JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 7669−7680 **7669**

Heat-Induced Redistribution of Disulfide Bonds in Milk Proteins. 2. Disulfide Bonding Patterns between Bovine β-Lactoglobulin and κ-Casein

EDWIN K. LOWE,*,† SKELTE G. ANEMA,† ANNIE BIENVENUE,‡
MICHAEL J. BOLAND,† LAWRENCE K. CREAMER,† AND RAFAEL JIMÉNEZ-FLORES‡

Fonterra Research Centre, Private Bag 11-029, Palmerston North, New Zealand, and Dairy Product Technology Center, California Polytechnic State University, San Luis Obispo, California 93407

Heat treatment of milk causes the heat-denaturable whey proteins to aggregate with κ-casein (κ-CN) via thiol−disulfide bond interchange reactions. The particular disulfide bonds that are important in the aggregates are uncertain, although Cys^{121} of β-lactoglobulin (β-LG) has been implicated. The reaction at 60 °C between β-LG A and an activated κ-CN formed small disulfide-bonded aggregates. The tryptic peptides from this model system included a peptide with a disulfide bond between a Cys residue in the triple-Cys peptide [β-LG(102−124)] and κ-CN Cys^{88} and others between κ-CN Cys^{88} or κ-CN Cys^{11} and β-LG Cys^{160}. Only the latter two novel disulfide bonds were identified in heated (90 °C/20 min) milk. Application of computational search tools, notably MS2Assign and SearchXLinks, to the mass spectrometry (MS) and collision-induced dissociation (CID)-MS data was very valuable for identifying possible disulfide-bonded peptides. In two instances, peptides with measured masses of 4275.07 and 2312.07 were tentatively assigned to β-LG(102−135):κ-CN(11−13) and β-LG A(61−69):κ-CN(87−97), respectively. However, sequencing using the CID-MS data demonstrated that they were, in fact, β-LG(1−40) and β-LG(41−60), respectively. This study supports the notion that reversible intramolecular disulfide-bond interchange precedes the intermolecular interchange reactions.

KEYWORDS: Heat interactions; disulfide bonds; β-lactoglobulin; κ-casein

INTRODUCTION

There are numerous studies examining the kinetics of the denaturation reactions for the major whey proteins when milk protein systems are heated under defined conditions (*1*−*4*), and models that allow prediction of the degree of denaturation of the individual whey proteins at any temperature and time combination have been generated (*1*, *2*). However, an understanding of the denaturation reactions of the individual whey proteins provides information on only the initial steps of a complex series of aggregation reactions that occur when milk is heated.

The major whey protein in milk is β-lactoglobulin (β-LG), and one of the interactions of interest is complex formation between denatured β-LG and the casein micelles. Early studies in protein model systems have suggested that there may be an interaction between β-LG and casein on heating (*5*, *6*); however, Zittle et al. (*7*) provided the first conclusive evidence of complex formation between β-LG and κ-casein (κ-CN). Sawyer et al. (*8*) demonstrated the involvement of thiol groups and suggested that the free thiol group of β-LG was involved in the interaction and that intermolecular disulfide bonds formed between κ-CN and β-LG.

Subsequent investigations have tended to focus on determining the types of bonding involved in complex formation and the stoichiometry of the complexes formed (*9*−*19*). Sawyer (*9*) and McKenzie et al. (*20*) showed that the self-aggregation of β-LG was limited when κ-CN was present and suggested that κ-CN formed complexes with intermediate species of aggregated β-LG. In contrast, Euber and Brunner (*16*) reported that aggregation of β-LG was not a prerequisite for interaction with κ-CN. Cho et al. (*19*) examined the interaction of β-LG with κ-CN using a "natural" κ-CN isolated without reduction or chromatography. Only denatured β-LG interacted with κ-CN, and many of the possible pathways involved in the aggregation of β-LG with κ-CN were elucidated. They proposed that, on heating of β-LG and κ-CN, the free thiol of β-LG was exposed and that this initiated a series of reactions with other denatured β-LG molecules or with κ-CN. The products formed were dependent on the ratio of κ-CN to β-LG and included 1:1 β-LG:κ-CN complexes and a range of large heterogeneous aggregates that were held together by disulfide bonding and/or hydrophobic interactions.

Milk is considerably more complex than purified protein model systems. Although β-LG is the major whey protein in milk, several other whey proteins with free thiol groups and/or

* Corresponding author (telephone +64-6-350-4649; fax +64-6-356-1476; e-mail edwin.lowe@fonterra.com).
† Fonterra Research Centre.
‡ California Polytechnic State University.

10.1021/jf0491254 CCC: $27.50
Published on Web 11/16/2004

disulfide bonds exist (*21*). Of the casein proteins, both κ-CN and α_{S2}-casein have disulfide bonds, and therefore both could participate in thiol−disulfide interchange reactions. As a consequence, many more thiol−disulfide interaction pathways exist, and the separation and the analysis of the reaction products are substantially more difficult. Despite this, it appears that reactions between β-LG and κ-CN similar to those observed in the model systems may occur when milk is heated, although, as expected, other denatured whey proteins are involved in complex formation (*13*, *17*, *18*, *22*−*24*).

The degree of interaction is dependent on many variables including the time, temperature, and rate of heating, the milk concentration and individual protein concentrations, the milk pH, and the concentration of the milk salts (*13*, *15*, *22*). For example, recent studies have shown that the rate of interaction of denatured β-LG and α-lactalbumin with the casein micelles is considerably slower than the denaturation reactions and that the level of denatured protein interacting with the casein micelles is markedly dependent on small shifts in the pH of the milk at heating (*17*, *18*, *24*). It remains unknown why such small shifts in pH affect these interactions; however, this behavior can have a marked effect on the physical and functional properties of the milk (*17*, *18*, *24*−*27*).

It is of interest to examine the specific thiol groups of κ-CN and, in particular, β-LG that are involved in the interaction between these proteins. On heating, β-LG can form a series of non-native monomers in addition to disulfide-aggregated complexes (*28*−*30*). These monomeric species are likely to be formed through intramolecular thiol−disulfide interchange reactions between the free thiol at position Cys^{121} and the two disulfide bonds in native β-LG and, therefore, could have a free thiol in any one of five positions. A recent study examining novel disulfide bonds in heat-induced β-LG aggregates (*31*) showed that ∼35% of the Cys^{160} was in a reduced form (free thiol) in the heated system, whereas all of the Cys^{160} residues were disulfide bonded to Cys^{66} in the native protein (*32*). These findings suggest that Cys^{160} may play a significant role in the inter-protein disulfide bonding that occurs in heat-induced whey protein aggregates.

Examining the specific thiol groups of β-LG involved in thiol−disulfide interchange reactions with κ-CN on heating milk is more difficult than it is in pure protein systems because of the large number of protein species with native disulfide bonds and, consequently, the large number of potential disulfide-bonded products. No studies on bovine milk have been reported; however, an interchain disulfide bond between Cys^{160} of β-LG and Cys^{88} of κ-CN formed on heating a model caprine milk consisting of resuspended casein micelles and β-LG (*33*). These results indicate that an intramolecular thiol−disulfide interchange reaction in β-LG precedes intermolecular complex formation with κ-CN when caprine milk is heated, as native caprine β-LG has Cys^{160} involved in an intrachain disulfide bond with Cys^{66} and the free thiol at $Cys^{119/121}$. Although other sulfhydryl groups may be involved in complex formation between β-LG and κ-CN, these were considered to be less abundant than those involving Cys^{160} (*33*). The heat stability characteristics of bovine milk and caprine milk are markedly different, and this has been attributed to different interaction behavior between β-LG and κ-CN (*34*, *35*); therefore, it is of interest to examine the disulfide groups involved in complex formation in heated bovine milk.

In the present work, we explore the chemistry of heat-induced interactions between β-LG and κ-CN, with the intention of identifying the specific thiol groups of β-LG and κ-CN involved in the intermolecular disulfide-bonded complexes. Initial experiments are conducted on a model system [heated solutions of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-activated κ-CN (TNB−κ-CN) and β-LG] to produce 1:1 and 2:1 β-LG:κ-CN disulfide-bonded complexes through an oxidative reaction (*36*). The study is then expanded to identify the disulfide-bonded complexes that are formed between β-LG and κ-CN when skim milk is heated.

A critical aspect of this work is the identification of the novel disulfide bonds created by the heat treatments. Simple peptides from tryptic hydrolysates can be readily identified by matching measured molecular mass with that of the expected peptide (*37*). Confirmation can be gained using tandem mass spectrometry (CID-MS), in which a single molecular ion is isolated in the first mass analyzer and passed into a collision cell where collision of the peptide ion with inert gas molecules provides sufficient energy to fragment the peptide by collision-induced dissociation (CID). The fragments are characterized using a second mass analyzer. The masses of fragments are used to confirm the peptide sequence.

CID-MS, when available, has become routine for the identification of non-disulfide-linked peptides (*37*, *38*), but it is not ideal for identifying disulfide-linked peptides (*39*). Commonly the identification of disulfide-linked peptides is by mapping the peptide masses found against the expected masses from a theoretical digest (*39*, *40*). However, the results need to be treated with caution (*40*). Consequently, we compared CID fragments after mapping peptide masses to theoretical fragmentation patterns using the analysis tools SearchXLinks (*41*) and MS2Assign (*42*), which have been created to cope with the complex fragmentation patterns of disulfide-linked peptides.

MATERIALS AND METHODS

Materials. Low-heat skim milk powder was obtained from Fonterra, New Zealand. Sequencing grade modified trypsin was obtained from Promega, Madison, WI. β-LG A was isolated from fresh milk of cows that were homozygous for β-LG A using the method described by Manderson et al. (*28*) and based on that of Mailliart and Ribadeau-Dumas (*43*). κ-CN was isolated from fresh milk using the method described by Cho et al. (*19*) and based on the method of Rasmussen and Petersen (*44*). The gel electrophoresis reagents and the Criterion gels (8−16% Tris-HCl) were obtained from Bio-Rad Laboratories, Hercules, CA. All other reagents were obtained from BDH Laboratory Supplies, Poole, U.K.

Methods. An overview of the experimental process is given in **Figure 1**.

Preparation of TNB−κ-CN. TNB−κ-CN was prepared using the method of Thresher (*36*). Reduced κ-CN was dissolved in 0.1 M phosphate buffer, pH 8.0. The cysteine residues were reacted by treatment with solid DTNB to a final concentration of 1 mM. TNB−κ-CN was dialyzed against a solution containing 1 mM acetic acid, 1 mM EDTA, and 3 M guanidine hydrochloride, pH 6.0, followed by a solution containing 1 mM acetic acid and 1 mM EDTA at pH 6.0, and then lyophilized and stored at −20 °C.

Sample Preparation and Reaction Conditions. Solutions of 2 mg/mL mixed AB variant TNB−κ-CN and 5 mg/mL pure β-LG, variant A, were made up in Milli-Q water and were mixed at ratios of 4:1, 2:1, and 1:1. The protein samples were heated for 60 min in test tubes in a water bath maintained at 60 °C. For experiments with skim milk, low-heat skim milk powder was reconstituted at 9.6% total solids and held for 24 h at 4 °C. The skim milk samples were heated for 20 min in plastic Eppendorf tubes (1 mL volume) in a water bath maintained at 90 °C, followed by rapid cooling in an ice/water slurry. After equilibrating to room temperature, the skim milk was centrifuged at 14000 rpm for 30 min in an Eppendorf centrifuge (model 5417C). Previous experiments by Anema et al. (*26*) had found these conditions to be suitable for separating the micelle and serum phases of skim milk.

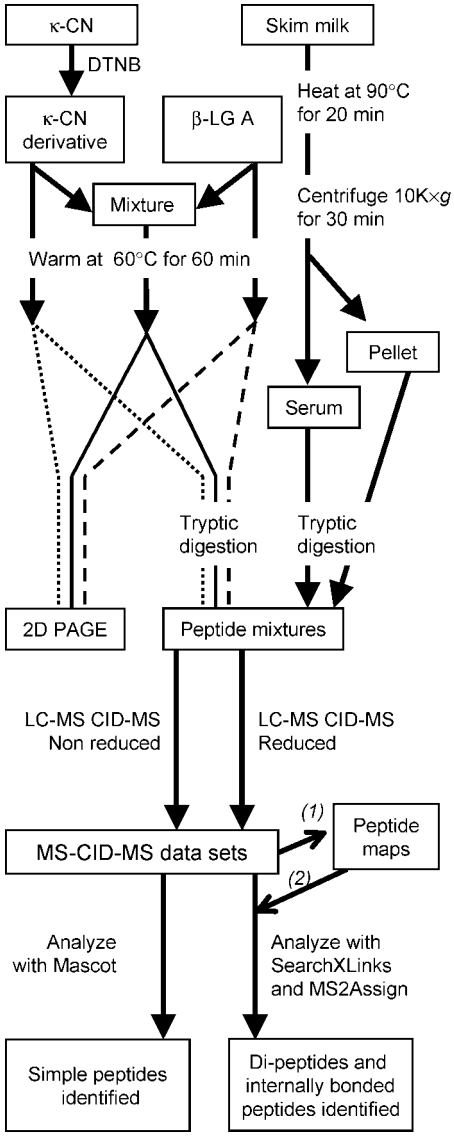

Figure 1. Diagram representing the protocol for sample preparation and analysis.

Two-Dimensional (2D) Polyacrylamide Gel Electrophoresis. The heated β-LG/TNB−κ-CN solutions were analyzed using the Criterion Precast Gel System (Bio-Rad Laboratories) and the method described by Havea et al. (*45*). For the first dimension [nonreduced sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE)], 10 μL samples of 0.1 mg/mL protein were loaded and run on an 8−16% T 12 lane Criterion Tris-HCl gel using SDS-PAGE (*46*). The lane of interest was excised from the gel for the second dimension (reduced SDS-PAGE) and soaked in reducing buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 10 mg/mL dithiothreitol, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 1 h at 20 °C. The gel strip was then placed on the top of an 8−16% T IPG comb Criterion gel and overlaid with 0.5% agarose. Molecular weight markers were also loaded, and the gel was run under SDS-PAGE (*46*). The 2D gels were stained using a silver stain protocol (*47*).

Tryptic Hydrolysis. The protein solutions were diluted to 0.1 mg/mL protein in 0.2 M ammonium bicarbonate, pH 8.0, and trypsin was added to give an enzyme-to-substrate ratio of 1:20 w/w in a sample volume of 100 μL. Digestion was carried out at 40 °C for 24 h in duplicate. To one replicate was added 1 μL of β-mercaptoethanol (BME), and the sample was held for 1 h at 25 °C to reduce the disulfide bonds in the peptides. Formic acid was added to the sample to give a concentration (after neutralization of the buffer) of 0.2%. The samples were centrifuged at 14000 rpm for 2 min, and the supernatant was held at −35 °C. If the reduced samples were held for >24 h, an extra 1 μL of BME was added.

Liquid Chromatography−Mass Spectrometry (LC-MS). A capillary reverse phase C18 150 mm × 0.3 mm column (Jupiter 4μ Proteo 90A, Phenomenex, Torrance, CA) was used with a gradient of acetonitrile in an Agilent 1100 capillary high-performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany). Eluent A was 0.2% formic acid in Milli-Q water; eluent B was 0.2% formic acid in HPLC grade acetonitrile. Aliquots (8 μL) were loaded on to the column and a gradient from 0 to 50% eluent B over 50 min at a flow rate of 3 μL/min was used, followed by a wash step at 80% eluent B for 20 min and a conditioning step at 0% eluent B for 30 min.

The eluting peptides were characterized by MS using a Perkin-Elmer Sciex (Thornhill, ON, Canada) $QSTAR_{XL}$ electrospray ionization (ESI) quadrupole/time-of-flight (Q-TOF) mass spectrometer. Mass spectra and collision-induced dissociation tandem mass spectra (CID-MS/MS) were recorded in positive ion mode with collection times of 1 and 8 s, respectively, using an information-dependent acquisition method. A "rolling collision energy" was selected for the CID-MS/MS. The mass spectrometer was calibrated using a polypropylene glycol standard as outlined in the Applied Biosystems/MDS SCIEX $QSTAR_{XL}$ software manual (*48*).

Data Analysis. Simple peptides were identified using the MS/MS pattern-matching tool Mascot (*38*). The MS/MS patterns were matched against a database of all *Bos taurus* proteins, using peptide and MS/MS tolerances of 0.2 Da. No enzyme cleavage sites were assumed, to allow for the identification of the atypical tryptic cleavage of the Tyr^{20}−Ser^{21} bond (*49*, *50*).

Disulfide-linked peptides were identified by peptide mapping, followed by confirmation by MS/MS spectral analysis. Using the LCMS Reconstruct tool in BioAnalyst (*48*), the masses of the observed peptides were calculated from the charge state of the ions observed. Only peaks with a signal-to-noise ratio >4 were considered for this analysis. The calculated peptide masses from the LCMS Reconstruct tool were compared using peptide mapping with theoretical tryptic digests of β-LG A and B and κ-CN, using the mapping modifications tool to search for disulfide bonds as well as the web-based tool SearchXLinks (*41*). Disulfide-linked peptides within and between other milk proteins were not searched for. The mass of the disulfide-bonded fragment was calculated by adding the masses of two cysteine-containing fragments from the predicted tryptic digest and subtracting 2.016 atomic mass units (amu) to account for the loss of two hydrogens during the reaction. To calculate the *m*/*z* of a disulfide-bonded charged peptide, the following equation was used:

$$m/z = \frac{(M_1 + M_2 - 2.016 + (z \times 1.0078))}{z}$$

M_1 and M_2 are the masses of the cysteine-containing fragments and *z* is the charge state of the peptide.

MS/MS spectra of disulfide-linked peptides were analyzed using the Web tools SearchXLinks (*41*) and MS2Assign (*42*). The fragmentation pattern observed for each suspected disulfide-linked peptide was compared against theoretical fragmentation patterns generated from the protein sequences.

RESULTS

Model System. Preliminary studies (*36*) showed that, if κ-CN was reduced, it would then be able to react with DTNB to form monomer κ-CN with two disulfide-attached thionitrobenzoate molecules, releasing equivalent quantities of the yellow thionitrobenzoate. It was also shown that TNB−κ-CN would react with β-LG at 60 °C to release the TNB ion and form a disulfide-bonded β-LG−κ−CN dimer.

Reaction of β-LG with TNB−κ-CN. Heating β-LG A with TNB−κ-CN at 60 °C at neutral pH gave a yellow solution, indicating that the TNB had been replaced by β-LG, thus forming a disulfide-bonded complex between β-LG and κ-CN. Heating TNB−κ−CN under the same conditions did not alter

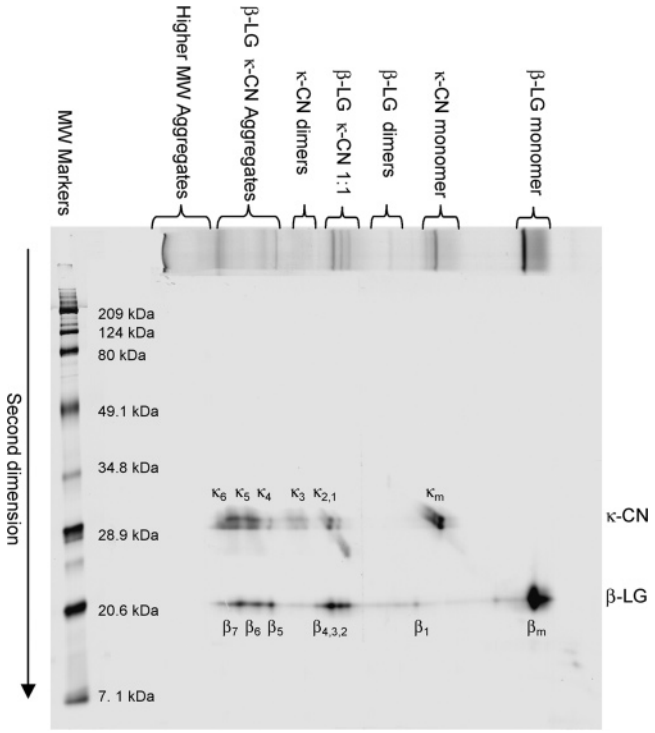

Figure 2. 2D SDS-PAGE (first dimension, nonreduced SDS-PAGE; second dimension, reduced SDS-PAGE) of the β-LG/TNB–κ-CN solution after heating at 60 °C for 60 min.

the color of the solution (light straw-colored). Likewise, heating the β-LG A solution under these conditions did not show any evidence of β-LG denaturation (results not shown.)

PAGE Analysis. The results of the 2D PAGE analysis of the warmed reaction mixture after silver staining of the protein are shown in **Figure 2**. The more mobile line of protein spots corresponds to reduced β-LG in terms of mobility, and the large spot β_m is unreacted native protein. There are similar lines of spots that correspond to κ-CN, with the spot κ_m corresponding to monomeric κ-CN. It is not clear why there are two lines of spots with similar mobilities for the κ-CN. One remote possibility is that, in this PAGE system, the two genetic variants had different mobilities. In the region corresponding to monomer κ-CN in both the reduced-SDS dimension and the SDS dimension of the gel, the spots are on a diagonal. This is probably a consequence of the variable glycosylation or phosphorylation of the κ-CN polypeptide in the expressed native proteins (*51*). The anomalously low migration of the monomer κ-CN in the SDS-PAGE system is a consequence of the unusual amino acid sequence of this protein (*52*, *53*).

To the left of the large monomer β-LG spot β_m, there is another clearly observable spot β_1 and a few lower mobility spots (in the SDS gel strip at the top of the picture), which are the various dimer β-LG species that were noted by Manderson et al. (*28*). Further to the left along the monomer β-LG series of spots, there are three moderately dense spots β_2, β_3, and β_4, with two corresponding spots κ_1 and κ_2 in the κ-CN series. The darkest β-LG spot β_4 aligns with the darker κ-CN spot κ_2 and indicates that the 1:1 dimer of κ-CN and β-LG is a significant product of the reaction between native β-LG and the activated κ-CN at 60 °C.

Even further to the left, β-LG spots β_5–β_7 and κ-CN spots κ_4–κ_6 align with some low-mobility bands in the SDS-PAGE pattern of the nonreduced samples shown at the top of **Figure 2**. No doubt, these are various higher polymers of β-LG and κ-CN and are probably formed from reactions of β-LG with higher oligomers of κ-CN that may be present in the TNB–κ–CN.

Tryptic Digestion. The mildly heated mixture of TNB–κ-CN and β-LG was readily converted into a mixture of peptides using trypsin, and the HPLC elution pattern (monitored by the total ion current) of the digest before (**Figure 3a**) and after (**Figure 3b**) reduction with BME showed many apparent similarities and some important differences. The reduced sample contained more material, particularly around 35–36.5 min (peaks 14 and 15), near 41 min (peak 20), and near 47 min (near peak 28). In contrast, peaks 1, 2, and 13 were present in **Figure 3a** but not **Figure 3b**, and peak 15 was present in **Figure 3b** but not **Figure 3a**. This was surprising because none of these peptides were expected to be altered by BME treatment as they contain no disulfide bonds. Peak 20, which was clearly visible in the reduced digest (**Figure 3b**) and was absent from **Figure 3a**, was identified as β-LG(149–162), which contains β-LG Cys^{160}.

Identification of Simple Peptides. Probing the MS/MS data from the HPLC run, using the sequence data of all *Bos taurus* proteins with the MS/MS pattern-matching tool Mascot (*38*),

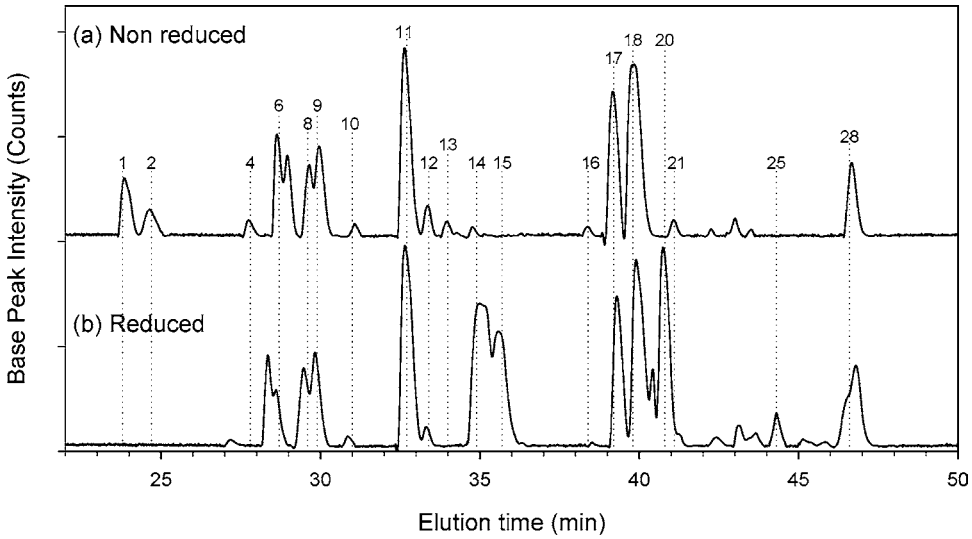

Figure 3. HPLC chromatogram of β-LG and κ-CN peptides from LC-MS of (**a**) nonreduced and (**b**) reduced tryptic digests. The elution time and the position of the identified and numbered β-LG and κ-CN tryptic peptides are listed in **Table 1**.

Table 1. Identity of β-LG and κ-CN Peptides Found by ESI-LC-MS/MS of Tryptic Digests of the Heated Model and Skim Milk Systems

peak	elution time (min)	observed masses (model system)		observed masses (milk system)		matched peptide	
		m/z[a]	mass[a]	*m/z*[a]	mass[a]	M[a]	peptide
1[c]	23.9	916.49^{+1}, 458.75^{+2}	915.49			915.47	β-LG(84–91)
2[c]	24.65	673.40^{+1}	672.39			672.38	β-LG(9–14)
2[c]	24.65	643.32^{+1}	642.31			642.30	κ-CN(17–21)
4[b,c]	27.2	419.25^{+2}	836.49	419.25^{+2}	836.41	836.47	β-LG(142–148)
6[b,c]	28.7	623.32^{+2}	1244.61	623.24^{+2}	1244.51	1244.58	β-LG(125–135)
8[b,c]	29.4	467.23^{+2}	932.56	467.23^{+2}	932.47	932.54	β-LG(1–8)
8[b,c]	29.5	903.59^{+1}, 452.30^{+2}	902.59	903.59^{+1}, 452.30^{+2}	902.50	902.56	β-LG(76–83)
9[b,c]	29.8	597.37^{+2}, 398.58^{+3}	1192.71	597.37^{+2}, 398.58^{+3}	1192.60	1192.67	β-LG(92–101)
10[b,c]	30.55	536.98^{+3}	1607.82	536.98^{+3}	1607.75	1607.84	κ-CN(98–111)
10[b,c]	30.9	674.43^{+1}	673.43	674.43^{+1}	673.36	673.42	β-LG(78–83)
11[b,c]	32.7	818.42^{+2}, 545.96^{+3}	1634.78	818.42^{+2}, 545.96^{+3}	1634.69	1634.77	β-LG(125–138)
12[c]	33.1	762.39^{+2}	1522.77	762.32^{+2}	1522.63	1522.71	κ-CN(35–46)
12[b,c]	33.35	533.32^{+2}	1064.62	533.32^{+2}	1064.51	1064.58	β-LG(92–100)
13[c]	33.85	697.75^{+3}	2090.23	697.75^{+3}, 523.50^{+4}	2090.23	2090.13	β-LG(84–101)
14[b,c]	34.7	601.03^{+3}	1800.07			1800.01	β-LG(76–91)
14	34.8	676.36^{+1}	675.35	676.28^{+1}	675.28	675.34	κ-CN(92–97)
15[b]	36.1	524.65^{+3}	1570.93			1570.87	β-LG(78–91)
17[c]	38.9	898.55^{+2}	1795.09	898.43^{+2}	1794.84	1795.00	κ-CN(71–86)
17[c]	39.1	975.57^{+1}, 488.30^{+2}	974.57	488.25^{+2}	974.48	974.55	κ-CN(27–34)
17[b,c]	39.35	626.38^{+2}	1250.72	626.38^{+2}	1250.60	1250.70	κ-CN(25–34)
18[b,c]	40.0	1157.16^{+2}, 771.77^{+3}	2312.26	1157.16^{+2}, 771.77^{+3}	2312.07	2312.25	β-LG(41–60)
18[b,c]	40.15	990.58^{+2}, 660.73^{+3}	1979.14	990.58^{+2}, 660.73^{+3}	1978.96	1979.08	κ-CN(69–86)
20[b]	40.8	829.89^{+2}	1657.84	829.89^{+2}	1657.67	1657.78	β-LG(149–162)
21[b,c]	41.3	1172.64^{+3}	3514.90	1172.54^{+3}	3514.63	3514.76	κ-CN(35–63)
25[b]	44.2			882.99^{+3}	2645.96	2646.19	β-LG B(102–124)
28[b,c]	46.7	1354.18^{+2}, 903.18^{+3}	2706.45	1354.18^{+2}, 903.18^{+3}	2706.20	2706.37	β-LG(15–40)
29[b,c]	48.4			1069.99^{+4}	4275.07	4275.26	β-LG(1–40)

[a] Monoisotopic masses. [b] Observed in reduced tryptic digests. [c] Observed in nonreduced tryptic digests.

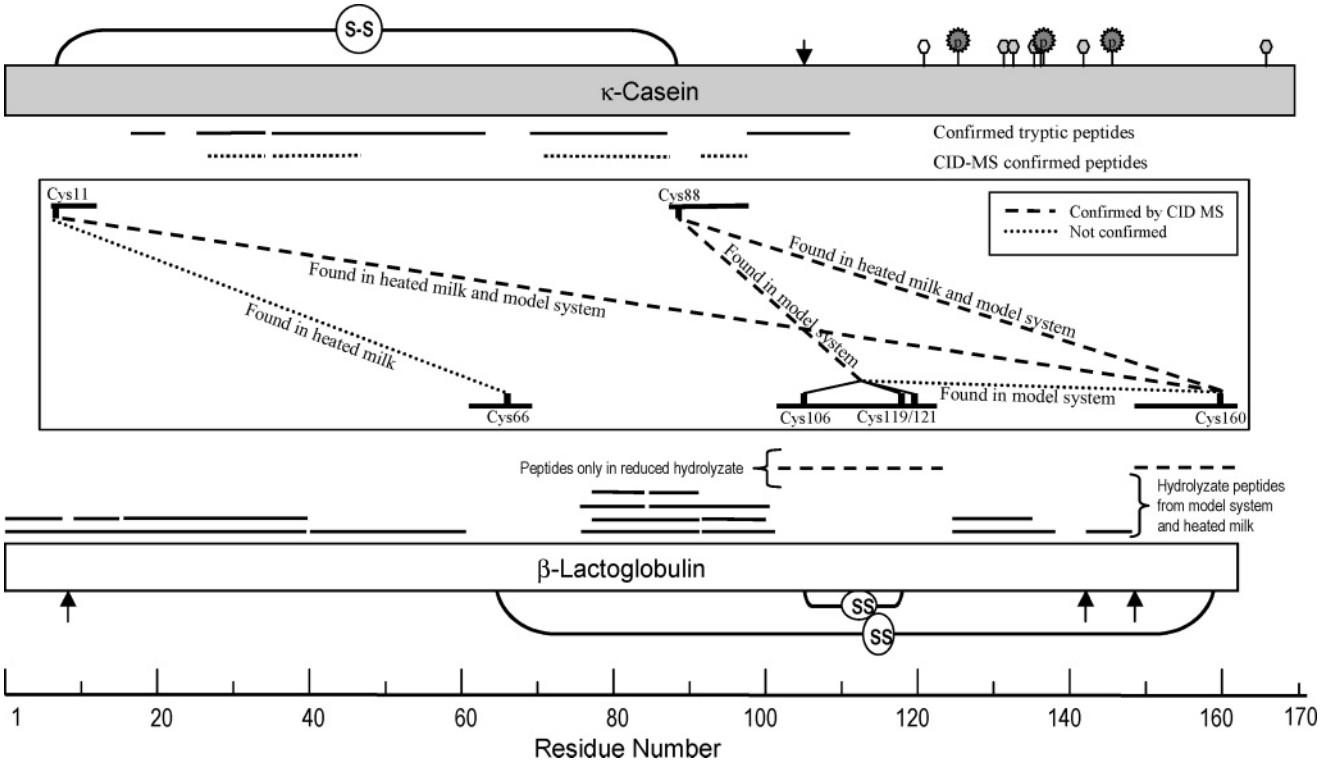

Figure 4. Diagram indicating the coverage of the identified peptides on the linear sequences of κ-CN and β-LG. The horizontal box lines represent the protein sequence, the lines over the boxes represent the peptides, and S–S indicates the presence of a disulfide bond. Arrows indicate the major proteolytic sites: the chymosin site for κ-CN and the rapid tryptic sites for β-LG. Potential glycosylation and phosphorylation sites are indicated in κ-CN according to the protocol of Farrell et al. (*51*).

identified a number of peptides. Those corresponding to the search rules for β-LG and κ-CN are shown in **Table 1**, and the identified β-LG and κ-CN peptides are shown in relation to the complete sequence of the proteins in **Figure 4**.

Comparing the present results with those obtained in an earlier study by our group (*31*) showed low concentrations of β-LG-(21−40) and a notable absence of β-LG(15−20) from the present set of results. Examination of the CID-MS spectra taken

Table 2. Potential Disulfide-Linked Peptides Found by Peptide Mapping of Tryptic Digests of the Heated Model and Skim Milk Systems

elution time (min)	observed masses (model system)		observed masses (milk system)		matched peptide	
	m/z[a]	mass[a]	*m/z*[a]	mass[a]	mass[a]	peptide
32.5	609.56^{+4}, 812.39^{+3}	2434.23	609.51^{+4}	2434.00	2434.09	**β-LG(149–162):κ-CN(11–16)**[b]
34.0	969.47^{+3}	2905.40	727.31^{+4}	2905.21	2905.30	**β-LG A(61–70):β-LG(149–162)**[b]
					2905.35	κ-CN(11–24):κ-CN(87–97)
34.8	713.12^{+4}, 950.48^{+3}	2848.46	713.05^{+4}, 950.40^{+3}	2848.20	2848.29	**β-LG(149–162):κ-CN(87–97)**[b]
35.4	695.35^{+4}, 926.78^{+3}	2777.38	695.28^{+4}	2777.09	2777.21	**β-LG A(61–69):β-LG(149–162)**[b]
39.5	966.74^{+4}, 1288.97^{+3}	3862.94			3862.73	**β-LG A(102–124):κ-CN(87–97)**[b,c]
42.9	1083.05^{+4}	4328.16			4327.97	β-LG A(102–124):β-LG(149–162)
46.7			865.02^{+3}	2592.05	2592.14	β-LG B(61–70):κ-CN(11–21)
46.7	565.08^{+5}, 705.85^{+4}	2819.39	705.79^{+4}	2819.12	2819.28	κ-CN(1–13):κ-CN(87–97)
48.35			907.44^{+3}	2719.31	2719.20	β-LG B(61–69):β-LG(149–162)

[a] Monoisotopic masses. [b] Peptide identity confirmed by MS/MS sequence analysis. [c] Potential peptide with internal disulfide link intact.

across peaks 14 and 15 in **Figure 3b** indicated that only small mass ions were present. Consequently, it was concluded that the slow cleavage rate of some trypsin-sensitive bonds did not give sufficient quantities of some peptides for reliable identification.

The peptides found in the hydrolysate that was not reduced and were identified as arising from β-LG covered most of the sequence (**Figure 4**). The exception was the peptides covering the regions that contained Cys residues, namely, β-LG(61−69) (Cys^{66}), β-LG(102−124) (Cys^{106}, Cys^{119}, and Cys^{121}), and β-LG(149−162) (Cys^{160}). One of these, β-LG(149−162) (elution time of 40.8 min, **Table 1**), was identified in the hydrolysate only after reduction. This suggests, on the basis of the result that heating β-LG gives products in which Cys^{160} is unbonded (*31*), that the presence of κ-CN alters the previously observed behavior.

The κ-CN peptides that were clearly tryptic peptides, that is, had an Arg or Lys residue at the C terminus, were identified as κ-CN(17−21), κ-CN(25−34), κ-CN(35−63), κ-CN(69−86), and κ-CN(98−111) (elution times of 24.65, 30.55, 39.35, 40.15, and 41.3 min, respectively; **Table 1**). There was also a series of peptides, κ-CN(27−34), κ-CN(35−46), κ-CN(71−86), and κ-CN(92−97), shown as dashed lines in **Figure 4**, that were unlikely to be tryptic peptides but may have been artifactual fragments from the tryptic peptides. [The sites for genetic variation and post-translational modification are between residues 120 and 169 (**Figure 4**), all of which would be on κ-CN(113−169), and did not cause any complications in the identification.] On the basis of the distribution of Lys and Arg residues, it might have been expected that κ-CN(1−10) and κ-CN(113−169) would have been observable in the nonreduced sample and that κ-CN(87−97) and κ-CN(11−16) would have been observable in the reduced sample. However, it is likely that κ-CN(1−10), with three acidic, one basic, four polar, and two hydrophobic residues, was not hydrophobic enough to attach to the HPLC column and that κ-CN(113−169) might have been hydrodynamically too large to enter the HPLC gel bead. Consequently, it is likely that all of the possible peptides that could be identified were identified, although it was a little surprising that κ-CN(87−97) was not identified in the reduced sample, but it is not very hydrophobic. Excluding the large glycosylated region, 74% of the sequence was accounted for.

Identification of Peptides Containing Disulfide Bonds. Peptide mapping using hypothetical peptide structures of β-LG and κ-CN (on the basis that only Lys-X and Arg-X bonds are cleaved by trypsin) against the MS data showed that some peptides fitted the following criteria: the mass of the peptide matched the calculated mass of the hypothetical disulfide-bonded peptide within 50 ppm; the peptide spectra showed typical carbon isotope patterns of a peptide of that number of carbon atoms; and the peptide was not present in the reduced sample.

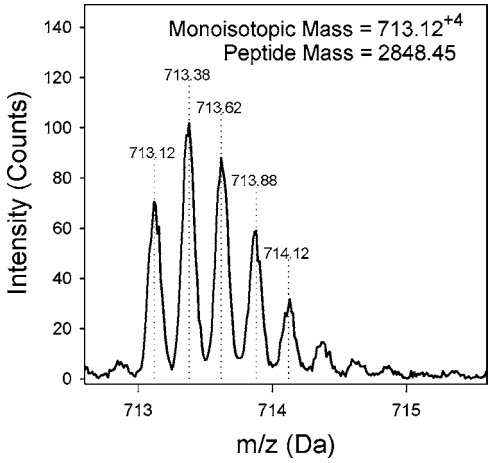

Figure 5. ESI-MS spectrum of possible disulfide-linked peptide β-LG(149–162): κ-CN(87–97) showing a +4 charged peptide.

As an example, the identification of the major peptide in a small peak that was observable in **Figure 3a** but not in **Figure 3b** with an elution time of ∼34.8 min is shown in detail. The measured and calculated masses are 2848.46 and 2848.29, respectively, and the *m*/*z* value for the quadruply charged ion is 713.12^{+4} (**Table 2**). **Figure 5** shows the measured mass distribution for this ion, with **Figure 6** showing the CID-MS spectrum that supports the assignment given [β-LG(149−162):κ-CN(87−97)]. The summarized information is shown in **Figure 7A**. Two other non-native heterologous disulfide-bonded peptides that were identified in the nonreduced tryptic digest [β-LG(149−162):κ-CN(11−16) and β-LG(102−124):κ-CN(87−97)] were analyzed similarly and are shown in **Figure 7B**,**C**.

To verify that the disulfide-bonded peptides identified were from β-LG and κ-CN, a BLAST similarity search (*54*) of the identified peptides was performed. This involved comparison of the peptide sequence against the Swiss-Prot protein database (*55*), which contains over 150,000 known and hypothetical protein sequences. The BLAST similarity search confirmed that the observed peptides are unique to β-LG and κ-CN.

Heated Milk. The centrifuged heated (90 °C for 20 min) reconstituted milk was separated into serum and colloidal phases. The essentially transparent serum contained ∼70% of the whey proteins and ∼30% of the κ-CN, as assessed by SDS-PAGE of reduced samples, in agreement with earlier work (*56*). As both serum and colloidal phases were analyzed and produced similar results, only the results of the serum phase are discussed. The identified simple (non-disulfide-bonded) peptides are shown in

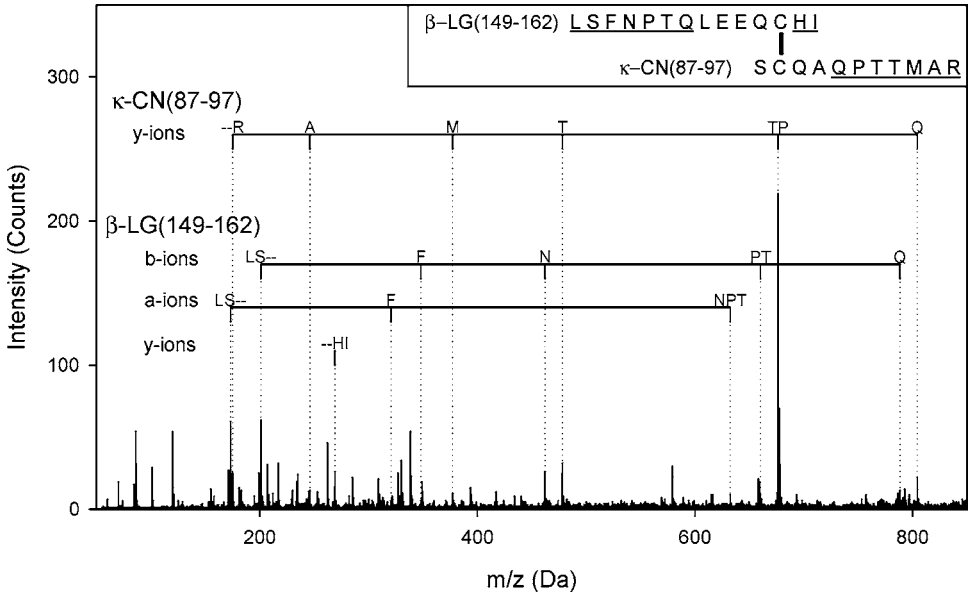

Figure 6. MS/MS spectrum of peptide 713.119^{+4} showing the assignment of the fragmentation pattern to the disulfide-bonded peptide β-LG(149–162):κ-CN(87–97) using SearchXLinks (*41*) and MS2Assign (*42*). Underlined amino acids indicate sequence matches with the MS/MS spectra.

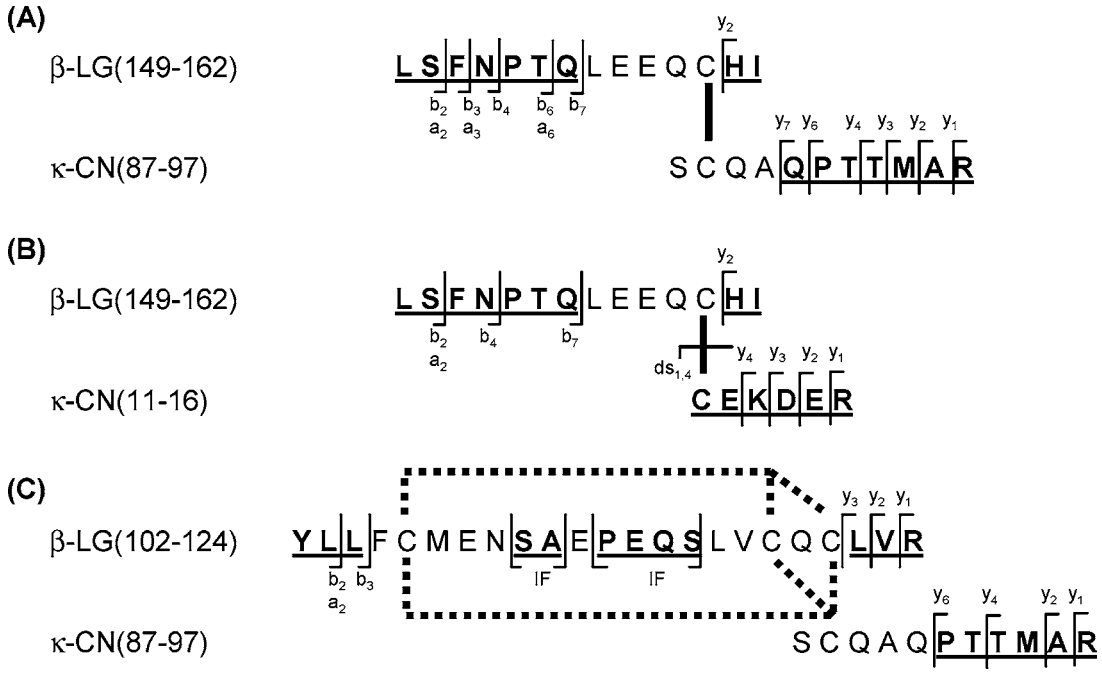

Figure 7. Peptide fragmentation results of CID-MS sequence analysis of three non-native disulfide-bonded peptides [β-LG(149–162):κ-CN(87–97); β-LG(149–162):κ-CN(11–16); β-LG(102–124–97)]. Underlined amino acids indicate sequence matches with the MS/MS spectra; IF = internal fragment.

Table 1. Most of the peptides that were identified in the hydrolysate of the model system were also found in the heated milk serum.

The potential disulfide-bonded peptides found in the hydrolysate of the heated milk serum are shown in **Table 2**. Of the disulfide-bonded peptides found in the heated model system, both β-LG(149−162):κ-CN(11−16) and β-LG(149−162):κ-CN(87−97) were found in the heated milk. Both of these peptides involve the Cys^{160} of β-LG. However, no evidence for the peptide β-LG(102−124):κ-CN(87−97) could be found in the heated milk, despite this peptide being readily located in the heated model system. In fact, no β-LG:κ-CN disulfide-bonded peptides involving the native free thiol of β-LG (Cys^{121}), or two other potential thiol groups (Cys^{106} and Cys^{119}), were found in the heated milk system. A peptide tentatively identified as β-LG B(61−70):κ-CN(11−21), involving Cys^{66} of β-LG, was identified in the heated milk but was not observed in the model system. The identity of this peptide has not been confirmed by sequencing using SearchXLinks (*41*).

It is unclear why the β-LG variant-specific disulfide-linked peptides in the heated milk system shown in **Table 2** do not occur in pairs. Of the possible peptides, only the peptides specific to β-LG A have been confirmed by CID-MS. One remote possibility is that under the digestion conditions used in this experiment, the aggregates containing β-LG A are more readily digested by trypsin and, hence, dominate in the LC-MS/MS run.

Identification of Potential Disulfide-Bonded Peptides. The ability to identify specific peptides in a hydrolysate could be valuable as a forensic tool. Examples might be the detection of

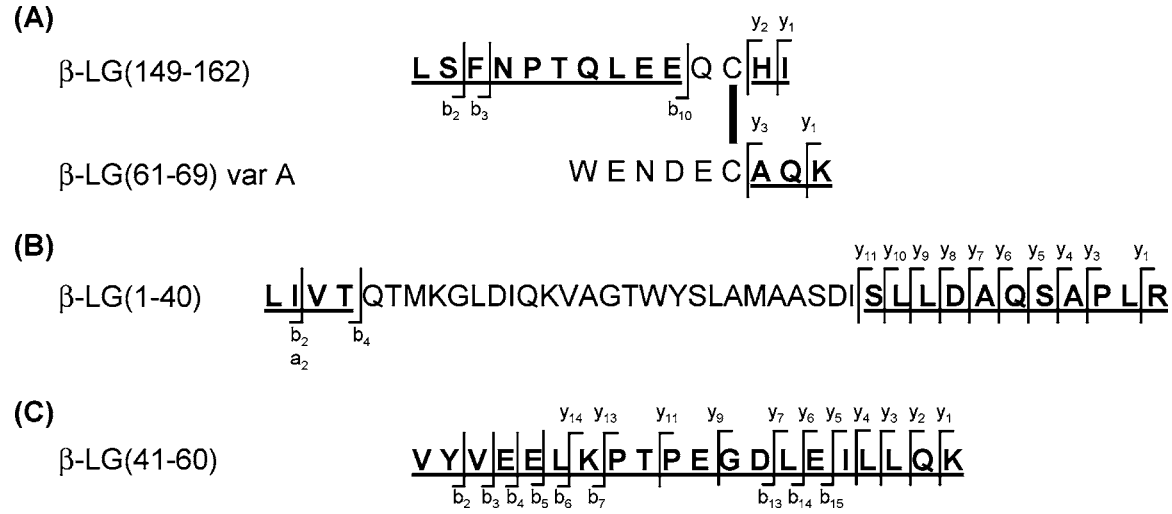

Figure 8. Peptide fragmentation results of CID-MS sequence analysis of three native peptides [β-LG A(61–69):β-LG(149–162); β-LG(1–40); β-LG-(41–60)]. Protein of origin and amino acid numbers are indicated. Underlined amino acids indicate sequence matches with the MS/MS spectra.

Table 3. Predicted Disulfide-Linked Peptides Found by Peptide Mapping of Tryptic Digests of the Heated Model and Skim Milk Systems Rejected Due to either Being Present in the Reduced Sample or the Peptide Spectra Not Showing the Typical Carbon Isotope Patterns of a Peptide

	observed masses (model system)		observed masses (milk system)		matched peptide	
elution time (min)	*m/z*[a]	mass[a]	*m/z*[a]	mass[a]	mass[a]	peptide
35.6			507.70^{+4}, 676.29^{+3}	2025.84	2025.85	β-LG A(61–70):κ-CN(11–16)
40.0	1157.16^{+2}, 771.77^{+3}	2312.26	579.04^{+4}, 771.70^{+3}	2312.07	2311.96	β-LG A(61–69):κ-CN(87–97)
					2312.25	**β-LG(41–60)**[b]
44.6			1340.66^{4}	5358.62	5358.63	β-LG(61–83):β-LG A(102–124)[c]
					5358.81	β-LG A(41–83):κ-CN(11–13)
44.7	509.53^{+4}, 679.05^{+3}	2034.12	509.73^{+4}, 678.98^{+3}	2033.91	2033.92	β-LG(149–162):κ-CN(11–13)
45.9			1330.39^{4}	5317.55	5317.56	β-LG A(61–69):κ-CN(1–34)
46.7			561.03^{+5}, 701.03^{+4}	2800.18	2800.31	β-LG A(101–124)[c]
48.35			856.03^{+5}, 1069.77^{+4}	4275.07	4274.92	β-LG A(102–135):κ-CN(11–13)[c]
					4275.26	**β-LG(1–40)**[b]

[a] Monoisotopic masses. [b] Peptide identity confirmed by MS/MS sequence analysis. [c] Potential peptide with internal disulfide link intact.

particular kinds of processing damage to proteins or the source of the protein. Such detection of low concentrations of a material with a specific mass and with an appropriate CID-MS pattern is almost facile using the powerful computational techniques that are now available and that were used in this study. These computational methods identified a number of possible peptides, but it was often necessary to apply further criteria to confirm the initial identification. Several examples arose in the present study.

Reducible β-LG:κ-CN Peptides. The peptide that eluted at 34.8 min (**Figure 3a**; **Table 2**, line 4) and that was not present in the BME-reduced peptide mix (**Figure 3b**) was tentatively identified as β-LG(149−162):κ-CN(87−97) by peptide mapping. Using the CID-MS data shown in **Figure 6** and summarized in **Figure 7A**, this was confirmed. Three other peptides that were not present in the reduced peptide mix, but were in the nonreduced sample, were analyzed similarly. The first two were β-LG(149−162):κ-CN(11−16) and β-LG A(102−124):κ-CN(87−97), as shown in parts **B** and **C**, respectively, of **Figure 7**. The third peptide was found to be a peptide with the native disulfide-bonded peptides that incorporated Cys^{66} and Cys^{160} and is shown in **Figure 8A**. It is not surprising to find this peptide because the PAGE pattern (**Figure 2**) indicated that there was an amount of monomeric (and probably, but not necessarily, native) β-LG in the model system.

Two other minor components were sequenced to check their identity. Although they seemed to be present in the reduced sample, they may have been there at low concentrations and had escaped reduction by BME or had reoxidized. The results of the sequence analysis of the peptides in the nonreduced sample showed that the peptides (**Figure 8B**,**C**) were in fact simple peptides and not disulfide-bonded dipeptides as implied by the computer-generated information—see lines 2 and 3 (40.0 min) and lines 9 and 10 (48.35 min) in **Table 3**. The potential disulfide-bonded peptide β-LG A(61−69):κ-CN(87−97) had a calculated mass of 2311.96, compared with a measured mass of 2312.07, and the calculated mass for β-LG(41−60) was 2312.25. The second potential disulfide-bonded peptide [β-LG A(102−135):κ-CN(11−13)] had a calculated mass of 4274.92, compared with a measured mass of 4275.07, and the calculated mass for β-LG(1−40) was 4275.26.

In another, more difficult, assignment, the software packages identified two different potential disulfide-bonded pairs of peptides that had very similar masses: 2905.35 for κ-CN(11−24):κ-CN(87−97) and 2905.30 for β-LG A(61−70):β-LG(149−162), and a measured mass of 2905.40 (**Table 2**, lines 2 and 3). In both cases, the CID-MS spectra (summarized in **Figure 9A**,**B**) were consistent with both of the suggested peptides. The "identified" b_1 fragment ion (**Figure 9A**) in reality is unstable and unlikely to have been present. The probability of κ-CN(11−24) being present in observable quantities is also low because cleavage at Arg^{16} is likely to be faster than cleavage at Lys^{24}. Taken together with the relatively low number of "identified" fragments for one of the three likely native internal

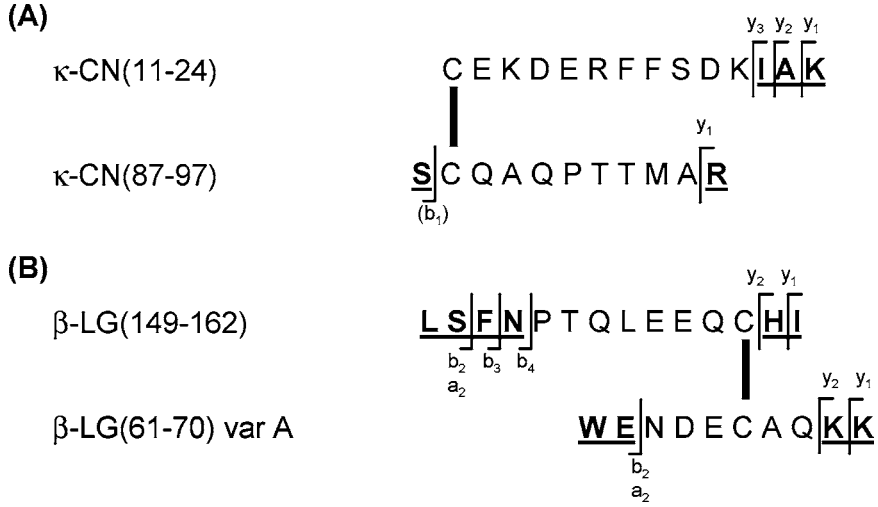

Figure 9. Peptide fragmentation results of CID-MS sequence analysis of one peptide with a monoisotopic mass of 2905.40, showing two possible native disulfide-bonded peptides and their match to the MS/MS spectra. Protein of origin and amino acid numbers are indicated. Underlined amino acids indicate sequence matches with the MS/MS spectra.

κ-CN disulfide bonds as opposed to one of the two major native disulfide-bonded β-LG A peptides and with expected trypsin sites (Lys^{60}, Lys^{70}, and Lys^{148}, **Figure 4**) cleaved, the peptide is probably β-LG A(61−70):β-LG(149−162).

When CID-MS data are matched to disulfide-linked peptide sequences, it would be advantageous if statistical analysis techniques could be applied to determine the significance of a sequence match. The MS/MS pattern-matching tool Mascot (*38*) uses a probability-based scoring algorithm for assessing the probability of a match for simple peptides. This is a combination of empirically determined weightings for the CID-MS fragmentation frequency and the probability that the observed match is a random event. SearchXLinks (*41*) uses a similar scoring system. Due to the limited information on CID-MS fragment frequency for disulfide-linked peptides, the SearchXLinks scoring system has assigned weightings on an ad hoc basis. This approach is flawed in that the peptide sequence itself can affect the CID-MS fragmentation frequency. An example of this is the CID-MS fragmentation pattern observed for the peptide β-LG(149−162):κ-CN(87−97) shown in **Figure 6**. Typically the smaller y ions dominate the CID-MS fragmentation. However, in this case the most prominent fragment observed was the y_6 ion (labeled TP in **Figure 6**) from the κ-CN peptide SCQAQPTTMAR. This is due to the effect of the Pro^{92} residue in the peptide, which will dominate CID-MS spectra (*57*). It was concluded that, despite the power of high-resolution liquid chromatography coupled with mass spectrometry with collision-induced dissociation and the associated software, it is necessary as well to apply experience-based logic to correctly identify the peptide products.

DISCUSSION

Heating TNB-κ-CN and β-LG A at 60 °C gave a more complex set of larger disulfide-bonded protein products than expected (**Figure 2**). Although some of the unusual pattern may have been partially a consequence of silver staining, which generally does not stain in proportion to the quantity of protein, it is likely that the derivatized κ-CN was more heterogeneous than anticipated. It is possible that the reduced κ-CN had been partially oxidized during preparation and prior to reaction with DTNB, thus generating disulfide-bonded κ-CN dimers and trimers. As a consequence, the β-LG and TNB−κ-CN model system was not as simple as expected. Nevertheless, a sufficient concentration of inter-protein κ-CN:β-LG disulfide bonds was formed to identify the novel bonding, and this model system was considerably less complex than milk.

Heating pure β-LG A and B at ∼60 °C (*58*−*60*) gave circular dichroism (CD) patterns that were consistent with a loss of helical structure. Edwards et al. (*61*) used H/D exchange to expand the study of Belloque and Smith (*62*) and showed that the FGH strand region of β-LG was very stable. These regions are shown in Figures 1 and 6 of the accompanying paper (*31*). These and other studies show that Cys^{121} is in the most stable region of β-LG and becomes reactive only after it has been exposed by changed environmental conditions. The recent studies of Croguennec et al. (*63*, *64*) show that, when β-LG is heated with *N*-ethylmaleimide (NEM) at 85 °C, two mono-NEM derivatives are formed, one with Cys^{119} blocked and the other with Cys^{121} blocked. This shows that disulfide interchange at 85 °C in this hydrophobic environment within a protein pocket is faster than reaction with the relatively small hydrophobic NEM molecule. Consequently, it might be expected that Cys^{119} and Cys^{121} would be the likely candidates for reaction with the reactive TNB−Cys residues of κ-CN. The present results partially support this expectation, as peptides involving Cys^{119} or Cys^{121} and the Cys residues of κ-CN were observed in the model system (**Table 2**; **Figure 7C**). However, further intramolecular thiol−disulfide exchange reactions in heated β-LG must precede intermolecular interactions as peptides involving Cys^{160} and the Cys residues of κ-CN were also observed as major products in the model system (**Table 2**; **Figure 7A**,**B**). On the basis of the results of Croguennec et al. (*63*, *64*), the peptide in **Figure 7C** would probably have κ-CN Cys^{88} linked to Cys^{119} or Cys^{121} but not to Cys^{106}.

In contrast, the heated milk system did not support this expectation as no β-LG:κ-CN peptides involving the native free thiol of β-LG (Cys^{121}), or two other potential thiol groups (Cys^{119} and Cys^{106}), were found in the heated milk system. Only β-LG:κ-CN peptides involving Cys^{160} and Cys^{66} were observed (**Table 2**; **Figure 7A**,**B**), and these two residues are linked by a disulfide bond in the native β-LG protein (*32*). This clearly indicates that intramolecular thiol−disulfide exchange reactions in β-LG precede the intermolecular thiol−disulfide exchange reactions. The reasons why the disulfide-bonded products in the model and milk systems are different are not clear; however, factors such as the heating conditions, the nature of the reactions

(oxidative interaction compared with thiol−disulfide interchange reactions), and the difference between micellar and nonmicellar κ-CN may influence the reaction pathways. The structure of κ-CN and those of its disulfide-bonded polymers are not well-defined: the possible monomer structure was reviewed in 1998 (*65*); the native polymeric state was investigated by Rasmussen et al. (*66*) and Farrell's group (*67*−*69*); the effects of heat on β-LG and κ-CN were investigated and discussed by Cho et al. (*19*). Native bovine κ-CN, which is mostly polymeric, contained Cys^{11}−Cys^{88}, Cys^{11}−Cys^{11}, and Cys^{88}−Cys^{88} disulfide bonds with an approximately random distribution of the disulfide bonds (*66*), and heating κ-CN (*19*, *67*−*69*) altered the size distribution of the κ-CN polymers.

Interestingly, caprine κ-CN differs from bovine κ-CN by having three Cys residues (Cys^{10}, Cys^{11}, and Cys^{88}) instead of two, and the sequence and the predicted structure (*70*) were very similar for the first 112 residues. No doubt, in the milk environment, the Cys residues would all be disulfide bonded, probably to one another and within the κ-CN polymers. Consequently, some structural features common to the κ-CN of each species affect the heat-induced reaction that leads β-LG Cys^{160} to react with κ-CN Cys^{88}. Any sensible native structure that could be applied to the κ-CN of both species would place most of the Cys residues in close proximity and close to the micellar surface if the κ-CN polymer is associated with the micelle, because there are only four hydrophobic residues in the N-terminal 22 residues. The region close to Cys^{88} is slightly polar and is about six residues away from the beginning of the unique chymosin-specific proteolysis site (*70*) available to an enzyme, and any other protein.

An examination of heat treatment of a model caprine milk system (*33*) gave a similar result to our heated model and milk systems; namely, a novel β-LG Cys^{160} and κ-CN Cys^{88} disulfide bond was found. However, no β-LG and κ-CN disulfide-bonded peptides involving other sulfhydryl groups were observed, although it was stated that these may exist at lower levels than that of the identified peptide. Taken altogether, these results give us confidence in the concept that heat treatment of β-LG allows the thiol of Cys^{121} to reversibly interchange with other Cys residues in β-LG, and for Cys^{121} to become part of a disulfide bond (*28*, *31*), and that the concept can be usefully applied to interactions of β-LG with κ-CN (*19*) and α-lactalbumin (*71*). When Cys^{160} of β-LG is the thiol and is not linked to Cys^{66}, it can productively react with the disulfide bonds of the native κ-CN, which is associated with the casein micelle at near-physiological conditions, to give stable κ-CN−whey protein aggregates that are the basis for generating processed milks with predictably different characteristics.

ABBREVIATIONS USED

2D, two-dimensional; amu, atomic mass units; BME, β-mercaptoethanol; CID, collision-induced dissociation; κ-CN, κ-casein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; β-LG, β-lactoglobulin; MS, mass spectrometry; MS-MS, tandem mass spectrometry; NEM, *N*-ethylmaleimide; Q-TOF, quadrupole/time of flight; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TNB−κ-CN, 5-thio-2-nitrobenzoic acid disulfide-bonded derivative of κ-CN.

ACKNOWLEDGMENT

We thank Carmen Norris, Keri Anne Higgs, and Julian Reid for assistance with mass spectral analysis in the early part of this project and Wayne Thresher for supplying the derivatized κ-casein.

LITERATURE CITED

(1) Dannenberg, F.; Kessler, H. G. Reaction kinetics of the denaturation of whey proteins in milk. *J. Food Sci.* **1988**, *53*, 258−263.
(2) Kessler, H. G.; Beyer, H. J. Thermal denaturation of whey proteins and its effect in dairy technology. *Int. J. Biol. Macromol.* **1991**, *13*, 165−173.
(3) Anema, S. G.; McKenna, A. B. Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. *J. Agric. Food Chem.* **1996**, *44*, 422−428.
(4) Bikker, J. F.; Anema, S. G.; Li, Y.; Hill, J. P. Thermal denaturation of β-lactoglobulin A, B and C in heated skim milk. *Milchwissenschaft* **2000**, *55*, 609−613.
(5) Slatter, W. L.; van Winkle, Q. An electrophoretic study of the protein in skimmilk. *J. Dairy Sci.* **1952**, *35*, 1083−1093.
(6) Della Monica, E. S.; Custer, J. H.; Zittle, C. A. Effect of calcium chloride and heat on mixtures of β-lactoglobulin and casein. *J. Dairy Sci.* **1958**, *41*, 465−471.
(7) Zittle, C. A.; Thompson, M. P.; Custer, J. H.; Cerbulis, J. κ-Casein-β-lactoglobulin interaction in solution when heated. *J. Dairy Sci.* **1962**, *45*, 807−810.
(8) Sawyer, W. H.; Coulter, S. T.; Jenness, R. Role of sulfhydryl groups in the interaction of κ-casein and β-lactoglobulin. *J. Dairy Sci.* **1963**, *46*, 564−565.
(9) Sawyer, W. H. Complex between β-lactoglobulin and κ-casein. A review. *J. Dairy Sci.* **1969**, *52*, 1347−1355.
(10) McKenzie, H. A. β-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1971; pp 257−330.
(11) Hill, A. R. The β-lactoglobulin-κ-casein complex. *Can. Inst. Food Sci. Technol. J.* **1989**, *22*, 120−123.
(12) Jang, H. D.; Swaisgood, H. E. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.* **1990**, *73*, 900−904.
(13) Corredig, M.; Dalgleish, D. G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res. Int.* **1996**, *29*, 49−55.
(14) Corredig, M.; Dalgleish, D. G. The binding of α-lactalbumin and β-lactoglobulin to casein micelles in milk treated by different heating systems. *Milchwissenschaft* **1996**, *51*, 123−127.
(15) Corredig, M.; Dalgleish, D. G. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.* **1999**, *9*, 233−236.
(16) Euber, J. R.; Brunner, J. R. Interaction of κ-casein with immobilized β-lactoglobulin. *J. Dairy Sci.* **1982**, *65*, 2384−2387.
(17) Anema, S. G.; Li, Y. Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *J. Agric. Food Chem.* **2003**, *51*, 1640−1646.
(18) Anema, S. G.; Li, Y. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *J. Dairy Res.* **2003**, *70*, 73−83.
(19) Cho, Y.; Singh, H.; Creamer, L. K. Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system. *J. Dairy Res.* **2003**, *70*, 61−71.
(20) McKenzie, G. H.; Norton, R. S.; Sawyer, W. H. Heat-induced interaction of β-lactoglobulin and κ-casein. *J. Dairy Res.* **1971**, *38*, 343−351.
(21) Swaisgood, H. E. Chemistry of the caseins. In *Advanced Dairy Chemistry−1. Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 63−110.
(22) Smits, P.; van Brouwershaven, J. H. Heat-induced association of β-lactoglobulin and casein micelles. *J. Dairy Res.* **1980**, *47*, 313−325.

(23) Singh, H.; Creamer, L. K. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *J. Dairy Res.* **1991**, *58*, 269−283.
(24) Vasbinder, A. J.; de Kruif, C. G. Casein-whey protein interactions in heated milk: the influence of pH. *Int. Dairy J.* **2003**, *13*, 669−677.
(25) Anema, S. G.; Lee, S. K.; Lowe, E. K.; Klostermeyer, H. Rheological properties of acid gels prepared from heated pH-adjusted skim milk. *J. Agric. Food Chem.* **2004**, *52*, 337−343.
(26) Anema, S. G.; Lowe, E. K.; Lee, S. K. Effect of pH at heating on the acid-induced aggregation of casein micelles in reconstituted skim milk. *Lebensm.-Wiss. -Technol.* **2004**, *37*, 779−787.
(27) Anema, S. G.; Lowe, E. K.; Li, Y. Effect of pH on the viscosity of heated reconstituted skim milk. *Int. Dairy J.* **2004**, *14*, 541−548.
(28) Manderson, G. A.; Hardman, M. J.; Creamer, L. K. Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *J. Agric. Food Chem.* **1998**, *46*, 5052−5061.
(29) Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.
(30) Schokker, E. P.; Singh, H.; Pinder, D. N.; Norris, G. E.; Creamer, L. K. Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *Int. Dairy J.* **1999**, *9*, 791−800.
(31) Creamer, L. K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; van Wanroij, M.; Lowe, E. K.; Anema, S. G.; Boland, M. J.; Jiménez-Flores, R. Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 7660−7668.
(32) Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure* **1997**, *5*, 481−495.
(33) Henry, G.; Molle, D.; Morgan, F.; Fauquant, J.; Bouhallab, S. Heat-induced covalent complex between casein micelles and β-lactoglobulin from goat's milk: identification of an involved disulfide bond. *J. Agric. Food Chem.* **2002**, *50*, 185−191.
(34) Anema, S. G.; Stanley, D. J. Heat-induced, pH-dependent behaviour of protein in caprine milk. *Int. Dairy J.* **1998**, *8*, 917−923.
(35) Morgan, F.; Micault, S.; Fauquant, J. Combined effects of whey protein and α_{s1}-casein genotype on the heat stability of goat milk. *Int. J. Dairy Technol.* **2001**, *54*, 64−68.
(36) Thresher, W. C. Interactions of β-lactoglobulin variants with κ-casein and DTNB: formation of a covalent complex. In *Milk Protein Polymorphism*; IDF Special Issue 9702; International Dairy Federation: Brussels, Belgium, 1997; pp 138−145.
(37) Gillece-Castro, B. L.; Stults, J. T. Peptide characterisation by mass spectrometry. *Methods Enzymol.* **1996**, *270*, 427−448.
(38) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551−3567.
(39) Gorman, J. J.; Wallis, T. P.; Pitt, J. J. Protein disulfide bond determination by mass spectroscopy. *Mass Spectrom. Rev.* **2002**, *21*, 183−216.
(40) Schurch, S.; Schaller, J.; Kampfer, U.; Kuhn-Nentwig, L.; Nentwig, W. Neurotoxic peptides in the multicomponent venom of the spider *Cupiennius salei.* Part II. Elucidation of the disulfide-bridge pattern of the neurotoxic peptide CSTX-9 by tandem mass spectrometry. *Chimia* **2001**, *55*, 1063−1066.
(41) Wefing, S.; Schnaible, V.; Hoffmann, D. "SearchXLinks" http://www.searchxlinks.de/; Center of Advanced European Studies and Research (Caesar): Bonn, Germany, 2001.
(42) Schilling, B.; Row: R. H.; Gibson, B. W.; Guo, X.; Young, M. M. MS2Assign, automated assignment and nomenclature of tandem mass spectra of chemically cross-linked peptides. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 834−850.
(43) Mailliart, P.; Ribadeau-Dumas, B. Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *53*, 743−745, 752.
(44) Rasmussen, L. K.; Petersen, T. E. Purification of disulphide-linked α_{s2}- and κ-casein from bovine milk. *J. Dairy Res.* **1991**, *58*, 187−193.
(45) Havea, P.; Singh, H.; Creamer, L. K.; Campanella, O. H. Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *J. Dairy Res.* **1998**, *65*, 79−91.
(46) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(47) Rabilloud, T. Silver staining of 2-D electrophoresis gels. In *Methods in Molecular Biology*; Link, A. J., Ed.; Humana Press: Totowa, NJ, 1999; pp 297−301.
(48) Anon Analyst QS/BioAnalyst. Applied Biosystems/MDS SCIEX, 2001.
(49) Dalgalarrondo, M.; Chobert, J. M.; Dufour, E.; Bertrand-harb, C.; Dumont, J. P.; Haertle, T. Characterization of bovine β-lactoglobulin B tryptic peptides by reversed-phase high-performance liquid chromatography. *Milchwissenschaft* **1990**, *45*, 212−216.
(50) Chobert, J.-M.; Dalgalarrondo, M.; Dufour, E.; Bertrand-Harb, C.; Haertlé, T. Influence of pH on the structural changes of β-lactoglobulin studied by tryptic hydrolysis. *Biochim. Biophys. Acta* **1991**, *1077*, 31−34.
(51) Farrell, H. M., Jr.; Jimenez-Flores, R.; Bleck, G. T.; Brown, E. M.; Butler, J. E.; Creamer, L. K.; Hicks, C. L.; Hollar, C. L.; Ng-Kwai-Hang, K. F.; Swaisgood, H. E. Nomenclature of the proteins of cows' milk—sixth revision. *J. Dairy Sci.* **2004**, *87*, 1641−1674.
(52) Green, M. R.; Pastewka, J. V. Molecular weights of three mouse milk caseins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and κ-like characteristics of a fourth casein. *J. Dairy Sci.* **1976**, *59*, 1738−1745.
(53) Creamer, L. K.; Richardson, T. Anomalous behavior of bovine αs1- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers. *Arch. Biochem. Biophys.* **1984**, *234*, 476−486.
(54) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403−410.
(55) Boeckmann, B.; Bairoch, A.; Apweiler, R.; Blatter, M. C.; Estreicher, A.; Gasteiger, E.; Martin, M. J.; Michoud, K.; O'Donovan, C.; Phan, I.; Pilbout, S.; Schneider, M. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res.* **2003**, *31*, 365−370.
(56) Anema, S. G.; Klostermeyer, H. Heat-induced pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100 °C. *J. Agric. Food Chem.* **1997**, *45*, 1108−1115.
(57) Kinter, M.; Sherman, N. E. *Protein Sequencing and Identification using Tandem Mass Spectrometry*; Wiley-Interscience: New York, 2000.
(58) Qi, X. L.; Holt, C.; McNulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochem. J.* **1997**, *324*, 341−346.
(59) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303−4308.
(60) Manderson, G. A.; Creamer, L. K.; Hardman, M. J. Effect of heat treatment on the circular dichroism spectra of bovine β-lactoglobulin A, B, and C. *J. Agric. Food Chem.* **1999**, *47*, 4557−4567.
(61) Edwards, P. J. B.; Jameson, G. B.; Palmano, K. P.; Creamer, L. K. Heat-resistant structural features of bovine β-lactoglobulin A revealed by NMR H/D exchange observations. *Int. Dairy J.* **2002**, *12*, 331−344.

(62) Belloque, J.; Smith, G. M. Thermal denaturation of β-lactoglobulin. A ^{1}H NMR study. *J. Agric. Food Chem.* **1998**, *46*, 1805−1813.

(63) Croguennec, T.; Bouhallab, S.; Mollé, D.; O'Kennedy, B. T.; Mehra, R. Stable monomeric intermediate with exposed Cys-119 is formed during heat denaturation of β-lactoglobulin. *Biochem. Biophys. Res. Commun.* **2003**, *301*, 465−471.

(64) Croguennec, T.; O'Kennedy, B. T.; Mehra, R. Heat-induced denaturation/aggregation of β-lactoglobulin A and B: kinetics of the first intermediates formed. *Int. Dairy J.* **2004**, *14*, 399−409.

(65) Creamer, L. K.; Plowman, J. E.; Liddell, M. J.; Smith, M. H.; Hill, J. P. Micelle stability: κ-casein structure and function. *J. Dairy Sci.* **1998**, *81*, 3004−3012.

(66) Rasmussen, L. K.; Hojrup, P.; Petersen, T. E. The multimeric structure and disulfide-bonding pattern of bovine κ-casein. *Eur. J. Biochem.* **1992**, *207*, 215−222.

(67) Groves, M. L.; Dower, H. J.; Farrell, H. M., Jr. Reexamination of the polymeric distributions of κ-casein isolated from bovine milk. *J. Protein Chem.* **1992**, *11*, 21−28.

(68) Groves, M. L.; Wickham, E. D.; Farrell, H. M., Jr. Environmental effects on disulfide bonding patterns of bovine κ-casein. *J. Protein Chem.* **1998**, *17*, 73−84.

(69) Farrell, H. M., Jr.; Wickham, E. D.; Groves, M. L. Environmental influences on purified κ-casein:disulfide interactions. *J. Dairy Sci.* **1998**, *81*, 2974−2984.

(70) Plowman, J. E.; Creamer, L. K.; Liddell, M. J.; Cross, J. J. Solution conformation of a peptide corresponding to bovine κ-casein B residues 130−153 by circular dichroism spectroscopy and ^{1}H-nuclear magnetic resonance spectroscopy. *J. Dairy Res.* **1997**, *64*, 377−397.

(71) Hong, Y.-H.; Creamer, L. K. Changed protein structures of bovine β-lactoglobulin B and α-lactalbumin as a consequence of heat treatment. *Int. Dairy J.* **2002**, *12*, 345−359.

Received for review May 30, 2004. Revised manuscript received September 19, 2004. Accepted September 20, 2004. This work was supported by funding from the New Zealand Foundation for Research, Science and Technology, Contracts DRIX0001 and DRIX0201, the CSU Agricultural Research Initiative, and Dairy Management Inc.

JF0491254