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Structural Characterization of Transglutaminase-Catalyzed Casein Cross-Linking

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Abstract

Microbial transglutaminase is used in the food industry to improve texture by catalyzing protein cross-linking. Casein is a well-known transglutaminase substrate, but the complete role of glutamine (Q) and lysine (K) residues in its cross-linking is not fully understood.

In this study, we describe the characterization of microbial Transglutaminase -modified casein using a combination of immunological and proteomic techniques. Using 5-(biotinamido)pentylamine as an acyl acceptor probe, three Q residues of β-casein and one of α_{s1}-casein were found to participate as acyl donors. However, no Q-residues were involved in network formation with κ-casein or α_{s2}-casein. Q and K residues in the ε-(γ-glutamyl)lysine-isopeptide bonds β-casein were identified by nanoelectrospray tandem mass spectrometry of the proteolytic digests.

This work reports our progress toward a better understanding of the function and mechanism of action of microbial transglutaminase-mediated proteins. The results suggest a possible role for transglutaminase in the formation of casein micelles.

Keywords: Microbial transglutaminase; Casein; Milk; ε-(γ -glutamyl)-lysine isodipeptide bond; 5-(biotinamido) pentylamine; Tandem mass spectrometry

Introduction

Transglutaminase (TG) is one of a family of enzymes that catalyzes an acyl transfer reaction in the presence of Ca^{2+}. This reaction forms amide bonds between the ε--carboxamide groups of peptide-bound glutamine (Q) residues and a variety of primary amines [1]. In the reaction catalyzed by TG, Q residues serve as the acyl donors and ε-amino groups of lysine (K) as the acyl acceptors. TG shows substrate selectivity for Q residues as the acyl donors. However, it is less selective with regards to the acyl acceptor (K-residue) [2,3]. The K-residue can be replaced by low molecular weight amines, such as monodansylcadaverine, fluoresceincadaverine [4,5] and 5-biotinamidopentylamine [6], and also by dansylated or biotinylated glutamine-containing peptides [7]. In the absence of a K-residue or other amines, water can act as the nucleophile, which results in the deamidation of Q-residues to glutamic acid [4,8,9]. The ability of TG to catalyze cross-linking, incorporate of amines, and deamidate modifies the functional properties of proteins [10-13]. Ca^{2+}-dependent mammalian TG (i.e., blood coagulation factor FaXIII and tissue TG) was the first TG to be investigated for application in the food industry [11,14,15], but the high cost of its extraction and purification limited its use. Hence, the major source of the enzyme has become Ca^{2+}-independent microbial TG (mTG), which is largely produced through fermentation technology utilizing Streptoverticullium species [16]. **Ca^{2+}**-independent mTG is useful for the modification of the functional properties of food proteins (i.e., milk proteins, soybean globulins, gluten, actin, myosin, and egg proteins) [17]. The amino acid sequence of mammalian TG is slightly different from that of mTG, although they both have Cys in the active site. mTG is 331 amino acids long with a molecular mass of 37,863 Da, and its optimum pH is in the range from 5-8 [18]. Digestibility of products containing ε-(γ-glutamyl) lysine isopeptide has been investigated in different circumstances [19,20]. After ingestion, ε-(γ-glutamyl) lysine isopeptide is somewhat resistant to digestive enzymes [21]. The ε-(γ-glutamyl) lysine isopeptide bond is cleaved by γ-glutamylcyclotransferase which releases L-Lys and 5-oxoproline. The 5-oxoproline is then further metabolized to glutamic acid by 5-oxoprolinase (5-OP) [1]. Foods treated with mTG have been approved for human consumption by the US Food and Drug Administration, and its application has expanded to all foods.

Many food proteins such as casein (CN) are excellent substrates for TG [22-24], primarily due to their highly accessible and flexible open chain structure. Milk with a high degree of cross-linking is unable to coagulate after the addition of rennet, probably due to failure during the primary phase of milk clotting. This hinders the nonenzymatic secondary phase that is initiated when a sufficient amount of κ-CN is hydrolyzed [25,26].

CN is a better substrate for TG than whey proteins. However, the denaturation of whey proteins makes their amino groups accessible to TG, which allows cross-linking to occur [27-29]. CN is an amphiphilic protein which can self-assemble into micelles stabilized by both hydrophobic interactions and calcium phosphate bridges. The amino acid sequences of α_{s1}-, α_{s2}-, β-, and κ-CN, their relative proportions and total CN vary in whole milk depending on the species.

TG-mediated modification of CN and its exact mechanisms have

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not been fully elucidated. The interaction of TG with its substrate depends not only on the primary amino acid sequence, but also the secondary and/or tertiary sequence. It also appears necessary that Q be exposed on the surface of CN fractions so that it can react with TG. It is possible for Q residues to be partially exposed on the outer CN fractions of micelles. However, other Q residues may be partially blocked by steric hindrance, which will make the protein substrate less accessible to TG. In any case, it seems that only a small fraction of Q residues is transamidated at any given time [30]. Because CN preparations incubated for different lengths of time present different rheological properties, the cross-linking of CN from four species (cow, sheep, goat, and buffalo) has not been investigated. Ovine milk in particular has received little attention, even though it possesses the highest level of CN. The present work aims to identify the *in vitro* susceptibility of CN fractions to mTG-mediated modification. Some Q-donor sites have been examined for their role in the assembly and structure of the CN micelle. This study reports the identification of several amino acid sequences of ovine CN that act as glutamine-donor substrates of mTG.

Materials and Methods

Trypsin, endoproteinase Glu-C, dithiothreitol (DTT), 5-(biotinamido) pentylamine (5-BP), α-cyano-4-hydroxycinnamic acid, and sinapinic acid, renin substrate tetradecapeptide porcine were purchased from Sigma (St. Louis, Missouri, USA). mTG from *Streptoverticillium mobaraense* was obtained from Ajinomoto (Tokyo, Japan).

Ovine CN from single milk was skimmed by centrifugation at 3000 rpm, and the isoelectric CN was prepared according to the procedure of Aschaffenburg [31]. β-CN was purified using the procedure of Ferranti et al. [32]. All other reagents and solvents were purchased from Carlo Erba in the highest purity available.

mTG reactions

1 mg of purified β-CN and whole CN were incubated with TG (1:50 enzyme: substrate ratio) at 37˚C for 0, 1, 2, 4, 6 and 24 h in 125 mM Tris-HCl and 10 mM EDTA at pH 7.6. Biotinylated CN was obtained by incubating whole CN and mTG in the same buffer in the presence of 5 mM 5-(biotinamido) pentylamine (5-BP). The reaction was stopped by heating in a bath of boiling water for 5 min. Gel filtration chromatography on a PD-10 column equilibrated with 20 mM ammonium bicarbonate at pH 7.8 was performed to remove salts and low MW peptides.

SDS gel electrophoresis and immunoblotting experiments

SDS-PAGE was carried out with 12% acrylamide using the GE 2/4 Pharmacia system. The membrane was stained with Coomassie Brilliant Blue R250. After electrophoresis, proteins were transferred from the gel to nitrocellulose paper as described by Addeo et al. [33] and then stained using polyclonal antibodies against single CN fractions [34].

Enzymatic reaction

Tryptic digestions were carried out in 0.4% ammonium bicarbonate (pH 8.5) at 37˚C for 4 or 18 h using an enzyme to substrate ratio of 1:50 (w/w). Endoproteinase Glu-C digestion was carried out in the same buffer at 40˚C for 18 h. Digestion was terminated by freeze-drying.

mTG-mediated biotin labeling

A sample containing 1 mg/ml of tryptic biotinylated CN digests was incubated for 30 min with 1 mg of streptavidin-coated Dynabeads in PBS/0.1%SDS, and the beads were washed as described in "*mTG reaction*" section. The enriched biotinylated peptides were eluted by adding 25 ml of 70% acetonitrile/2% formic acid/0.2 mM biotin and incubating for 30 min at 37˚C. The assay was performed in parallel on a sample incubated with no mTG and 5BP.

RP- HPLC and LC-ES-MS analysis

Liquid chromatography was performed using a 2.1 mm i.d. x 250 mm, C18, 5 μm reverse-phase column (Vydac, Hesperia, CA, USA) with a flow rate of 0.2 ml/min on an Agilent 1100 modular system (Palo Alto, CA, USA). Solvent A was 0.1% trifluoroacetic acid (TFA) (v/v) in water, and solvent B was 0.1% TFA in acetonitrile. The proteins were separated using a gradient from 35% to 50% B over 60 min. Peptide separation was instead performed using a gradient from 5% to 70% B over 90 min. Peaks were monitored by UV detection at 220 nm. Mass spectrometry (MS) was performed using a Platform (Micromass, Manchester, UK) equipped with standard electrospray. The eluent from the Vydac column chromatography was injected into the mass spectrometer online with the UV detector via a 75 μm i.d. fused silica capillary. The mass spectra were scanned from 2000 to 400 atomic mass units at a scan cycle of 5 s/scan. The source temperature was held at 200˚C and the cone voltage at 40 V. Mass scale calibration was obtained using myoglobin as the reference compound.

MALDI -TOF MS analyses

MALDI-MS TOF experiments were carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO instrument equipped with a N2 laser (337 nm, 3 ns pulse width). Each spectrum was taken with the following procedure: a 0.5 μL-aliquot of sample was loaded on a stainless steel plate together with 0.5 μL of 4-HCCA matrix (10 mg in 1 mL of 50% acetonitrile) or 1 μL of 3,4-dihydroxybenzoic acid (10 mg in 1 mL Milli-Q water). The mass spectrum was acquired by accumulating 200 laser pulses, in linear or in reflectron mode for the analysis of protein or peptide, respectively. The accelerating voltage was 25 kV. External mass calibration was performed with high or low-mass standards (SIGMA).

Nano ESI-TOF-MS/MS

MS/MS spectra of normal and m-TG modified peptides were performed with a Q-STAR mass spectrometer (Applied Biosystems) equipped with a nanospray interface from Protana (Odense, Denmark). Samples were sprayed from gold-coated 'medium length' borosilicate capillaries (Protana). The capillary voltage used was 800 V. Multi-charged ion isotopic clusters were selected by the quadrupole mass filter (MS1), and then, fragmentation was induced by collision. The collision energy varied between 20 and 40V depending upon the mass and charge state of the peptides. TOF analyzer was calibrated using Renin Substrate Tetradecapeptide porcine. The fragmentation spectra of the cross-linked peptides were obtained by combining 5 minutes of acquisition from the $+4^{th}$ and $+5^{th}$ charges. The resulting spectra were summed using the "add data" feature in Analyst software (Applied Biosystems) to obtain a single collision-induced dissociation spectrum. Spectra were manually interpreted with the help of MS-product software (http:/prospector.edu),

Results

TG-catalyzed cross-linking of whole CN

Ovine CN incubated at pH 8 with mTG was submitted to SDS-PAGE analysis. We observed CN cross-linking whose band **intensity**

increased as a function of time, as shown in Figure 1 (panel a). The intensity of native CN bands decreased with time as the relative bands clearly decreased because of the progressive polymer formation. Incubation for up to 24 h altered the electrophoretic profile, showing the loss of native CN and the formation of polymers that partially migrated through the high porous stacking gel but did not completely enter the gel top.

At shorter incubation times, a limited number of high molecular weight bands was apparent, indicating that glutamyl-lysine cross-linking involved only a few Q-and K- residues. Some Q-residues are in the interior or core of the protein structure and have limited accessibility to mTG. The CN polymers could still contain most of the reactive Q- and K-residues nearby or those that are preferred as substrates for covalent protein cross-linking catalyzed by microbial transglutaminase. The molecular weight of the formed polymers increased to between ~50 and ~70 kDa during incubation periods of up to 4 h. This indicates that the K- and Q-residues formed a limited number of the possible transamidated linkages in the presence of mTG. Therefore, a number of Q-residues may not enhance the reactivity of CN to mTG. SDS-PAGE analysis is unable to identify the exact composition of protein polymers because they are separated by mass. One possible solution to this issue is to utilize a modified staining procedure with polyclonal antipeptide antibodies that specifically target CN fractions (Figure 1).

Immunoblotting assay

To assess the ability of the CN fractions to crosslink among themselves, the protein was incubated with mTG, and then, immunoblotting was carried out using antibodies against α_{s1}-, β-, α_{s2}- and κ-CN. (Figure 1, panel b, c, d, and e, respectively). Because immunoblotting can be 100 times more sensitive than traditional Coomassie Blue stain, hydrolytic CN fragments arising from each CN were detected simultaneously. This phenomenon is a consequence of the CN hydrolysis by endogenous protease, resulting in the degradation of ovine CN. In all cases, other high molecular weight bands were detected in addition to the monomers not incubated with mTG. Based on the apparent molecular weight, the coexistence of dimer and oligomer CN was reproducibly observed after a 4 h incubation. At all incubation times other than 24 h, there was incomplete CN cross-linking, which indicates that the process is progressive; the Q- and K-residues aggregated by TG according to their propensity to polymerize. After incubation for 24 h, no monomer was detected because all the CN was incorporated into the polymer network. At shorter times, β-CN formed bands arranged in dimer and tetramer.(panel c), whereas α_{s1}- and α_{s2}-CN essentially formed dimers. Notably, two polyclonal antibody preparations simultaneously recognized a resolved band via SDS-PAGE electrophoresis, meaning that at least two fractions participated in CN cross-linking during polymerization. During the initial stage of the experiment, κ-CN molecules did not interact with any other CN fraction (panel e). This is in contrast with the results from the 24 h incubation. Individual κ-CN, which has unique properties, seemed more resistant to cross-linking than the other CN. This may be due to the polymerization of Q-residues with limited accessibility to mTG, while κ-CN was targeted similarly to the other CN fractions.

Identification of Q susceptible to transamidation in whole CN

To identify the location of K- and Q-residues available for mTG-mediated isopeptide bond formation, a free amine was initially used as the mTG protein substrate. This was accomplished with streptavidin-biotin technology that incorporates a biotinylated primary amine (5-BP) for protein transamidation. Subsequently, CN was digested with trypsin, reacted with biotinylated peptides fixed to streptavidin-coated magnetic beads, and eluted with acidic acetonitrile (Figure 2). The addition of mTG to four CN solutions resulted in different HPLC profiles (panel a) than the control sample (panel b). The two intense pairs of peaks dominating the HPLC profile result from peptides that were not incubated with biotin. The numbered peaks in panel (a) were singly collected and submitted to nano ESI-MS/MS.

The spectrum obtained by selecting the appropriate positively charged precursor displayed a complete '*b*-' and '*y*-ion' series (not shown). The location of the mTG-susceptible Q-residue within the peptide chain was indicated by the loss of biotinylated Q (439.22 Da) instead of native Q (128.05 Da). The biotin-containing tryptic peptides identified in the MS/MS spectra are listed in Table 1. Three Q transamidated biotin-containing tryptic peptides were found at positions 71 87 and 94 of the β-CN (64-112) peptide (fraction 5). The results were confirmed by the retrieval of the two peptides 64-83 (peak 3) and 84-112 (fraction 4), which possibly resulted from non-specific hydrolysis of β-CN (64-112).

Tryptic α_{s1}-CN (f119-129) peptide (peak 2) had a single biotin-binding Q-123 residue (fraction 2), similarly to α_{s1}-CN (f118-129) peptide (fraction 1). We did not observe any other biotinylated Q residue of α_{s1}- and β-CN that acted as an amine acceptor for mTG.

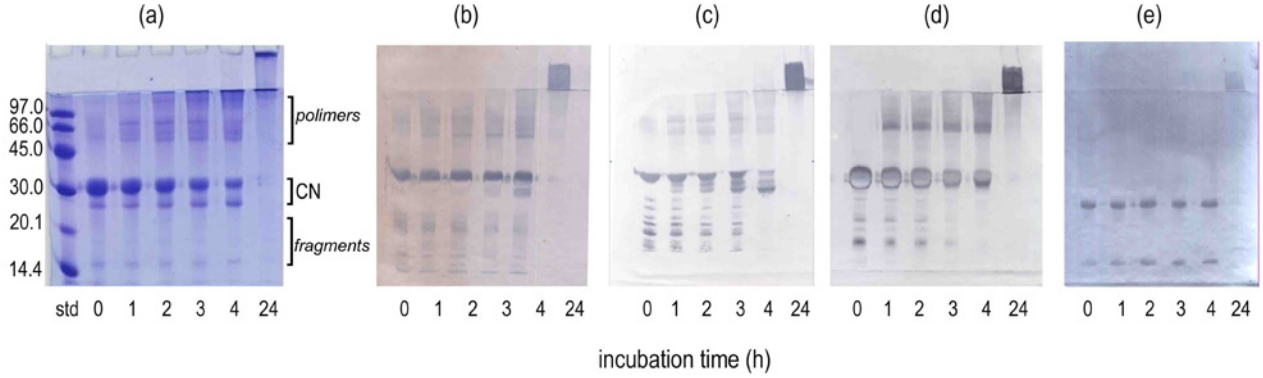

Figure 1: The time course of cross-linking of whole ovine CN and mTG incubated for 0, 1, 3, 4 and 24 h. Proteins separated on SDS-PAGE were stained with Coomassie brilliant blue R-250 (panel a). Individual CN were detected with anti-peptide antibodies against αs1-(b), β- (c), αs2- (d) and κ-CN (e).

It is noteworthy that κ-CN and α_{s2}-CN were both unable to react with 5-BP in the presence of mTG. As observed in the immunoblotting assay, κ-CN was the sole protein not modified by mTG (except after lengthy incubation), indicating that the protein did not function either as an acyl donor or acceptor (Figure 2). Under the same conditions, the immunoblotting assay showed that α_{s2}-CN was susceptible to mTG-mediated cross-linking (Figure 1, panel d). We assume that α_{s2}-CN participated in mTG-mediated CN-polymerization by means of an acyl acceptor (K-residue), as no Q-residue served as the acyl donor.

Structural analysis of polymer β-CN species

Our research focused on how β-CN develops cross-linkages, and we also studied the number of **Q-residues that** can react with amino moieties and the location of reactive Q-residues. The RP-HPLC profile of the mixture of 4 h-incubated protein and mTG confirmed that β-CN is a good substrate for mTG (Figure 3).

HPLC peaks were analyzed by MALDI-TOF. The peak at t_R 41.7 min (Figure 3, panel a) showed the mass spectrum in Figure 4, panel a, which supports the presence of a mixture of monomer and dimer β-CN. Analysis of the peak at t_R 56.1 by MALDI-TOF showed three additional proteins possessing the expected sizes of trimers, tetramers and pentamers of β-CN (Figure 4, panel b). Real intensity of aggregates could be underestimated in comparison to that of the monomers because of the different ionization efficiency.

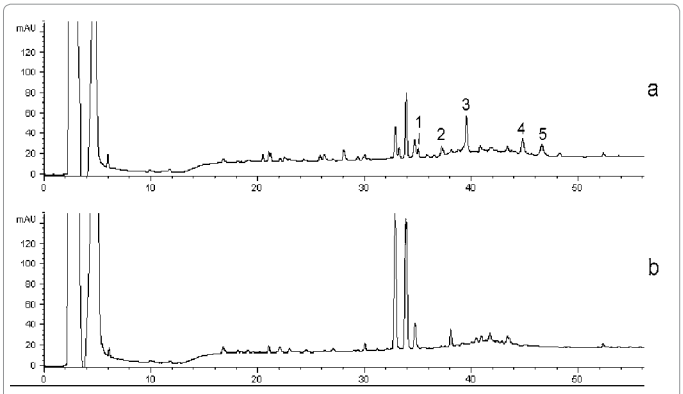

Figure 2: RP-HPLC chromatogram of enrichment CN biotinylated CN tryptic peptides (panel a) compared to control (b). Numbered peaks (panel a) were collected and analyzed by MS/MS (Table 1).

(MH+)[a]	HPLC fraction[b]	Amino acid sequence	peptide[d]	Biotinylated residue
1738.98	1	KYNVPQLEIVPK	α_{s1}-CN (118-129)	Q_{123}
1610.89	2	YNVPQLEIVPK	α_{s1}-CN (119-129)	Q_{23}
2508.30	3	IHPFAQA**Q**SLVYPFTG-PIPN	β-CN (64-83)	Q_{71}
3775.11	4	SLP**Q**NILPLT**Q**TPVVVP-PFLQPEIMGVPK	β-CN (84-112)	Q_{87}, Q_{94}
5956.73	5	IHPFAQA**Q**SLVYPFTG-PIPNSLP**Q**NILPLT**Q**TPV-VVPPFLQPEIMGVPK	β-CN (64-112)	Q_{71}, Q_{87}, Q_{94}

Table 1:
[a]*MH+* values observed by MALDI-TOF MS in reflectron mode, but fraction 5 in linear mode
[b]The numbered peaks in Figure 2 (panel a) were singly collected and submitted for nano ESI-MS/MS
[c]Amino acid sequence determined by MS/MS experiment. Biotinylated Q-residues are indicated in bold
[d]Numbers indicate the amino acid residues at the extremities of each peptide refers to αs1-CN (P04653) and β-CN (P11839)

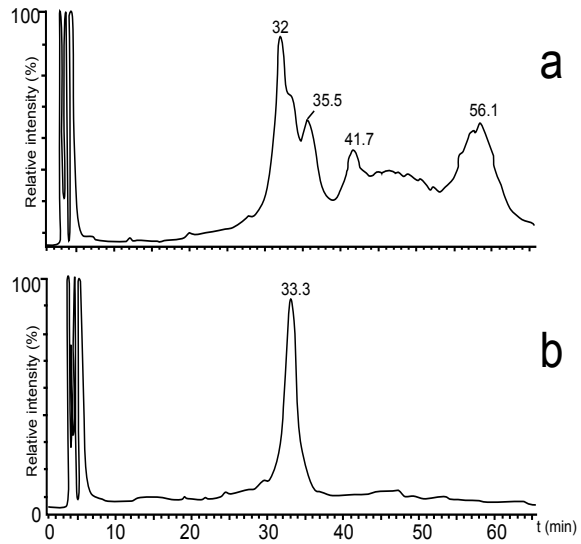

Figure 3: HPLC chromatogram of purified β-CN (panel b) and β-CN incubated with mTG for 4 h (panel a). Peaks at 41.7 and 56.1 min (panel b) were collected and analyzed by MALDI-TOF MS (Figure 4).

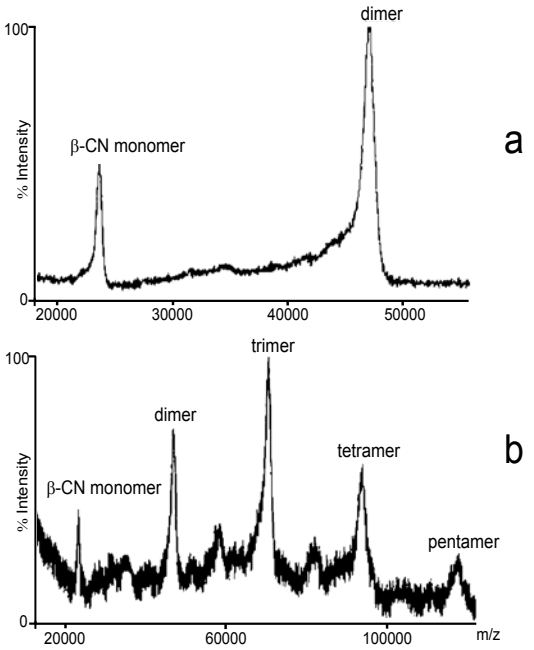

Figure 4: MALDI TOF MS analysis of HPLC fractions (Figure 3) at 41.7 min (panel a) and 56.1 min (panel b).

The location of amino acid residues that form ε-(γ-glutamyl)-lysine isodipeptide bonds was determined with endoproteinase Glu-C digestion. Enzyme digests of the CN and mTG mixture after a 4 h incubation period were fractionated by HPLC. The HPLC peaks were analyzed by MALDI-TOF (not shown). The molecular weight distribution of signals before and after the 4 h incubation period was dominated by two β-CN peaks at m/z 6141.3 and 13669.9. Because these two observed molecular masses did not correspond to any known β-CN-derived peptide in the databases, identification was achieved by the mass fingerprinting of enzyme-mediated peptides.

MS/MS analysis of peptide MH$^+$ 6143, 27

The reverse-phase HPLC fraction from the Glu-C digestion of the polymerized β-CN and the corresponding control digest containing the native β-CN (60-106) Glu-C peptide were analyzed by nano-ESI-TOF MS/MS. The resulting spectra are shown in Figure 5, panels a and b**.** Prior to fragmentation, mass measurement was performed on the peptides in the HPLC fraction, whose main component was 6140.22 Da in size (data not shown). Despite the great complexity of the mass spectral data, the comparison between the MS/MS spectrum of the native β-CN peptide (60-106) (Figure 5, panel a) with that of the transamidated peptide at ~6-kDa (Figure 5, panel b) allowed the identification of the cross-linked Q- and K-residues incubated with mTG.

As shown in Figure 5, some *y*-ion fragments allowed straightforward matching of the fragments of the cross-linked peptide with those of the native β-CN (60-106) peptide. This result indicates that the β-CN (60-106) peptide is part of the 6-kDa cross-linked peptide. Moreover, subtraction from the measured molecular mass of the longer cross-linked peptide (6140.22 Da) of that of the native β-CN (60-106) peptide (5188.80 Da) and adding 17 Da to allow for the ammonia molecule lost during transamidation, the obtained mass was in very good agreement with that of the β-CN Glu-C fragment (116-123).

MS/MS data analysis showed that the triply charged ion series b_{38}, b_{39}, and b_{40} of the β-CN (60-106) peptide, the three of which correspond to V-97, V-98, and V-99, respectively (Figure 6, panel a), are missing in the transamidated peptide (Figure 6, panel b). At the same time, the MS/MS fragmentation spectrum of the ~6-kDa peptide showed the same group of *b* signals (marked with an asterisk in Figure 6, panel b) along with a shift in mass corresponding to that of the triply charged ion of the β-CN (116-123) peptide minus the mass of the ammonia lost in the cross-linking reaction. This finding suggests that MS/MS fragmentation of the cross-linked peptide takes place only on the β-CN (60-106) backbone, leaving the attached peptide (116-123) intact. (((Following this criterion,))) After de novo sequence analysis, it was possible to infer nearly the complete sequence of the peptide. Figure 7 reports all the ion fragments identified in the MS/MS spectra of the cross-linked peptide along with their corresponding charge states. As expected for a Glu-C peptide, a prevalence of *b* signals and a limited amount of *y* signals were observed. Some fragments contain the mass shift of the intact β-CN (116-123) fragment (indicated with an asterisk), but others are referable to the native β-CN (60-106) sequence. For example, the ion fragment having a mass of 1475.74 Da reported in Figure 6, panel b was identified as the quadruple-charged b^*_{45} ion of the cross-linked peptide; at the same time, a clear *y* series referable to the native β-CN (60-106) sequence and showing the same Q-residue free of cross-linking was found (Figure 5 and 7). Taken together, the data from the *b*/*y* and *b**/*y** ion series permitted identification of four co-existing isobaric cross-linked peptides possessing isopeptidyl bonds linking the Glu-C fragment β-CN (116-123) to the Q-71, Q-87, Q-94 Glu-C β-CN (60-106) fragments (Table 2).

Fragment (1116-123) of the cross-linked peptide did not fragment, thus helping to identify the location of the Q-residue involved in transamidation. It was not possible to identify the acyl acceptor because of the presence of the K-residues 120 and 122. Due to the proximity of these residues, both could be engaged in cross-links; however, the spectral data indicated possible involvement of K-120 in the reaction. The fragmentation spectrum of the cross-linked peptide contains a weak y_2 signal coming from the fragmentation of peptide (116-123) that is missing in the native Glu-C peptide (60-106) spectrum used as

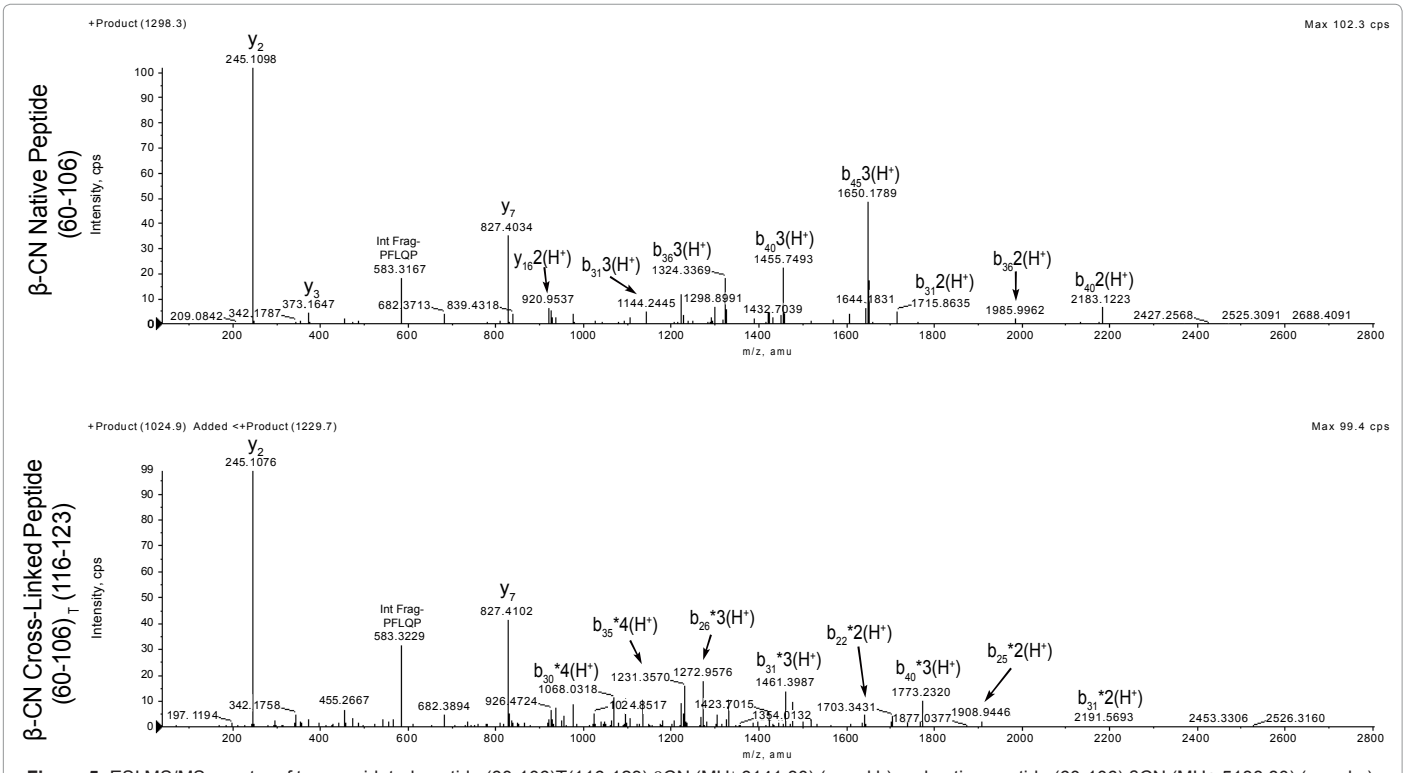

Figure 5: ESI MS/MS spectra of transamidated peptide (60-106)T(116-123) βCN (MH$^+$ 6141.30) (panel b) and native peptide (60-106) βCN (MH+ 5188.80) (panel a). The signals of the fifth charged parent ions were selected for CID (MH^{+5} 1229.06 Da, panel a; MH^{+5} 1038.56, panel b).

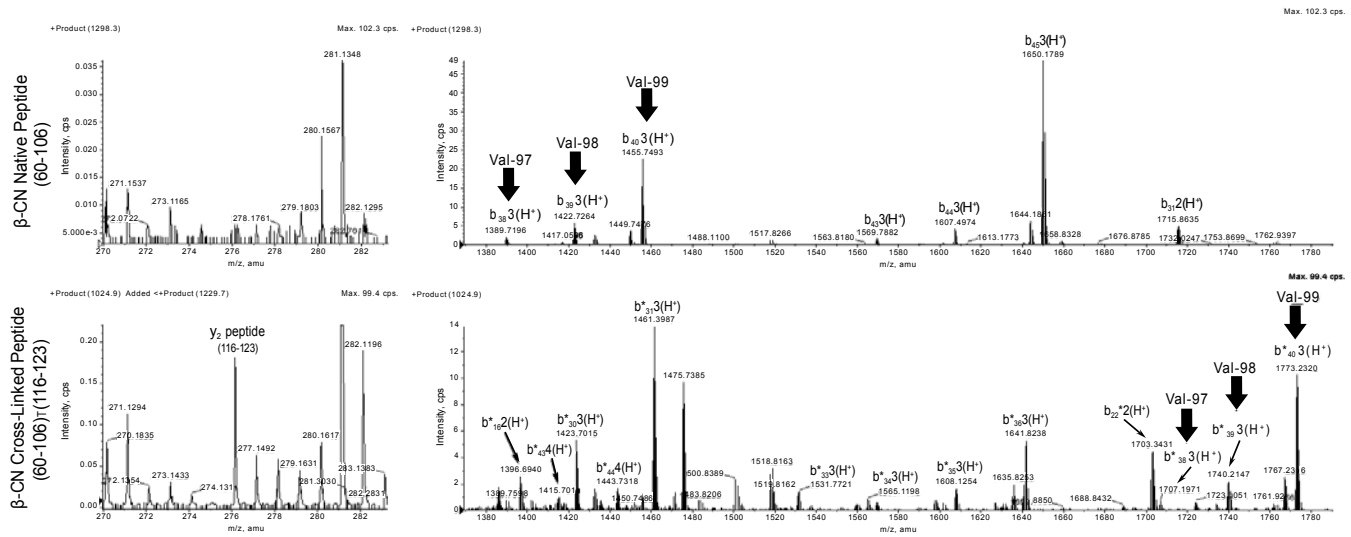

Figure 6: An expansion of the MS/MS spectra shown in Figure 5. The fragmentation spectrum of the β-CN (60-106) peptide shows that the triply charged ion series b_{38}, b_{39}, and b_{40}, corresponding to V-97, V-98, and V-99 (panel a), is missing from the spectrum of the transamidated peptide (panel b). The signals marked with an asterisk (panel b) indicate a shift in mass corresponding to that of the triply charged ion of the β-CN (116-1123) peptide minus one ammonia mass lost in the cross-linking reaction.

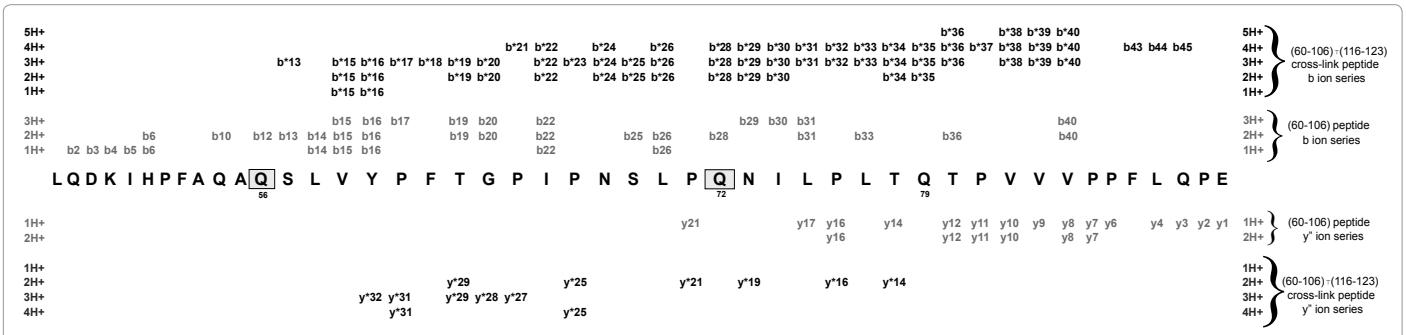

Figure 7: Schematic illustration of observed daughter ions in the nanoESI MS/MS spectra shown in Figures 5 and 6.

a control (Figure 6). The presence of the y_2 signal indicates that the K-122 is not transamidated; however, because no other fragments were identified, we cannot therefore rule out the possibility that K-122 may be involved in all cross-links.

MS/MS analysis of peptide MH$^+$ 13669

The mTG-treated β-CN giving rise to a peptide with a molecular mass of 13669.9 Da was also subjected to nano-ESI-TOF MS/MS analysis. Due to the high complexity of the fragmentation spectrum, the exact location of the isopeptide bond remains to be determined. However, some of the ion fragment series detected were easily assigned to the β-CN (60-106) and the (147-222) Glu-C peptides (data not shown). To this end, comparative LC-ESI-MS analysis of the chymotryptic hydrolysate of the 13669.9 Da peptide and the β-CN native peptides (60-106) and (147-222) was performed (Figure 8). Of the three digests accounting for the 13-kDa peptide, the chymotryptic digest of the cross-linked peptide gave more complex LC-MS patterns (panel b) than single peptides 60-106 (panel a) and 147-222 (panel c). In particular, the chymotryptic digest of the 13669.9 Da peptide had six peptides in common with β-CN (30-106) and twelve with β-CN (147-222). Two unknown peptides with molecular masses of 2607.2 and 3036.4 Da co-eluted with the peak at t_R 48.1 min. The first mass peak consisted of peptide 68-77 cross-linked with peptide 191-203. This result means that an isopeptidyl bond is formed by K-191 and Q-71 residue. The LC-MS analysis showed that the two individual chymotryptic peptides 93-106 (parent peptide 60-106) and 191-203 (parent peptide 147-222) cross-link to form the mass peak of 3036.4 Da consisting of an aggregate. The K-191 residue reacted with Q-94, resulting in the two major cross-linking sites. MS experiments showed that the cross-linked peptide (60-106)-T-(147-222) contained three K- and nine Q-residues. The glutamine/lysine pairs 87/120, 94/120, 94/191, and 71/191 are significant due to the formation of multimers (Table 2).

Discussion

Conformational factors as well as the primary structure of the substrate play an important role in biochemical activities such as the transglutamylation of CN with TG. Recently, considerable efforts have been undertaken to determine the preferred location of the reactive Q-residue. A series of studies on proteins and peptides have demonstrated that the transamidation of Q-residues is strongly influenced by the position of P-residues in the C-terminal region in the

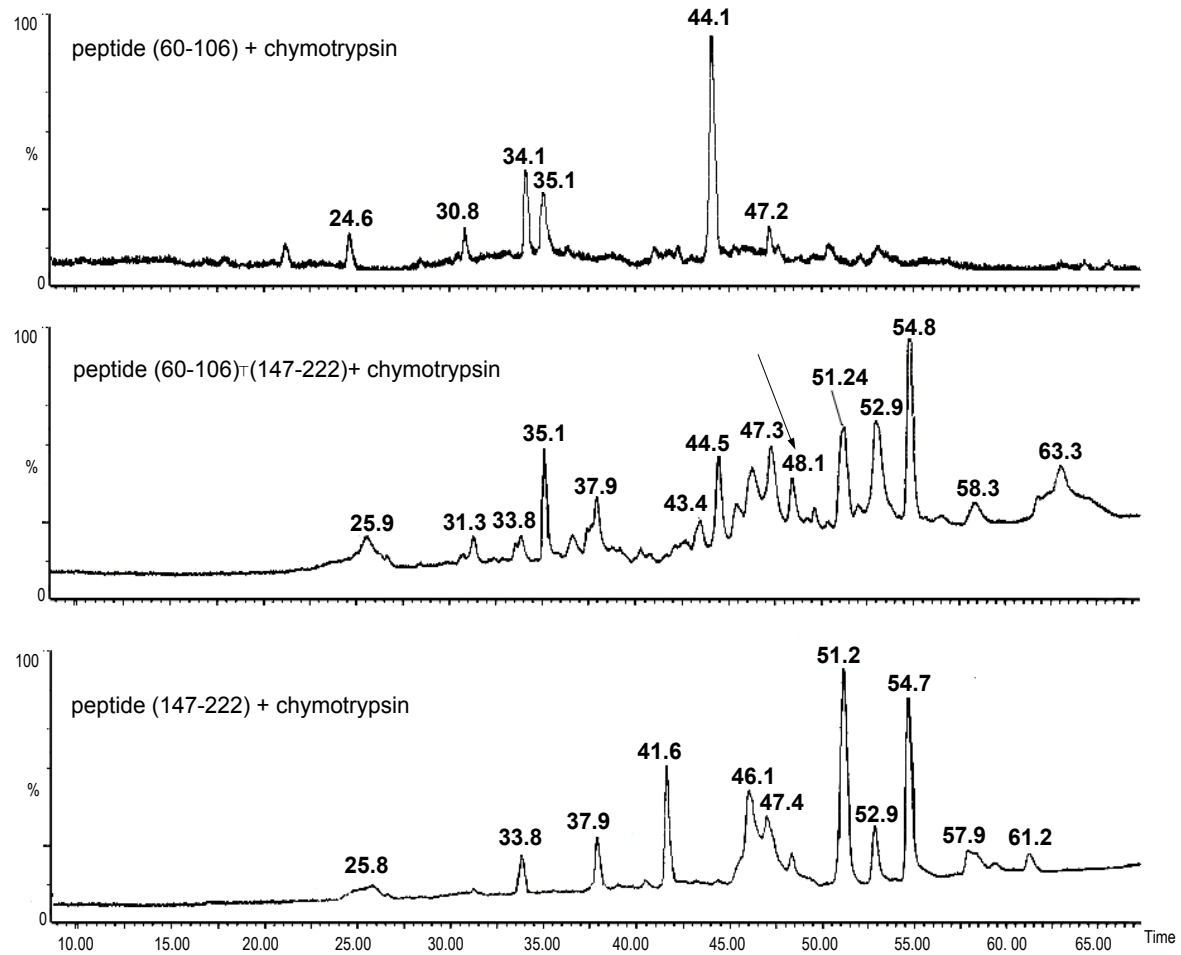

Figure 8: LC-ESI-MS analysis of chymotryptic hydrolysate of transamidated peptide (60-106)T(147-222) (panel b) compared to chymotryptic hydrolysate of native peptides 60-106 (panel a) and 147-222 (panel c). The peak at t_R 48.1 min (marked with an arrow) contains two transamidated peptides identified as (68-77)T(191-203) (MH+ 2607.2) and (93-106)T(191-203) (MH+ 3036.4 Da) (Table 2).

MH+ [a]	Cross-linked β-CN *via* isopeptide bonds	Q- and K- residue involved in cross-linking	MS experiment
6141.30[b]	([60]LQDKIHPFAQAQSLVYPFTGPIPNSLP**Q**NILPLT**Q**TPVVVPPFLQPE[106]) T ([116]TMVPKHKE[123])	(Q_{87}or Q_{94})-(K_{120} or K_{122})	ESI-MS/MS
13688.52[b]	([60]LQDKIHPFA**Q**AQSLVYPFTGPIPNSLP**Q**NILPLTQTPVVVPPFLQPE[106]) T ([147]KLHLPLPLVQSWMHQPPQPLPPTVMFPPQSVLSLSQPKVLPVPQ**K**AVPQRDMPIQAFLLYQEPV-LGPVRGPFPILV[222])	(Q_{71}or Q_{94})-(K_{120} or K_{122})	ESI-MS/MS
2606.47[d]	([68]AQA**Q**SLVYPF[77])T([191]**K**AVPQRDMPIQAF[203])	(Q_{71})-(K^{191})	LC-ESI-MS
3034.62[d]	([93]T**Q**TPVVVPPFLQPE[106])T([191]**K**AVPQRDMPIQAF[203])	(Q^{94})-(K^{191})	LC-ESI-MS

Table 2:
[a] *MH+* values observed by MALDI-TOF MS; Peptide generated from: [b]Glu-C hydrolysis of polymer β-CN at 4-h incubation time; [c] chymotryptic cleavage of peptide at MH+ 6141.30; [d] chymotryptic cleavage of peptide at MH+ 13668.52. Numbers indicate the amino acid residues at the extremities of each peptide refers to β-CN (P11839). Transamidated Q- and K-residues are indicated in bold.

specific sequence Q-X-P (X is any amino acid residue) [4,9,35]. While these Q residues are targeted by tissue TG, any Q-residue belonging to the motif QP or QXXP is not a substrate for TG [36-40].

The majority of biochemical studies in recent years has examined mammalian TG, whereas there are limited data concerning the sites in the CN sequence recognized by mTG. Despite the relatively high numbers of Q-residues in CN, there is a limited number that effectively acts as amine donor. As shown in the result section, using an excess of free 5-BP as the acyl acceptor made possible the identification of three transamidated Q-residues in β-CN (Q-71, -87 and -94) and one in α_{s1}-

CN (Q-123) (Table 1). Among possible transamidation sites of CN, only Q-94 and Q-207 of β-CN belonged to the TG-sequence Q-X-P. Even with this common sequence, Q-207 did not serve as acyl donor, most likely due to steric hindrance on the surface of β-CN.

Interestingly, separation of CN by SDS-PAGE showed, in addition to the monomer, high molecular weight proteins migrating either in the stacking gel, at the borderline between the stacking and separating gels, or along the latter (Figure 1). Polymers of CN generated by m-TG action are preserved in this part of the gel even in the presence of strong reducing and denaturing agents, indicating the covalent nature of their bonds. Individual CN incubated for a short time with mTG polymerize at different rates. However, in all the CN fractions, even poor substrates of mTG such as κ-CN and α_{s2}-CN were included in a polymer network after 24 h. Incorporation of α_{s2}-CN into cross-linked polymers suggests that this protein functions merely as the acyl acceptor via its K-residue, resulting in the formation of intermolecular ε-(γ-glutamyl) lysine isopeptide bonds (Figure 1, panel d).

Fragmentation of large peptides by MS/MS proved to be effective in the characterization of transamidated residues. High-complexity spectra caused by overlapped fragment ion series were observed; however, the sequence and modifications of the transamidated Glu-C peptides were clearly identified with the mass accuracy and resolution of a TOF analyzer. Fragmentation of larger peptides/proteins, such as the 13-kDa transamidated peptide, generates high-molecular-weight fragments that can be very difficult to annotate with confidence. In this case, it is necessary to sub-digest the peptides using other enzymes.

The results reported here are similar to results obtained using mammalian tissue TG and bovine CN [41], especially β-CN, despite the different sequence, conformation and/or specificity of ovine CN and the mammalian enzyme. Residues Q-71, -87, -94 and -89, which are involved in cross-linking with K-120 and/or -122 and -191 of ovine β-CN, are located in a relatively small stretch of the β-CN sequence, suggesting that this portion of the protein may be exposed on the surface and facilitate reaction with mTG. The β-CN monomer is progressively polymerized into dimers, trimers, tetramers and pentamers, and the rate of polymerization could depend on the propensity of the different Q- or K- amino acid residues to form intermolecular cross-links along the protein chains while the polymer network is growing.

Gaining insight into the covalent linkage of CN multimers has been a long-term goal of our research. Purification of native whole casein by RP-HPLC always shows unknown peaks eluting later than β-CN, the most hydrophobic of all the CN. Moreover, MALDI-TOF analysis of these proteins indicates the co-presence of β-CN with other species with the expected molecular weights of β-CN oligomers (S. Lilla, unpublished data). It is known that dimer formation can occur as an artifact of MALDI-TOF ionization of proteins, and high-molecular-weight forms are in some cases more intense than the β-CN monomer. The nature of these unknown protein species has never been established due to their low abundance. Their observed behavior in RP-HPLC and MS closely resembles that of the β-CN polymer generated by mTG (Figures 3 and 4). However, except for transamidating activities like blood coagulation factor XIII (FXIII), TG have not been found in milk. The former could pass into milk from the blood through the lactating mammary gland.

Characterization of ovine β-CN polymers that have not been typified previously may help us understand how CN fractions aggregate in supramolecular structures. The observations reported here cannot be compared with any of the dangling CN micelle models reported in the literature [42]. The so-called 'colloidal' calcium phosphate clusters or, more likely, chains of colloidal calcium phosphate (CCP) particles would be surrounded by external hydrophobic regions.

In conclusion, in this work, we have identified several sequences recognized by mTG for CN cross-linking. The observations reported here provide important insight into the self-assembly of sodium caseinate by mTG. Our observations indicate that casein sub-micelles assemble into micelles upon *in vivo* cross-linking of the casein sub-micelles by transglutaminase. However, in contrast to native casein micelles, the casein-nanogel particles described herein would be expected to be stable either in urea solution or/and in the absence of calcium.

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