactive sites were affected by enzymatic cross-linking, indicated by primary formation of casein dimers shown via SEC (Fig. 1). After incubation, MC suspensions were ultracentrifuged to isolate the extra-micellar fraction (EC) for separate analysis. Extra-micellar casein (EC) accounts for 6.0–7.2% of total casein and consists of all caseins with β-casein as the major component, as determined in our previously published study [10].

The degree of oligomerization, calculated via SEC as the area of cross-linked molecules related to the whole area under the curve, was determined to 14.9%, 13.5% and 13.9% in MC, EC and SC, respectively (Fig. 1). To concentrate the isopeptide content within the samples for further analysis via HRMS, dimer fractions were separated from monomer and trimer fractions via semi-preparative SEC. As proof of concept, the collected fractions were analyzed via SDS polyacrylamide electrophoresis according to Schägger & von Jagow (1987) with a Coomassie Brilliant Blue staining according to Radola (1980) to ensure the correct assignment of SEC peaks to oligomer type (see Supplementary Data: Fig. S4) [23, 24]. The purified dimer fractions were digested by trypsin and resulting tryptic peptides were sequenced by HRMS.

As blanks, non-cross-linked MC and sodium caseinate were hydrolyzed similarly and obtained peptides were utilized as a standard for normally formed peptides due to tryptic digestion of caseins. For sequencing those peptides, HRMS in Auto-MSMS mode combined with data analysis software PEAKS 8.5 was applied. By doing so, the coverage of amino acid sequence reached 77%, 55%, 88% and 75% for αs1-, αs2-, β- and κ-casein, respectively. These ordinarily formed peptides were afterward defined as markers in the data analysis software. To consider the loss of ammonia during enzymatic cross-linking, the mass of ammonia (17.0264 Da) was subtracted from the original peptide masses. A summary of these marker peptides with their amino acid sequence, original and corrected monoisotopic mass and a coverage map for each casein is shown in the supplementary material (Tables S1-S4, Figure S1). Within this group of marker peptides, 103 and 63 peptides contained glutamine and/or lysine, respectively. Accordingly, there are at least 6,489 possible dimer combinations without considering posttranslational modifications and multiple glutamine or lysine moieties within one peptide.

Identification of reactive sites

After defining marker peptides, the software was able to detect automatically isopeptides in the cross-linked samples. As discussed by Iacobucci and Sinz [25], automatic identification of peptide cross-links implies the high probability of false-positive results. They recommended five guidelines to avoid misinterpretations of cross-links. Although our study is somewhat different from the studies discussed by these authors, because we did not use any cross-linker substance, we likewise formulated some requirements, which should be fulfilled to state a result from the automatic detection by the software as a real, identified cross-link. Accordingly, we evaluated the software results manually and accepted them as isopeptides only if they would have met the following conditions:
were usually differentiated with Greek letters [25, 27, 28]. The absence of this peptide mass with a charge of 4 in the blank samples was checked manually and confirmed as shown in Fig. 2, meeting the above-defined 1st condition for data quality. The theoretical mass of this isopeptide would be 1774.9799 Da. With a detected molecular mass of 1775.0060 Da, the mass accuracy of detection is 14.7 ppm meeting the abovementioned 2nd condition of a mass accuracy within ± 20 ppm. The software assigned this isopeptide mass to a cross-link formed between two peptides originating from β-casein, namely peptides β106-K107 and βQ190–βK122, which were highlighted in red and blue according to the respective peptide chain. Because this could be easily mistaken for αS- and β-caseins, we decided on Roman numerals for the differentiation of reactive moieties from unreactive sites.

All likewise 15 identified isopeptides are summarized in Table 3. Respective detailed analysis data including detected m/z ratio, charge z, retention time, theoretical molecular mass of each isopeptide, and the mass accuracy of detection in each casein sample are given in the supplements (Tables S5–S6). In the last column of Table 3, results were graded in terms of the data quality obtained from the targeted analysis. Hence, 10 isopeptides with verified reactive sites and fragmentation ions of both peptide chains were marked with three stars. One isopeptide (#6) with fragmentation ions from both peptide chains, but multiple possible reactive sites, was marked with two stars, and four isopeptides (#7, #12, #14, #15) with fragmentation ions from only one peptide chain were labeled with only one star. The latter were detected only with a low abundance of precursors and thus can be considered as less important reactive sites, at least for the early stage of reaction.

### Evaluation of the identified isopeptides

Detected isopeptides were also categorized in two groups: (I) homogenous isopeptides, which were formed between molecules of one casein type, and (II) heterogeneous isopeptides, resulting from two different casein types. These groups include a similar number of isopeptides indicating that there is no preferred building of isopeptides. According to the steric arrangement of caseins in the casein micelle, this result indicates that caseins are not organized in uniform blocks consisting only of one casein type. The casein micelle can be imagined as a protein aggregate of a more random shape and inner arrangement. However, the group of homogeneous isopeptides mainly consists of linkages between β-caseins. For sodium caseinate, this could be explained by the self-associating behavior of free β-caseins, which was previously reported [29–31]. These β/β-cross-links were also detected in MC, indicating that β-casein molecules neighbor each other also in the casein micelles, which complies with the proposed formation of core polymers as initial protein aggregates for casein micelle formation [32, 33].

Undeniably, especially partially sequenced isopeptides (marked with one or two stars in Table 3) could raise the question if their masses result really from isopeptide bond formation or just from incomplete tryptic digestion. In particular, incomplete digestion is highly probable because of increasing steric hindrance due to isopeptide formation. Because the used data analysis software is able to consider non-specific as well as missed enzymatic cleavages, some isopeptides were detected via two different molecular masses due to the performance of digestion. For instance, isopeptide #4 (Table 3) was identified as a cross-link between β-casein K191 and β-casein Q190, but was detected with the two different peptide masses 1541.9739 Da (#4(I)) and}

---

(1) absence of the peptide mass in blank samples, (2) mass accuracy should be ± 20 ppm, and (3) amino acid sequence of the isopeptide was accurately sequenced by targeted HRMS.

The procedure of this data analysis should be explained in more detail through an example. According to the software analysis, an isopeptide with a mass-to-charge ratio of m/z 444.7515 and a charge of z = 4 was identified in micellar as well as free casein (Table S5, isopeptide #2). The absence of this peptide mass with a charge of 4 in the blank samples was checked manually and confirmed as shown in Fig. 2, meeting the above-defined 1st condition for data quality. The theoretical mass of this isopeptide was 1774.9799 Da. With a detected molecular mass of 1775.0060 Da, the mass accuracy of detection is 14.7 ppm meeting the abovementioned 2nd condition of a mass accuracy within ± 20 ppm. The software assigned this isopeptide mass to a cross-link formed between two peptides originating from β-casein, namely peptides β106-K107 and βQ190–βK122, which were highlighted in red and blue according to the respective peptide chain. Because this could be easily mistaken for αS- and β-caseins, we decided on Roman numerals for the differentiation of reactive moieties from unreactive sites.

All likewise 15 identified isopeptides are summarized in Table 3. Respective detailed analysis data including detected m/z ratio, charge z, retention time, theoretical molecular mass of each isopeptide, and the mass accuracy of detection in each casein sample are given in the supplements (Tables S5–S6). In the last column of Table 3, results were graded in terms of the data quality obtained from the targeted analysis. Hence, 10 isopeptides with verified reactive sites and fragmentation ions of both peptide chains were marked with three stars. One isopeptide (#6) with fragmentation ions from both peptide chains, but multiple possible reactive sites, was marked with two stars, and four isopeptides (#7, #12, #14, #15) with fragmentation ions from only one peptide chain were labeled with only one star. The latter were detected only with a low abundance of precursors and thus can be considered as less important reactive sites, at least for the early stage of reaction.

### Evaluation of the identified isopeptides

Detected isopeptides were also categorized in two groups: (I) homogenous isopeptides, which were formed between molecules of one casein type, and (II) heterogeneous isopeptides, resulting from two different casein types. These groups include a similar number of isopeptides indicating that there is no preferred building of isopeptides. According to the steric arrangement of caseins in the casein micelle, this result indicates that caseins are not organized in uniform blocks consisting only of one casein type. The casein micelle can be imagined as a protein aggregate of a more random shape and inner arrangement. However, the group of homogeneous isopeptides mainly consists of linkages between β-caseins. For sodium caseinate, this could be explained by the self-associating behavior of free β-caseins, which was previously reported [29–31]. These β/β-cross-links were also detected in MC, indicating that β-casein molecules neighbor each other also in the casein micelles, which complies with the proposed formation of core polymers as initial protein aggregates for casein micelle formation [32, 33].

Undeniably, especially partially sequenced isopeptides (marked with one or two stars in Table 3) could raise the question if their masses result really from isopeptide bond formation or just from incomplete tryptic digestion. In particular, incomplete digestion is highly probable because of increasing steric hindrance due to isopeptide formation. Because the used data analysis software is able to consider non-specific as well as missed enzymatic cleavages, some isopeptides were detected via two different molecular masses due to the performance of digestion. For instance, isopeptide #4 (Table 3) was identified as a cross-link between β-casein K191 and β-casein Q190, but was detected with the two different peptide masses 1541.9739 Da (#4(I)) and
Fig. 2 Representative extracted ion chromatograms and respective mass spectra of isopeptide #2 (m/z 444.75; z = 4) in incubated casein and blank samples (EC extra micellar casein, MC micellar casein, SC sodium caseinate)
2353.4088 Da (#4(II)) regarding to complete and incomplete tryptic digestion, respectively.

Posttranslational modifications alter the monoisotopic mass of a peptide. Particularly, phosphorylation is a common modification of caseins, but sample preparation could evoke additional modifications like deamination or dehydration. The calculation software also considered those modifications. In the supplementary data, Figure S1 shows the identified modifications in the ordinarily formed casein peptides as yellow squares in the respective amino acid sequence. Also some of the detected isopeptides, as for example isopeptide #7 (Table 3), show phosphorylated serine residues, which were indicated as “J” (phosphoserine) instead of “S” (serine) in the respective amino acid sequence.

So far, we were able to identify 15 isopeptides formed in the early stage of mTG cross-linking of caseins and furthermore specify the exact lysine and glutamine residues involved in the reaction. In general, β-casein has mainly participated in the formation of the most isopeptides identified in this study. This underlines the high flexible structure of the β-casein molecule. However, the most interesting result might be that nearly all of the identified isopeptides were found in free and MC. Hence, it can be concluded that, in the initial phase of mTG cross-linking, the same reactive sites are available in casein micelles as well as in sodium caseinate, thus emphasizing the high porosity of this natural nanostructure. Only the isopeptide #14, formed between

![Fig. 3](image-url)  
Annotated fragmentation spectrum of isopeptide #2, blue: ions originated from peptide chain I (β-170VLPVPQK176), red: ions originated from peptide chain II (β-106HKEMPFPK113), grey: immonium ions; Nomenclature of fragmentation ions: [ion type (y/b)] + [charge, if not +1] + [peptide chain (I/II)] + [number of fragmentation ion according to Table 2]

### Table 2
Comparison between theoretically formed and detected fragmentation ions of isopeptide #2; grey: unspecific ions; black: specific ions, blue: detected ions for peptide chain I (β-170VLPVPQK176); red highlighted: detected ions for peptide chain II (β-106HKEMPFPK113)

| Fragmentation ions of peptide I: 170VLPVPQK176 | Fragmentation ions of peptide II: |
|-----------------------------------------------|---------------------------------|
| β (170-176) | β (106-113) |
| l | b(1+) | y(1+) | b(2+) | y(2+) | l | b(1+) | y(1+) | b(2+) | y(2+) |
| V | 100.0757 | 1775.9875 7 | 50.5415 | 888.4974 7 | 138.0662 | 1775.9875 8 | 69.5368 | 888.4974 8 |
| L | 213.1598 | 1676.9191 6 | 107.0836 | 838.9632 6 | 106.9161 | 1638.9286 7 | 69.5368 | 888.4974 8 |
| P | 310.2126 | 1563.8351 5 | 155.6099 | 782.4212 5 | 1358.7608 | 488.2686 4 | 693.3841 | 244.6471 4 |
| V | 409.2810 | 1466.7923 4 | 205.1442 | 733.8948 4 | 1532.8293 | 391.2340 3 | 766.9183 | 196.1207 3 |
| P | 506.3337 | 1367.7139 3 | 253.6705 | 684.3606 3 | 1629.8820 | 1270.6611 2 | 815.4447 | 635.8342 2 |
| Q+β(106-113) | 1757.9770 | 147.1129 1 | 879.4922 | 74.0601 1 | 1757.9770 | 147.1129 1 | 879.4922 | 74.0601 1 |
Table 3 Isopeptides identified in micellar (MC) and extra-micellar (EC) casein as well as sodium caseinate (SC) via HRMS; data quality classes: *** isopeptides with verified reactive sites and fragmentation ions of both peptide chains; ** isopeptides with fragmentation ions from both peptide chains, but multiple possible reactive sites; * isopeptides with fragmentation ions from only one peptide chain

| #  | Isopeptide | Peptide 1 (reactive K) | Peptide 2 (reactive Q) | Detected in | z | MC | EC | SC | M_theoretical (DA) | Data Quality |
|----|------------|------------------------|------------------------|-------------|---|----|----|----|---------------------|--------------|
|    |            |                        |                        |             |   |     |     |     |                     |              |
| HOMOGENEOUS ISOPEPTIDES |            |                        |                        |             |   |     |     |     |                     |              |
| 1  | β K176 - β Q54 | β175LVPVPQA KPVPQR185 | β175HPFAGQSLVYPFPGPNP186 | 4           | + | +  | +  |     | 3796.0481           | ***          |
| 2  | β K107 - β Q175 | β207HEMP FPQ175 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 174.9799           | ***          |
| 3 (I)| β K113 - β Q175 | β207HEMP FPQ175 | β175LVPVPQA KPVPQR185 | 2           | + | -  | -  |     | 1509.8261           | ***          |
| 3 (II)| β K113 - β Q175 | β207HEMP FPQ175 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 4957.5840           | ***          |
| 4 (I)| β K176 - β Q175 | β175LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 3           | - | -  | -  |     | 1941.9540           | ***          |
| 4 (II)| β K176 - β Q175 | β175LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 2353.3878           | ***          |
| 5 (I)| β K169 - β Q175 | β207SLSQ546LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 2172.2874           | ***          |
| 5 (II)| β K169 - β Q175 | β207SLSQ546LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 2172.2874           | ***          |
| 6 (I)| αs1 K7 - αs1 Q130/131 | αs1-104βHPK123GQLPQVLNFLRL20 | αs1-104βHPK123GQLPQVLNFLRL20 | 4           | + | +  | +  |     | 3126.6657           | **           |
| 6 (II)| αs1 K132 - αs1 Q9 | αs1-104βHPK123GQLPQVLNFLRL20 | αs1-104βHPK123GQLPQVLNFLRL20 | 4           | + | +  | +  |     | 3126.6657           | **           |
| 7  | αs1 K105 - αs1 Q130/131 | αs1-104βKVPQLEIPVPJEERR119 | αs1-104βKVPQLEIPVPJEERR119 | 4           | + | +  | +  |     | 2843.3875           | *            |
|    |            |                        |                        |             |   |     |     |     |                     |              |
| HETEROGENEOUS ISOPEPTIDES |            |                        |                        |             |   |     |     |     |                     |              |
| 8 (I)| αs1 K13 - β Q176 | β175LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 2996.6894           | ***          |
| 8 (II)| αs1 K13 - β Q176 | β175LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 5           | + | +  | +  |     | 2996.6894           | ***          |
| 9 (I)| αs1 K105 - β Q54 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 3           | - | -  | -  |     | 2580.2493           | ***          |
| 9 (II)| αs1 K105 - β Q54 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 4156.0714           | ***          |
| 9 (III)| αs1 K105 - β Q54 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 4           | - | -  | -  |     | 3961.8075           | ***          |
| 10 (I)| αs1 K105 - β Q175 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 4           | + | +  | +  |     | 2713.4112           | ***          |
| 10 (II)| αs1 K105 - β Q175 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 2713.4112           | ***          |
| 10 (III)| αs1 K105 - β Q175 | αs1-104βKVPQLEIPVPJEERR119 | β175LVPVPQA KPVPQR185 | 3           | - | -  | -  |     | 266.2405            | ***          |
| 11 | αs1 K124 - β Q175 | αs1-123L HSMEGHAQDK132 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 266.2405            | ***          |
| 12 (I)| αs1 K191 - β Q54/56 | αs1-104βKMPW103 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 2836.4391           | *            |
| 12 (II)| αs1 K191 - β Q54/56 | αs1-104βKMPW103 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 2342.1751           | *            |
| 13 | β Q175 - β Q24 | β175LVPVPQA KPVPQR185 | β175LVPVPQA KPVPQR185 | 3           | + | +  | +  |     | 1870.0962           | ***          |
| 14 | αs1 K34 - αs1 Q101 | αs1-104βKVPQLEIPVPJEERR119 | αs1-104βKVPQLEIPVPJEERR119 | 4           | - | -  | -  |     | 2724.3247           | *            |
| 15 | αs1 K32 - β Q54/56 - β Q175 | αs1-104βKVPQLEIPVPJEERR119 | αs1-104βKVPQLEIPVPJEERR119 | 4           | + | +  | +  |     | 3181.6471           | *            |
αs1 K34 and αs2 Q101, was identified as an exclusive cross-link in sodium caseinate, indicating that these moieties are located in a less reachable region or not near to each other in the casein micelle.

Comparison to previously reported reactive sites

The identified reactive moieties αs1 Q13, αs1 Q130, β Q54/56, β Q175, κ K24 were already reported by other studies as substrates for mTG (see Table 4) [12–14, 34]. However, there are more contradictions revealed in the comparison of our results to the literature reports. We identified 14 other casein moieties, which were not previously described as reactive sites of the enzymatic cross-linking in caseins, whereas 17 other moieties, reported as reactive sites of caseins in the literature, were not confirmed in this study. This huge difference is most probably due to the different analytical techniques. In the abovementioned studies, labeling substances with structures similar to glutamine (e.g. [14C]-putrescine, [14C]-monodansylcadaverine, N-(glucose-glucose-glucitol-1)-cadaverine) were used as a reaction partner for the identification of reactive glutamine residues [12–14]. For suppression of protein cross-linking, lysine residues were sometimes amidinated or succinylated, which could evoke electrostatic repulsions and thus might influence the native protein structure to a considerable amount [13]. Therefore, reaction sites reported in the literature are highly influenced by marker solubility as well as protein stability in the presence of a high marker concentration. Moreover, the identification of reactive lysine residues was previously hindered by the lack of appropriate marker substances. Christensen et al. [12] described two reactive lysine residues in κ-casein (κ K21, κ K24) only based on mass calculations according to a detected isopeptide mass. On the contrary, by using HRMS, we were able to identify precisely also the reactive lysine residues. Furthermore, the source of transglutaminase might as well affect preferred reactive sites. Finally, the stage of the enzymatic reaction might not be comparable between different studies, as we focused on the early stage of protein cross-linking. Accordingly, in the present study, the enzymatic cross-linking of casein micelles was performed under native conditions (no marker and SMUF as solvent) to preserve the native protein structure as well as under mild reaction conditions (1 U/g casein; 40 °C, 60 min) to identify only the “hottest” reactive sites. The formed isopeptides were precisely identified by peptide sequencing via HRMS, which enabled information not only about the reactive moieties, but also about the residues located perfectly arranged near to each other to form an isopeptide. Based on the HRMS method as well as the data analysis and data control management, also one trimer formed between αs2-casein K47, β-casein Q69/71 and β-casein Q190/K191 was identified in our study (see Table 3, isopeptide #15).

| Table 4 Summary of previously reported and in this study identified reactive sites in caseins |
|-------------------------------|-----------------|-----------------|
| Literature | Protein | Enzyme | Reactive Q | Reactive K |
| --- | --- | --- | --- | --- |
| Christensen et al. [12] | αs1-casein | TG (guinea pig) | 13 | 130 |
| Our study | SC, MC | mTG | 7 | 13 |
| Christensen et al. [12] | αs2-casein | TG (guinea pig) | 79 | 131 |
| Our study | SC, MC | mTG | 101 | 130 |
| Yan and Wold [13] | β-casein | TG (guinea pig) | 54 | 182 |
| Christensen et al. [12] | β-casein | TG (guinea pig) | 54 | 182 |
| Gorman and Folk [14] | β-casein | Factor XIIIa | 54 | 182 |
| Tokai et al. [34] | tryptic casein | mTG | 54 | 182 |
| Our study | SC, MC | TG (guinea pig) | 29 | 114 |
| Christensen et al. [12] | κ-casein | mTG | 29 | 114 |
| Our study | SC, MC | mTG | 29 | 114 |

© Springer
Influences of neighboring amino acids on enzymatic activity

To draw some conclusions concerning substrate specificity of mTG, Table 5 summarizes all reactive glutamine and lysine residues identified in this study with their neighboring five amino acids on N-terminal as well as C-terminal side. Consecutive glutamine residues, which were in other proteins previously reported as good substrates for transglutaminases, were also in our study found within the reactive sequences (αs1 Q130/131; β Q54/56) [35–38]. The glutamine β Q175 was found as a major reactive site involved in the formation of homogenous isopeptides (#2, #3, #4, #5) as well as cross-links to all other casein types (#10, #11, #13, #15). Tokai et al. [34] also reported the tryptic peptide $^{170}$VLPVPQK$^{176}$ of β-casein as the most reactive one in the enzymatic cross-linking of caseins by mTG. Our study, moreover, revealed that this protein region delivers not only an appropriate amino acid sequence for optimal substrate recognition but should be also located in a highly flexible protein region due to enabling cross-linking to all other caseins. The reactivity of the directly neighboring lysine residue β K176 supports that finding by the building of homogenous isopeptides (#1, #4) and heterogeneous isopeptides with αs-caseins (#8, #15). Interestingly, Q175 is substituted by E175 in the genetic variant A1 of β-casein, tempting to speculate that β-casein A1 could be a less efficient substrate by E175 in the genetic variant A1 of β-casein, tempting to speculate that β-casein A1 could be a less efficient substrate for mTG. Our study, moreover, revealed that this protein region delivers not only an appropriate amino acid sequence for optimal substrate recognition but should be also located in a highly flexible protein region due to enabling cross-linking to all other caseins. The reactivity of the directly neighboring lysine residue β K176 supports that finding by the building of homogenous isopeptides (#1, #4) and heterogeneous isopeptides with αs-caseins (#8, #15). Interestingly, Q175 is substituted by E175 in the genetic variant A1 of β-casein, tempting to speculate that β-casein A1 could be a less efficient substrate for mTG. To the best of our knowledge, no experimental recommendations to predict the reactivity of glutamine residues in new proteins, but does not replace experimental data. Considering the vicinity of reactive lysines, our results show that positively as well as negatively charged moieties are randomly present or absent near to reactive lysine residues, so that they should be neither a discouraging nor a promoting feature for cross-linking. Besides, multiple proline residues predominantly surround cross-linked regions indicating that reactive sites could be located within loop structures.

Table 5 Summary of identified reactive sites and their ±5 neighbouring amino acids; blue: positive charges; red: negative charges; yellow: hydrophobic sites; grey: proline residues

| Isopeptide Sequence | Reactive site |
|---------------------|--------------|
| -5 -4 -3 -2 -1 0 +1 +2 +3 +4 +5 | |
| reactive glutamine residues: | |
| #1, #9 | I H P F A Q T Q S L V | β Q54 |
| #2, #3, #4, #5, #10, | V L P V P Q K A V P Y | β Q175 |
| #11, #13, #15 | | |
| #6 | H P I K H Q G L P Q E | αs1 Q9 |
| #6, #7 | E G I H A QQ K E P M I | αs1 Q130/131 |
| #8 | H Q G L P Q E V L N E | αs1 Q13 |
| #12, #15 | L Q Y L Y Q G P I V L | β Q54/56 |
| #14 | I H P F A QTQ S L V Y P | αs2 Q1 |
| reactive lysine residues: | |
| #1, #4, #8, #15 | L P V P Q K A V P Y P | β K176 |
| #2 | M A P K H K E M F F P Y P | β K107 |
| #3 | E M F F K Y P V Q P | β K113 |
| #5 | S L S Q S K V L P V P | β K169 |
| #6 | P K H P I K H Q G L P | αs1 K7 |
| #6 | I H A Q Q K E P M I G | αs1 K132 |
| #7, #9, #10 | R L K K Y K V P Q L E | αs1 K105 |
| #11 | R L H S M K E G I H A | αs1 K124 |
| #12 | H Q K A M K P W I Q P | αs2 K191 |
| #13 | S D K I A K Y I P I Q | k K24 |
| #14 | P E V F G K E K V N E | αs1 K34 |
| #15 | A I N P J K E N L C S | αs2 K32 |
Localization of reactive sites within casein molecules

To obtain information about the steric localization of reactive sites, Fig. 4 visualizes the predicted 3D models of individual caseins as they were described elsewhere [32, 40–43]. Most obviously, identified reactive sites are primarily placed in less structured regions or near β/γ-loops. Exceptions are αs2 K32 and κ K24, which are located in an α-helix and a β-sheet structure, respectively. Notwithstanding, all of the reactive sites seem to be exposed to the solvent, which was previously reported as a requirement for cross-linking [39, 44]. The molecular model of β-casein was described as “crab-like” by Kumosinski et al. [40] due to two hydrophilic “crab arms” (I: 28–55; II: 85–119) and the hydrophobic protein backbone as “crab body.” The hydrophobic backbone forms many loop structures through which water can easily diffuse. Within that protein region, the reactive lysine K169, as well as the most reactive lysine K176 and glutamine Q175, is located. As plasmin can hydrolyze the hydrophilic crab arms in the positions 28–29, 105–106 and 107–108 [45], good accessibility also for mTG can be imagined and is demonstrated by the reactive residues located in those regions (K107, K113, Q54, Q56). In contrast, reactive sites of αs1-casein are not spread over the whole molecule, but located in the N-terminal region (1–133). This region is followed by two “dog-leg structures,” which contain antiparallel β-sheet structures (Fig. 4) [32]. The first includes the sequence 136–159 and promote αs1-casein dimerization by the formation of sheet-sheet-interaction as marked by one asterisk in Fig. 4 [32]. Consequently, dimerization might bring the region K105-K132 of two αs1-caseins close enabling the development of homogenous isopeptide #7 (αs1 K105–αs1 Q130/131). Besides, the nearness of αs1-caseins would be further supported by linking of phosphoserine residues mainly concentrated in the region 41–75 (marked by two asterisk in Fig. 4) to colloidal calcium phosphate. Via the second dog-leg structure of αs1-casein (162–175; three asterisk in Fig. 4), two αs1-casein dimers are expected to associate with one κ-casein, each with one of the two dog leg-structure of κ-casein; **region for self-association of αs1-casein. 3D models were created via UCSF Chimera version 1.13.1 based on previously published data [32, 40–43].
leg structures in κ-casein (I: 21–34, II: 39–55) [32]. This αs1-κ-associate is described as intermediate in the formation of a submicellar structure, offering four open sectors in the interior. Two of these sectors are hydrophobic and may be used for hydrophobic incorporations of four β-casein molecules, whereas the other two sectors enable a diffusion of water and enzymes through the associate because of their hydrophilic character [32]. The single reactive site identified in κ-casein (K24) lies in that “dog-leg” structure, which comes into close proximity to other caseins especially in sub-micellar formation.

**Weighting the identified reactive sites**

As mentioned above, most of the identified reactive sites were equally detected in non-micellar, extra micellar as well as in MC. So far, the micellar structure has no pronounced effect on enzymatic cross-linking in the initial phase. One exception is isopeptide #14, which is formed by a linkage between αs1 K34 and αs2 Q101 and was found only in sodium caseinate. In casein micelles, especially αs-caseins interact via their multiple phosphoserine residues with colloidal calcium phosphate forming a skeletal structure. Accordingly, αs-caseins were reported as less accessible for enzymatic cross-linking in micellar than in non-micellar casein [10, 11, 46].

For further differentiation of the reactive sites and to obtain more information about differences in the cross-linking between non-micellar versus MC, the amount of individual isopeptides formed with increasing time of incubation was estimated. MC, as well as sodium caseinate, was incubated with mTG over 15, 30, 45 and as used before over 60 min. EC was again obtained by ultracentrifugation of cross-linked casein micelles. All samples were digested by trypsin, but this time without any former purification of dimers via SEC. Subsequently, the above identified isopeptide masses were searched and detected via HRMS. The increase of peak areas with increasing reaction time represents additional proof of the obtained results: Only the peak areas of isopeptides are expected to increase with reaction time due to an advanced formation, whereas other peak areas should stay nearly constant. Accordingly, Fig. 5 shows the extracted ion chromatograms of isopeptide #10 (I) over incubation time as an example, and in the supplementary data, Figures S2 and S3 summarize the formation of each identified isopeptide over reaction time. However, every peptide or isopeptide has a different ionization ability resulting in unequally intensive peak areas hampering direct comparison of the results. Hence, standardization of peak areas was performed by setting the peak area of each isopeptide in ratio to the peak area of the peptide αs1-145FYELFR151, which can neither be affected by enzymatic cross-linking nor by posttranslational modification and thus represents an efficient “internal” reference peptide. Figure 6 shows a heat map visualizing the obtained ratios of isopeptide to reference peptide, whose exact values are summarized in the supplementary data in Table S7. Most obviously, most isopeptides (#1, #3, #4, #6, #9, #11, #12, #13, #14, #15) are no longer detected or only identified with a small peak area as indicated by the grey areas in the map. This was because the isolation step for dimers was omitted. Consequently, the purification of cross-linked proteins is an important and necessary step in particular for the identification of “hot” reactive sites, which are formed in the initial phase of the reaction. Notwithstanding, the tracking of peak areas over reaction time gives additional evidence for the accurate identification of reactive sites. The results revealed that there are two groups of isopeptides, which might be described as the “hottest” reactive sites due to good detection even without purification. These two groups include isopeptides.

---

**Fig. 5** Extracted ion chromatograms of isopeptide #10 (I) (m/z 679.3572) in micellar casein (MC), extra-micellar casein (EC) and sodium caseinate (SC) incubated with mTG (1 U/g casein; E/S = 1/80) at 40 °C for 0–60 min.
which were favorably built either in EC or in SC. Particularly, isopeptides #2 and #7 as predominantly formed in EC demonstrate that ECs do not fully react like free caseins in sodium caseinate, and thus, the equilibrium between colloidal and soluble caseins in casein micelle suspensions somehow influences the cross-linking reaction. It can be hypothesized that these reactive sites might be well accessible for mTG in casein micelles. After the cross-linking, the corresponding proteins might no longer be in an appropriate steric conformation for incorporation into the micelle, thus resulting in a release of that cross-linked micellar region to the soluble phase. There are no reactive sites which showed a preferred cross-linking in MC confirming that micellar structure somehow might have an impact on the process of enzymatic cross-linking although this is at least in the initial phase not as apparent as beforehand expected. Moreover, the isopeptides #5, #8 and #10 showed a favorable formation in sodium caseinate, indicating that the reactive sites of the cross-links β K107–β Q175, αs1 Q13–β K176 and αs1 K105–β Q175 might be located in micelle structure either in an inappropriate arrangement to each other or in a for mTG less accessible subunit.

### Concluding remarks

In conclusion, our study demonstrates an appropriate analysis method for evaluating reactive sites of enzymatic cross-linking without labeling and while maintaining the natural protein conformation. Moreover, lysine and glutamine sites of a cross-link were identified, which delivers more information about steric arrangement and interactions of individual caseins to each other. The two moieties β Q175 and β K176 were identified as the most flexible reactive sites for the formation of isopeptides in micellar as well as sodium caseinate in the initial phase of the reaction. The residues αs1 K34 and αs2 Q101 seemed to be less accessible for mTG in casein micelles, but in general potential reactive sites identified in sodium caseinate. Most notably, our study revealed that casein micelle structure does not affect the enzymatic cross-linking in the initial phase of the reaction as dramatically as hypothesized in this study.

![Heat map showing the ratios of the peak areas of each isopeptide and the respective peak area of reference peptide αs1-145FYPELFR151 multiplied by factor 1,000. Results were classified into six groups marked shades ranging from 0 (grey) till over 20 × 10^3 (bright red) in steps of 5 × 10^3](image-url)

| Nr. | Isopeptide | MC 15 | MC 30 | MC 45 | MC 60 | SC 15 | SC 30 | SC 45 | SC 60 | EC 15 | EC 30 | EC 45 | EC 60 |
|-----|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1   | β K176 - β Q54 |       |       |       |       |       |       |       |       |       |       |       |       |
| 2   | β K107 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 3 (I)| β K113 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 3 (II)| β K113 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 4 (I)| β K176 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 4 (II)| β K176 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 5 (I)| β K169 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 5 (II)| β K169 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 6 (I)| αs1 K7 - αs1 Q130/131 // αs1 K132 - αs1 Q9 |       |       |       |       |       |       |       |       |       |       |       |       |
| 6 (II)| αs1 K105 - αs1 Q130/131 |       |       |       |       |       |       |       |       |       |       |       |       |
| 7   | αs1 K105 - αs1 K105/130/131 |       |       |       |       |       |       |       |       |       |       |       |       |
| 8 (I)| αs1 Q13 - β K176 |       |       |       |       |       |       |       |       |       |       |       |       |
| 8 (II)| αs1 Q13 - β K176 |       |       |       |       |       |       |       |       |       |       |       |       |
| 9 (I)| αs1 K105 - K54 |       |       |       |       |       |       |       |       |       |       |       |       |
| 9 (II)| αs1 K105 - K54 |       |       |       |       |       |       |       |       |       |       |       |       |
| 10 (I)| αs1 K105 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 10 (II)| αs1 K105 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 11   | αs1 K124 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |
| 12 (I)| αs2 K191 - β Q54/56 |       |       |       |       |       |       |       |       |       |       |       |       |
| 12 (II)| αs2 K191 - β Q54/56 |       |       |       |       |       |       |       |       |       |       |       |       |
| 13   | β Q175 - κ K24 |       |       |       |       |       |       |       |       |       |       |       |       |
| 14   | αs1 K34 - αs2 Q101 |       |       |       |       |       |       |       |       |       |       |       |       |
| 15   | αs2 K32 - β Q54/56 - β Q175 |       |       |       |       |       |       |       |       |       |       |       |       |

Peak area ratio isopeptide to reference peptide αs1-145FYPELFR151.
as expected emphasizing the porosity of this nanocarrier that enables a similar reaction behavior like in non-micellar casein.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-022-04069-w.

Acknowledgements We thank Prof. Dr. Michael Mertig, Chair of Physical Chemistry, Measurement and Sensor Technology, TU Dresden, for permission to utilize his ultracentrifuge, and Prof. Dr. Eike Brunner, Chair of Bioanalytical Chemistry, TU Dresden, for permission to utilize his mass spectrometer temporarily. Furthermore, the authors are indebted to Dr. Paul D’Agostino (Chair of Technical Biochemistry, TU Dresden) for his critical and helpful suggestions.

Funding Open Access funding enabled and organized by Projekt DEAL. We thank the German Research Foundation (DFG) for financial support (HE 2306/12–1).

Declarations

Conflict of interest The authors declare no competing financial interests.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Mostafa HS (2020) Microbial transglutaminase: an overview of recent applications in food and packaging. Biocatal Biotransformation 38:161–177
2. Miwa N (2020) Innovation in the food industry using microbial transglutaminase: keys to success and future prospects. Anal Biochem 597:113638
3. Dell’Olmo E, Gaglione R, Arciello A et al (2021) Transglutaminase-mediated crosslinking of a host defence peptide derived from human apolipoprotein B and its effect on the peptide antimicrobial activity. Biochim Biophys Acta Gen Subj 1865:129803
4. Akbari M, Razavi SH, Kieliszek M (2021) Recent advances in microbial transglutaminase biosynthesis and its application in the food industry. Trends Food Sci Technol 110:458–469
5. Darasch A, Wissel J, Henle T (2018) Reassembling of alkali-treated casein micelles by microbial transglutaminase. J Agric Food Chem 66:11748–11756
6. Sharma R, Lorenzen PC, Qvist KB (2011) Influence of transglutaminase treatment of skim milk on the formation of ε-(γ-glu-tylamyl)lysine and the susceptibility of individual proteins towards crosslinking. Int Dairy J 11:785–793
7. Christensen BM, Sorensen ES, Hinjrup P et al (1996) Localization of potential transglutaminase cross-linking sites in bovine caseins. J Agric Food Chem 44:1943–1947
8. Yan SB, Wold F (1984) Neoglycoproteins: In vitro introduction of glycosyl units at glutamines in beta-casein using transglu-taminase. Biochemistry 23:3759–3765
9. Gorman JJ, Folk JE (1980) Structural features of glutamine substrates for human plasma factor XIIIa (activated blood coagulation factor XIII). J Biol Chem 255:419–427
10. Moecckel U, Duerasch A, Weiz A et al (2016) Glycation reactions of casein micelles. J Agric Food Chem 64:2953–2961
11. Duerasch A, Herrmann P, Hogh K, Henle T (2020) Study on β-casein depleted casein micelles: Micellar stability, enzymatic cross-linking, and suitability as nanocarriers. J Agric Food Chem 68:13940–13949
12. Moon JH, Hong YH, Huppertz T et al (2009) Properties of casein micelles cross-linked by transglutaminase. Int J Dairy Technol 62:27–32
13. Hinz K, Huppertz T, Kelly AL (2012) Susceptibility of the individual caseins in reconstituted skim milk to cross-linking by transglutaminase: influence of temperature, pH and mineral equilibria. J Dairy Res 79:414–421
14. Recio I, Oliemo L (1996) Determination of denatured serum proteins in the casein fraction of heat-treated milk by capillary zone electrophoresis. Electrophoresis 17:1228–1233
15. Jenness R, Koops J (1962) Preparation and properties of a salt solution which simulates milk ultrafiltrate. Ned Melk-En Zuiveltechnol 16:153–164
16. Lauber S, Henle T, Klostermeyer H (2000) Relationship between the crosslinking of caseins by transglutaminase and the gel strength of yoghurt. Eur Food Res Technol 210:305–309
17. Henle T, Schwarzenbolz U, Klostermeyer H (1996) Irreversible crosslinking of casein during storage of UHT-treated skim milk. In: Heat treatments and alternative methods—IDF symposium, pp 290–298
18. Schägger H, von Jagow G (1984) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 150:76–89
19. Lauber S, Henle T, Klostermeyer H (2000) Relationship between the crosslinking of caseins by transglutaminase and the gel strength of yoghurt. Eur Food Res Technol 210:378–383
20. Henle T, Schwarzenbolz U, Klostermeyer H (1996) Irreversible crosslinking of casein during storage of UHT-treated skim milk. J Agric Food Chem 64:2953–2961
21. Akbari M, Razavi SH, Kieliszek M (2021) Recent advances in microbial transglutaminase biosynthesis and its application in the food industry. Trends Food Sci Technol 110:458–469
22. Järner D, Heidig C, Rohm H (2007) Enzymatic modification through microbial transglutaminase enhances the viscosity of stirred yogurt. J Texture Stud 38:179–198
23. Lorenzen PC, Neve H, Mautner A, Schlümmer E (2002) Effect of enzymatic cross-linking of milk proteins on functional properties of set-style yoghurt. Int J Dairy Technol 55:152–157
24. Bönisch MP, Heidebach TC, Kulozik U (2008) Influence of transglutaminase protein cross-linking on the rennet coagulation of casein. Food Hydrocoll 22:288–297
25. Partschfelder C, Schwarzenbolz U, Richter S, Henle T (2007) Crosslinking of casein by microbial transglutaminase and its resulting influence on the stability of micelle structure. Biotechnol J 2:456–461
26. Smidy MA, Martin J-EGH, Kelly AL et al (2006) Stability of casein micelles cross-linked by transglutaminase. J Dairy Sci 89:1906–1914
27. Roepstorff P, Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 11:601–601
27. Lexhaller B, Ludwig C, Scherf KA (2019) Comprehensive detection of isopeptides between human tissue transglutaminase and gluten peptides. Nutrients 11:1–25
28. Götze M, Pettolkau J, Schaks S et al (2012) StavroX-A software for analyzing crosslinked products in protein interaction studies. J Am Soc Mass Spectrom 23:76–87
29. Andrews AL, Atkinson D, Evans MTA et al (1979) The conformation and aggregation of bovine β-casein A. I. molecular aspects of thermal aggregation. Biopolymers 18:1105–1121
30. Tai M, Kegeles G (1984) A micelle model for the sedimentation behavior of bovine β-casein. Biophys Chem 20:81–87
31. Takase K, Niki R, Arima S (1980) A sedimentation equilibrium study of the temperature-dependent association of bovine β-casein. Biochim Biophys Acta 622:1–8
32. Farrell HMJ, Kumasinski TF, King G (1994) Three-dimensional molecular modeling of bovine caseins. Energy-minimized submicelle structure compared with small-angle X-ray scattering data. In: Kumasinski TF, Liebmann MN (eds) Molecular modeling. From virtual tools to real problems. ACS symposium series. American Chemical Society, Washington DC, pp 392–419
33. Waugh DF, Creamer LK, Slattery CW, Dresdner GW (1970) Core polymers of casein micelles. Biochemistry 9:786–795
34. Tokai S, Uraji M, Hatanaka T (2020) Molecular insights into the mechanism of substrate recognition of Streptomyces transglutaminases. Biosci Biotechnol Biochim 84:575–582
35. Hohenadl C, Mann K, Mayer U et al (1995) Two adjacent N-terminal glutamines of BM-40 (Osteonectin, SPARC) act as amine acceptor sites in transglutaminase-catalyzed modification. J Biol Chem 270:23415–23420
36. Chen R, Doolittle RF (1971) γ-γ Cross-linking sites in human and bovine fibrin. Biochemistry 10:4486–4491
37. Berbers GAM, Feenstra RW, van Den Bos R et al (1984) Lens transglutaminase selects specific β-crystallin sequences as substrate. Proc Natl Acad Sci U S A 81:7017–7020
38. Simon M, Green H (1988) The glutamine residues reactive in transglutaminase-catalyzed cross-linking of involucrin. J Biol Chem 263:18093–18098
39. Coussons PJ, Price NC, Kelly SM et al (1992) Factors that govern the specificity of transglutaminase-catalyzed modification of proteins and peptides. Biochem J 282:929–930
40. Kumasinski TF, Brown EM, Farrell HM (1993) Three-dimensional molecular modeling of bovine caseins: an energy-minimized β-casein structure. J Dairy Sci 76:931–945
41. Kumasinski TF, Brown EM, Farrell HM (1993) Three-dimensional molecular modeling of bovine caseins: a refined, energy-minimized κ-casein structure. J Dairy Sci 76:2507–2520
42. Farrell HM, Brown EM, Kumasinski TF (1993) Three-dimensional molecular modeling of bovine caseins. Food Struct 12:235–250
43. Farrell HM, Malin EL, Brown EM, Mora-Gutierrez A (2009) Review of the chemistry of αS2-casein and the generation of a homologous molecular model to explain its properties. J Dairy Sci 92:1338–1353
44. Aeschlimann D, Paulsson M, Mann K (1992) Identification of Gln726 in nidogen as the amine acceptor in transglutaminase-catalyzed cross-linking of laminin-nidogen complexes. J Biol Chem 267:11316–11321
45. Bastian ED, Brown RJ (1996) Plasmin in milk and dairy products: an update. Int Dairy J 6:435–457
46. Bönisch MP, Lauber S, Kulozik U (2004) Effect of ultra-high temperature treatment on the enzymatic cross-linking of micellar casein and sodium caseinate by transglutaminase. J Food Sci 69:398–404

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Anja Duerasch1 · Maja Konieczny1 · Thomas Henle1

1 Technische Universität Dresden, 01062 Dresden, Germany

© Springer