JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Specificity of Disulfide Bond Formation during Thermal Aggregation in Solutions of *β*-Lactoglobulin B and *κ*-Casein A

YOAV D. LIVNEY AND DOUGLAS G. DALGLEISH*

Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Heat-induced (90 °C, 10 min, pH 6.7) intermolecular disulfide bond formation in 1:1 mixtures of *β*-lactoglobulin B (*β*-Lg) and *κ*-casein A (*κ*-CN) was studied by enzymatic digestion with trypsin or glu-C, reverse-phase HPLC, and MALDI-TOF-MS. Observed masses were compared to theoretically calculated masses of disulfide-bonded peptide dimers and trimers, and the number of different masses matching peptide combinations involving each bond was used as a measure of confidence of identification. The *β*-Lg cysteine residues 121 or 119 were involved in bonds with both cysteines of *κ*-CN and all cysteines of *β*-Lg. This agrees with the supposed initiatory role of *β*-C121 in heat-induced SH/SS interchange. The largest numbers of matches corresponded to bonds linking *β*-C119/C121 with *κ*-C11 or with *β*-C66. Multiple matches were recorded for *β*-C119/C121 bonding with *β*-C119/C121, with *β*-C160, or with *κ*-C88. However, *β*-C106 was observed only in bonds with *β*-C119/C121 and did not appear to bond to *κ*-CN, suggesting it remains buried in the core of the protein.

KEYWORDS: Milk proteins; *β*-lactoglobulin; *κ*-casein; heat-induced aggregation; disulfide bonds; thiol; cysteine; cystine; mass spectrometry

INTRODUCTION

One of the most important heat-induced interactions occurring during milk processing is that between *β*-lactoglobulin (*β*-Lg) and *κ*-casein (*κ*-CN). It is central to the improved acid gelation of heated milk (*1*−*3*), which is crucial in yogurt production. Conversely, it dramatically decreases the rennetability of milk by creating an alternative steric barrier to micelle aggregation (*4*), which makes it very difficult to use heated milk for cheesemaking.

In milk, the globular protein *β*-Lg (3.2 g/L) normally exists as a dimer (*5*). Each monomer (M_w = 18.3 kDa) is made of 162 amino acids and contains two cystines, or intramolecular disulfide bridges (C106−C119, C66−C160), and one free cysteine (C121) (*5*, *6*). Its native tertiary structure is a *β*-barrel composed of nine strands (A−I), and there is a three-turn α-helix on the surface (*6*−*8*). The free cysteine is on *β*-strand H and is buried underneath the α-helix, ∼9 Å from the dimer interface and close to the C106−C119 disulfide bridge, which connects strands G and H. The other disulfide bridge connects the C terminus to strand D, at the surface of the globular protein (*6*). In several differential scanning calorimetry (DSC) studies, the reported denaturation temperature (temperature of maximum heat flow) of *β*-Lg at pH 6.6 ranges between 72 and 79 °C (*9*). During heating at neutral pH, the native dimer first dissociates into native monomers (*10*), which become reactive by partial unfolding of the EFGH *β*-strand region and the α-helix, leading to exposure of the free sulfhydryl group and the adjacent disulfide bond. The now solvent-accessible thiol group (*10*, *11*) starts a chain of sulfhydryl−disulfide interchange reactions, which may be analogous to radical polymerization (*12*), leading to aggregation (*13*−*15*).

κ-CN [3−4 g/L in milk (*16*)] is the protective colloid forming a hairy layer that stabilizes the casein micelles (*17*). It is made of 169 amino acids (M_w = 19.0 kDa) and has two cysteine residues in positions 11 and 88, which do not form an intramolecular disulfide bond (*16*). It is a calcium-insensitive glycoprotein, of an extremely amphiphilic nature, with a hydrophobic N-terminal part and a hydrophilic glycosylated C-terminal part, the cleavage of which by rennet, at the F105−M106 position, initiates aggregation and curd formation (*16*, *18*). When isolated in the native state from milk without the use of disulfide-bond-breaking reagents such as 2-mercapto-ethanol, *κ*-CN shows a range of intermolecularly disulfide-linked oligomers having molecular weights that appear to be rather evenly distributed across a range from monomer to octamer and above (*19*, *20*). Rasmussen et al. (*21*) showed that both C11 and C88 were involved in the intermolecular bond formation, with an apparently random distribution of bonds (C88−C88, C11−C11, and C11−C88).

Heat induces sulfhydryl−disulfide interchange interactions of *β*-Lg and *κ*-CN, leading to their aggregation (*22*). However, it has not been established which of the five cysteines of bovine *β*-Lg and the two of *κ*-CN participate in the reaction and whether there is any steric specificity to the interaction. In goat's milk, one of the disulfide bonds involved in the binding of *β*-Lg to casein micelles was identified as *κ*-C88−*β*-C160, although the

* Corresponding author [telephone (519) 824-4120, ext. 56929; fax (519) 824-6631; e-mail ddalglei@uoguelph.ca].

10.1021/jf049955h CCC: $27.50 © 2004 American Chemical Society
Published on Web 07/28/2004

authors state it was possibly only one of several different bonds occurring (*23*).

The current study aimed to identify whether specific intermolecular disulfide bonds form during heating of model solutions of mixed β-Lg and κ-CN, at conditions (90 °C, 10 min, pH 6.7) typical of industrial heat treatments. The ratio of 1:1 was chosen, as it is similar to the ratio of these proteins in milk. The κ-CN concentration was chosen to be ~10 times higher than its critical micellization concentration [cmc = 0.53 mg/mL (*16*)] so that most of the κ-CN would be in micellar aggregate form, thus presumably exposing to the water its hydrophilic parts, which would normally be exposed when it is covering natural casein micelles. We used reverse-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses of both tryptic and glu-C digests of the heated mixed protein solutions to allow determination of the specificity of disulfide interactions at the molecular level and to identify the various cysteine residues involved.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI, Alacen 895) was obtained from New Zealand Milk Proteins (USA) Inc. (Lemoyne, PA). Bis-tris-propane (99%), 2-mercaptoethanol (98%), sinapinic acid, equine cytochrome *c* (97%), adrenocorticotropic hormone (ACTH) clipped 18−39 (99%), α-cyano-4-hydroxycinnamic acid, iodoacetamide of 97% purity, trypsin (type T-0303), and glu-C endoproteinase (P6181) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada); ammonium acetate (HPLC grade), ammonium bicarbonate (certified), acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) (99.8%) were obtained from Fisher Scientific (Nepean, ON, Canada).

Methods. *Protein Purification.* κ-CN (Var A) was isolated from fresh milk (in the presence of 2-mercaptoethanol) according to the preparative method described by Dalgleish (*24*), which had been based on the analytical method of Hollar et al. (*25*). For practical reasons we used monomeric κ-CN, which was reduced by mercaptoethanol to facilitate its isolation from other caseins. This is a limitation of this model system, as in casein micelles some of the κ-CN exists in disulfide-bonded oligomers, and the significance of this difference remains to be determined. Residual mercaptoethanol was exhaustively dialyzed before the protein was freeze-dried, and β-Lg B was isolated from commercial WPI, as described by Livney et al. (*26*). To avoid complications arising from mixtures of genetic variants, we used only the B variant of β-Lg in our experiments. The identity and purity of both isolated proteins (~95% each) were determined by polyacrylamide gel electrophoresis (PAGE) using a Phast System (Amersham Pharmacia Biotech AB, Baie d'Urfé, PQ, Canada).

Solution Preparation and Reaction Conditions. Standard solutions of 0.6 mM of each of the two proteins (11.4 mg/mL κ-CN; 10.98 mg/mL β-Lg; molar concentration based on monomer M_w) in 5 mM ammonium acetate in Milli-Q water were prepared, and their pH was adjusted to 6.7 with 1 M ammonium bicarbonate. A 1:1 mixture of the β-Lg and κ-CN solutions was prepared, in a total volume of 1.5 mL per sample. All experiments were performed in duplicate. The protein solutions were heated for 10 min in test tubes immersed in a water bath maintained at 90 °C, followed by a rapid cooling in ice and equilibration at room temperature. Following the heat treatment, the samples were treated with iodoacetamide (50 mM at 30 °C for 30 min while agitating at 14 krpm in a Thermomixer). This was intended to inactivate any residual free thiol groups and to minimize possible SH−SS interactions between peptides formed during enzymatic digestion.

Enzymatic Digestion and Subsequent Fractionation. The pH of the samples was adjusted to 7.80 ± 0.05 by the addition of 1 M ammonium bicarbonate, and the samples were divided into two aliquots for digestion by two different enzymes: trypsin and glu-C. Trypsin attacks proteins at the C-terminal side of lysine (K) and arginine (R), wheras glu-C attacks at the C-terminal side of glutamic acid (E) and aspartic acid (D). Each enzyme was initially added at a 1:20 w/w ratio (enzyme to protein) into a sample volume of 0.4 mL. Digestions were carried out at 37 °C for 24 h, with the addition of a second aliquot of enzyme after the first 12 h. The digests were vortexed, and 20 μL of each was collected and refrigerated for subsequent whole-digest MS analysis.

Both digests appeared to have small amounts of undigestible precipitate, and the supernatants after centrifugation at 10000*g* for 10 min were collected for further analysis. Because RP-HPLC analysis requires clear samples acidified with 0.05% TFA, acidification may be done before or after centrifugation, which may result in different peptide compositions of the supernatant. To maximize the number of peptides observed, samples were split and both strategies executed. Supernatants were collected and refrigerated for subsequent MS analysis, and portions of them were analyzed by RP-HPLC.

RP-HPLC. A μRPC C2/C18 ST 4.6/100 column (Pharmacia Biotech) was used with a gradient of acetonitrile, in an HPLC system (BioLogic Duo-Flow, Bio-Rad Inc., Hercules, CA). Detection was done by absorbance at 214 nm. Aliquots (250 μL) of the supernatants from enzymatic digestion were loaded on the column. Eluant A was 0.05% TFA in Milli-Q water; eluant B was 0.05% TFA in a solution of 9:1 v/v HPLC grade acetonitrile/Milli-Q water. A gradient of 0−56% eluant B over 56 min at a flow rate of 0.75 mL/min was used. To cover the entire range of peptides, a fraction collector was used, continuously collecting 8 mL fractions. These fractions were vortexed, and 1.5 mL of each was vacuum-concentrated and dried by a Labconco CentriVap centrifugal concentrator at 45 °C. Then 20 μL of each unconcentrated fraction was added to maintain the solvent characteristics and vortexed to redissolve the concentrated peptides. These peptide concentrates were then analyzed by MALDI-TOF-MS.

MALDI-TOF-MS was used to identify peptides and disulfide-linked peptides in the digests of the heated proteins. A 4 μL aliquot of each sample of either whole digest, whole-digest supernatant, or an RP-HPLC fraction was mixed with 4 μL of a saturated solution of matrix in 1:1 H_2O/acetonitrile with 0.1% TFA and was spotted onto a stainless steel MALDI plate and then air-dried. Samples for lower mass analysis were prepared with a matrix of α-cyano-4-hydroxycinnamic acid and calibrated internally with ACTH clipped 18−39. Samples for higher mass analysis were prepared with sinapinic acid matrix and calibrated internally with ACTH (18−39) and equine cytochrome *c*. All samples were spotted in duplicate onto the plate. Mass spectra were acquired using a Bruker Reflex III MALDI-TOF-MS (Bruker, Billerica, MA) in positive ion mode. The lower mass spectra were acquired operating in reflectron mode in a mass to charge range of 0−4 kDa with a laser energy of 26%. The higher mass spectra were acquired operating in linear mode from 4 to 20 kDa with laser energy of 29%. In both cases, 300 scans were summed and then processed manually using XTOF software (Bruker). Mass error reporting limits were 50 and 100 ppm for masses below and above 4 kDa, respectively. Only peaks having a signal-to-noise ratio of five or more were reported. Because molecular mass was the only criterion used to identify the peptide products (as their sequencing by further degradation is not possible with the equipment available to us), we used the number of different masses (due to different cleavage locations or to different dimers/trimers formed) matching peptide combinations involving each bond as a measure of confidence of identification of this specific bond.

Theoretical Digestion and Analysis of Results. The primary sequences of the two proteins (κ-CN, P02668; and β-Lg, P02754) were obtained from the Swiss-Prot Protein knowledgebase (*27*). Theoretical digestions of both proteins with each of the enzymes (trypsin and glu-C) were carried out by the web-program MS-Digest (*28*). Common isotopic masses were used for the calculations. Scanning for peptide combinations of matching masses was performed by an MS Excel-based application we developed for this purpose. To minimize ambiguity, masses matching more than two possibilities of peptide combinations indicating different disulfide bonding were disregarded.

RESULTS AND DISCUSSION

Tables 1 and **2** detail the results of MS analysis of trypsin and glu-C digests (respectively) of heated (90 °C 10 min, pH 6.7) mixtures of κ-CN and β-Lg, each at 0.3 mM (based on

Table 1. Observed Disulfide-Bonded Peptides in Heated κ-CN/β-Lg Solutions (90 °C, 10 min) Treated with Iodoacetamide and Digested with Trypsin

obsd *m*/*z* (Da)	apparent identities of bonded peptides[a]				calcd M + H+ (Da)
2125.8[b]	ββ	β61–69 (C66)	β61–69 (C66)		2125.8
3706.7[c]	ββ	β102–124 (C106, C119, C121)[d]	β61–69 (C66)		3706.6
4270.9[e]	κβκ	κ11–13 (C11)	β102–124 (C106, C119, C121)[f]	κ87–97 (C88)	4270.9
4383.2[c]	κβ	κ1–13 (C11)	β101–124 (C106, C119, C121)[d]		4383.0
4480.8[b]	κβκ	κ11–13 (C11)	β102–124 (C106, C119, C121)[f]	κ11–21 (C11)	4481.0
4555.6[b]	κκ	κ1–21 (C11)	κ69–97 (C88)		4555.2
5143.0[b]	ββ	β41–70 (C66)	β149–162 (C160)		5142.6
5431.2[b]	ββ	β102–124 (C106, C119, C121)[d]	β139–162 (C160)		5431.7
or	κβκ	κ11–16 (C11)	β92–124 (C106, C119, C121)[f]	κ11–16 (C11)	5431.5
5496.4[c]	κβ	κ1–16 (C11)	β41–70 (C66)		5496.7
or	κββ	κ11–13 (C11)	β102–135 (C106, C119, C121)[f]	β61–70 (C66)	5496.4
5505.0[e]	κβκ	κ11–21 (C11)	β102–124 (C106, C119, C121)[f]	κ11–21 (C11)	5505.4
or	κβκ	κ1–13 (C11)	β102–124 (C106, C119, C121)[f]	κ87–97 (C88)	5504.5
5531.9[b]	ββ	β102–124 (C106, C119, C121)[d]	β102–124 (C106, C119, C121)[g]		5531.5
or[h]	ββ	β102–124 (C106, C119, C121)[g]	β102–124 (C106, C119, C121)[d]		5531.5
5628.5[b]	κβ	κ69–97 (C88)	β142–162 (C160)		5628.8
5722.0[b]	κββ	κ11–13 (C11)	β102–124 (C106, C119, C121)[f]	β102–124 (C106, C119, C121)[d]	5722.5
5963.3[c]	κββ	κ11–13 (C11)	β102–135 (C106, C119, C121)[f]	β149–162 (C160)	5962.7
6517.4[e]	κβκ	κ11–16 (C11)	β101–124 (C106, C119, C121)[f]	κ87–112 (C88)	6517.1
6632.5[b]	κβκ	κ11–16 (C11)	β102–124 (C106, C119, C121)[f]	κ69–97 (C88)	6632.1
7596.6[b]	κββ	κ1–16 (C11)	β102–135 (C106, C119, C121)[f]	β149–162 (C160)	7596.5
8314.6[b]	κββ	κ87–111 (C88)	β92–124 (C106, C119, C121)[f]	β149–162 (C160)	8315.0

[a] In parentheses are the cysteines. [b] Observed in an RP-HPLC fraction of the tryptic digest supernatant. [c] Observed in supernatant of tryptic digest. [d] The internal disulfide bridge is probably intact. [e] Observed in whole tryptic digest. [f] One carbamidomethyl group attached (following iodoacetamide reaction with a free thiol). [g] Two carbamidomethyl groups attached. [h] This and the previous combination are indistinguishable (by this methodology), and they indicate the same disulfide bonds; thus they are regarded as a single observation.

monomer M_w). The tables list all of the peptide combinations identified as disulfide bonded in the whole digests, in the supernatants, and in the fractions of supernatants analyzed by RP-HPLC, based on the matching of theoretically calculated and observed masses. Each mass was listed only once, even when it was observed several times.

In addition to the various κκ, κβ, and ββ peptide−dimer combinations, we found a significant number of disulfide-bonded mixed trimers of peptides. Because the use of iodoacetamide to block free thiols should preclude SH/SS shuffling during and after proteolysis, the finding of these trimers implies that considerable unfolding of β-Lg occurred during heating, allowing intimate contact, and formation of closely spaced disulfide bonds between two and sometimes three protein molecules. We have previously demonstrated this in mixtures of β-Lg with α-lactalbumin (*26*).

The RP-HPLC-MS analysis of the supernatants was complementary to MS analysis of the whole digests, in which the large number of peptides and peptide combinations created a difficulty for analysis, because of crowded spectra and the hindrance of better ionizing peptides with the identification of others. Because only filtered (or centrifuged) solutions can be run through the RP-HPLC column, only the supernatants of the centrifuged digests were analyzed by RP-HPLC, giving information about the soluble fraction of the digest. It is important to note that a major limitation of this technique is that any large polymers or aggregates, which survive digestion, would not be analyzed by the method employed here and that only dimers and trimers of the peptides would be identifiable. The use of two different enzymes in parallel partly overcomes this problem, as their different cleavage rules create different peptides with different tendencies to cross-link or to aggregate non-covalently. Trypsin cannot cleave between β-C106, β-C119, and β-C121, thus forming a trifunctional peptide, theoretically capable of forming branched polymers by disulfide interactions. Glu-C can detach β-C106 from β-C119 and β-C121, but leaves the latter two connected and theoretically capable of forming linear polymers. However, their very close proximity should make it sterically quite difficult for many protein molecules to come in close enough proximity to form such polymers, which would be later uncleavable by glu-C. A disadvantage of glu-C in the digestion of κ-CN is that it leaves a large uncleavable peptide (κ21−115), which includes κ-C88. This peptide, which is quite hydrophobic, might be more likely to precipitate, especially in disulfide-bonded homodimers or when aggregated with bifunctional β-Lg peptides as cross-linkers. Trypsin, on the other hand, has more uniformly distributed cleavage sites, making the study of κ-C88 interactions in this system easier. Therefore, the two enzymes are complementary, and by combining their results, we can get a more comprehensive and genuine picture of the studied interactions.

The detailed data in **Tables 1** and **2** are further analyzed and summarized in **Tables 3** and **4**, respectively, to map and identify any specificity of disulfide bonding between the studied proteins in the mixture and to measure the degree of confidence with which each bond was identified, based on the number of observations. The numbers in **Tables 3** and **4** were obtained by counting the masses in **Table 1** or **2** respectively corresponding to peptide combinations involving each specific bond. Number ranges describe uncertainty when more than one alternative interpretation exists. For example, bonds between tryptic peptides containing β-C106/119/121 and κ-C88 are involved in combinations corresponding to the masses (**Table 1**), 4270.9, 6517.4, 6632.5, and 8314.6, but also as one of two alternative interpretations of 5505.0; therefore, the corresponding box in **Table 3** is given the range 4−5. Additionally, in the glu-C digest (**Table 2**) there is one more mass confirming that a bond formed between β-C119/121 and κ-C88, which is marked as 1 in the appropriate box in **Table 4**. The larger the number of different masses a certain bond appeared in, the higher the degree of confidence of its identification. Moreover, if a bond was

Table 2. Observed Disulfide-Bonded Peptides in Heated κ-CN/β-Lg Solutions (90 °C, 10 min) Treated with Iodoacetamide and Digested with Glu-C

obsd *m/z* (Da)		apparent identities of bonded peptides[a]			calcd M + H+ (Da)
2485.3[b]	κβ	κ1–12 (C11)	β66–74 (C66)		2485.2
2599.3[b]	κκ	κ1–12 (C11)	κ7–15 (C11)		2599.2
2930.4[b]	κβ	κ7–14 (C11)	β113–129 (C119, C121)[c]		2930.4
3778.0[b]	βββ	β66–74 (C66)	β115–130 (C119, C121)	β66–74 (C66)	3777.9
3818.0[b]	κββ	κ7–12 (C11)	β109–127 (C119, C121)	β66–74 (C66)	3817.8
4460.6[b]	κββ	κ7–12 (C11)	β115–130 (C119, C121)	β113–129 (C119, C121)[c]	4461.1
or[d]	κββ	κ7–12 (C11)	β113–129 (C119, C121)	β115–130 (C119, C121)[c]	4461.1
4515.7[b]	κββ	κ7–15 (C11)	β113–127 (C119, C121)	β113–127 (C119, C121)[c]	4516.1
4556.8[b]	βββ	β99–108 (C106)	β113–129 (C119, C121)	β99–108 (C106)	4557.2
or	κββ	κ1–12 (C11)	β109–127 (C119, C121)	β66–74 (C66)	4557.1
4589.0[e]	κββ	κ7–14 (C11)	β115–129 (C119, C121)	β113–129 (C119, C121)[c]	4589.2
or[d]	κββ	κ7–14 (C11)	β113–129 (C119, C121)	β115–129 (C119, C121)[c]	4589.2
5024.1[f]	βββ	β66–85 (C66)	β115–127 (C119, C121)	β99–108 (C106)	5023.6
5077.9[f]	βββ	β109–127 (C119, C121)[c]	β115–127 (C119, C121)	β115–127 (C119, C121)[c]	5078.4
or[d]	βββ	β115–127 (C119, C121)[c]	β109–127 (C119, C121)	β115–127 (C119, C121)[c]	5078.4
5100.0[e]	κββ	κ3–14 (C11)	β113–127 (C119, C121)	β113–129 (C119, C121)[c]	5100.4
or[d]	κββ	κ3–14 (C11)	β113–129 (C119, C121)	β113–127 (C119, C121)[c]	5100.4
or	βββ	β56–74 (C66)	β113–129 (C119, C121)	β66–74 (C66)	5099.6
5132.4[f]	κββ	κ7–15 (C11)	β115–130 (C119, C121)	β66–85 (C66)	5132.6
5238.2[e]	βββ	β66–85 (C66)	β115–129 (C119, C121)	β99–108 (C106)	5237.7
5275.4[b]	κββ	κ3–14 (C11)	β115–129 (C119, C121)	β109–127 (C119, C121)[c]	5275.4
or[d]	κββ	κ3–14 (C11)	β109–127 (C119, C121)	β115–129 (C119, C121)[c]	5275.4
5294.3[b]	βββ	β90–108 (C106)	β115–129 (C119, C121)	β63–74 (C66)	5294.6
5316.9[f]	βββ	β66–85 (C66)	β109–127 (C119, C121)	β66–74 (C66)	5316.8
5348.1[e]	κββ	κ7–14 (C11)	β115–127 (C119, C121)	β138–162 (C160)	5347.7
5357.8[b]	κββ	κ3–14 (C11)	β115–129 (C119, C121)	β56–74 (C66)	5357.6
or[d]	κββ	κ3–12 (C11)	β115–127 (C119, C121)	β66–89 (C66)	5357.7
5556.5[b]	βββ	β90–108 (C106)	β113–127 (C119, C121)	β97–108 (C106)	5556.7
or	βββ	β66–89 (C66)	β113–129 (C119, C121)	β66–74 (C66)	5556.9
6419.1[f]	βββ	β138–162 (C160)	β113–127 (C119, C121)	β115–130 (C119, C121)[c]	6419.2
or[d]	βββ	β138–162 (C160)	β115–130 (C119, C121)	β113–127 (C119, C121)[c]	6419.2
6497.3[e]	βββ	β56–74 (C66)	β109–127 (C119, C121)	β56–74 (C66)	6497.3
6558.9[b]	βββ	β66–85 (C66)	β115–130 (C119, C121)	β63–85 (C66)	6559.5
6642.8[f]	βββ	β138–162 (C160)	β115–130 (C119, C121)	β97–112 (C106)	6643.2
14727.5[e]	κββ	κ16–115 (C88)	β115–127 (C119, C121)	β99–112 (C106)	14726.5

[a] In parentheses are the cysteines. [b] Observed in an RP-HPLC fraction of the glu-C digest supernatant. [c] One carbamidomethyl group attached (following iodoacetamide reaction with a free thiol). [d] This and the previous combination are indistinguishable (by this methodology), and they indicate the same disulfide bonds; thus, they are regarded as a single observation. [e] Observed in supernatant of glu-C digest. [f] Observed in whole glu-C digest.

Table 3. Number of Different Masses Corresponding to Tryptic Peptide Combinations in Which Each Disulfide Bond Was Observed To Participate

	κ-C11	**κ-C88**	**β-C66**	**β-C106, C119, C121**	**β-C160**
κ-C11					
κ-C88	1				
β-C66	0–1		1		
β-C106, C119, C121	9–11	4–5	1–2	2	
β-C160		1	1[a]	3–4	

[a] A native intramolecular bond location. The other such bond is β-C106–C119 (*5*, *6*).

Table 4. Number of Different Masses Corresponding to Glu-C Peptide Combinations in Which Each Disulfide Bond Was Observed To Participate

	κ-C11	**κ-C88**	**β-C66**	**β-C106**	**β-C119, C121**	**β-C160**
κ-C11	1					
κ-C88						
β-C66	1					
β-C106						
β-C119, C121	9–11	1	10–13[a]	5–7	6–7	
β-C160			b		3	

[a] In four of these observed masses both β-C119 and β-C121 are simultaneously connected to two peptides each containing only β-C66. These four masses provide evidence that each of the two, β-C119 and β-C121, interacts with β-C66 during heating. [b] A native intramolecular bond location. The other such bond is β-C106–C119 (*5*, *6*).

identified by both enzymes, the confidence of its identification is further enhanced.

Because nonexistence of evidence is not evidence of nonexistence, the bonds that were not observed by any of the methods employed here may only be considered rare or unlikely to form, but not necessarily impossible, considering the limitations of the methods used and the nature of the MALDI-TOF analysis.

Several representative mass spectra showing peaks of interest are presented in **Figures 1** and **2** for tryptic and glu-C digests, respectively. For example, panels B−D of **Figure 1** show evidence for bond formation between β-C106/119/121 and κ-C11. Whereas **Figure 1C** suggests two of these three β-cysteines can interact with κ-C11, **Figure 1D** provides an example of evidence for interaction of β-C106/119/121 with both κ-C11 and κ-C88 (see **Table 1** for details). Panels A and C of **Figure 2** provide evidence for bond formation between κ-C11 and β-C119/121, whereas **Figure 2D** shows evidence for bond formation between κ-C88 and β-C119/121. Examples of evidence for the main ββ bonds identified in this work are presented in **Figures 1A** and **2A−D** (see **Tables 1** and **2** for details). Noteworthy is the evidence of trimers of β-C119/121-containing peptides (e.g., **Figure 2B**, 5077.9 Da), which suggests that considerable unfolding is required for three β-Lg molecules

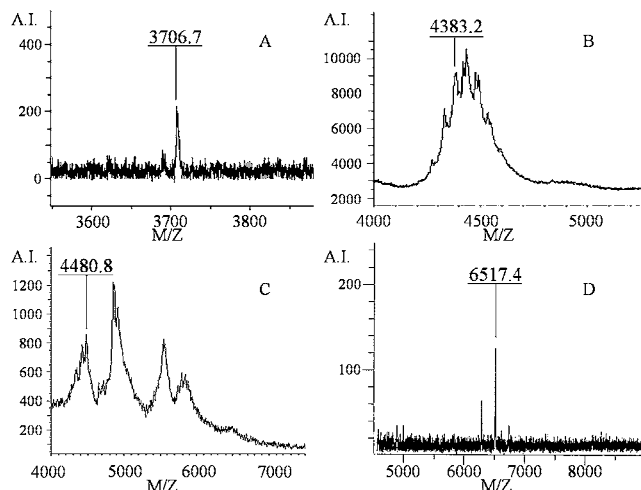

Figure 1. Examples of representative mass spectra of tryptic digests with several masses of interest (bond evidenced in parentheses; see **Table 1** for details): (**A**) 3706.7 Da (*β*-C106/C119/C121–*β*-C66); (**B**) 4383.2 Da (*κ*-C11–*β*-C106/C119/C121); (**C**) 4480.8 Da (*κ*-C11–*β*-C106/C119/C121–*κ*-C11); (**D**) 6517.4 Da (*κ*-C11–*β*-C106/C119/C121–*κ*-C88).

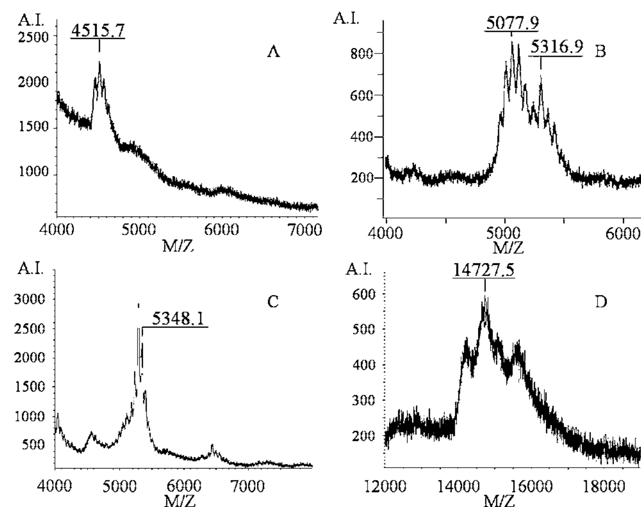

Figure 2. Examples of representative mass spectra of glu-C digests with several masses of interest (bond evidenced in parentheses; see **Table 2** for details): (**A**) 4515.7 Da (*κ*-C11–*β*-C119/C121–*β*-C119/C121); (**B**) 5077.9 Da [*β*-C119/C121 trimer(s)], 5316.9 Da (*β*-C66–*β*-C119/C121–*β*-C66); (**C**) 5348.1 Da (*κ*-C11–*β*-C119/C121–*β*-C160); (**D**) 14727.5 Da (*κ*-C88–*β*-C119/C121–*β*-C106).

to be able to come in close enough proximity to form such trimers, as C119 and C121 are very close together in the protein structure.

The results presented in **Tables 3** and **4** clearly suggest that the distribution of intermolecular disulfide bond locations is not random. The results, taken as a whole, suggest that although quite a few of the possible intermolecular disulfide bonds are formed during heating, they are not all formed with equal efficiency. Some of the bonds predominate, whereas others were not observed at all. It is clear, however, that there is no single specific site for interaction between the two proteins during their heat-induced aggregation.

Table 3 (summary of trypsin digestion results) clearly shows that the *β* peptides containing C106, C119, and C121 form disulfide bonds with both *κ*-C11 and *κ*-C88, as well as with all of the *β*-Lg cysteines. **Table 4** (glu-C digestion results) shows that by cleaving *β*-C106 from *β*-C119/C121 we can learn that apparently it is only *β*-C119/C121 that are predominant in forming the intermolecular disulfide bonds. These observations are in accord with the supposed role of *β*-C121 as the initiator of the sulfhydryl−disulfide interchange chain reaction, as it becomes solvent-exposed upon *β*-Lg dimer dissociation and the unfolding of the protein during heat denaturation (*29*). On the other hand, *β*-C106 is not observed in bonds with any other *β*-Lg cysteine except for *β*-C119/C121 (e.g., **Figure 2D**), nor is it observed bonded with any of the cysteines of *κ*-CN. This suggests that *β*-C106, which is located in the core of the native protein tertiary structure and engaged in the native *β*-C106-C119 intramolecular disulfide bond (*6*), remains inaccessible and buried in the core of *β*-Lg even when the protein unfolds and that it engages in intramolecular bonds only with either *β*-C119 or *β*-C121 or remains as a free thiol. It has previously been reported that during heating, some interchange of the *β*-C106−C119 bond occurs, to form a *β*-C106−C121 bond (*30*).

Moreover, in some cases it was possible to gain evidence for the participation of each of the two cysteines, *β*-C119 and *β*-C121, in disulfide bonds with another *β*-cysteine. This was possible when peptides containing both *β*-C119 and *β*-C121 were linking two peptides, each containing only *β*-C66. Four such *βββ* trimers were observed [**Table 2**: 3778.0, 5316.9 (**Figure 2B**), 6497.3 and 6558.9 Da]. Considering the close proximity of *β*-C119 and *β*-C121, it is plausible that they would both be considerably involved in disulfide bonding, although without cleaving between them (which is not easily achievable) no distinction can be made between their specificities.

The relatively large number of observations here of peptides containing *β*-C106/C119/C121 or just *β*-C119/C121 provides reassurance that not much information was lost by precipitation of disulfide-linked polymers during proteolysis, because these peptides should theoretically comprise the backbone of such polymers.

The data show some single-observation (therefore, low confidence) evidence for bond formation between *κ*-C11 and *β*-C66 and between *κ*-C88 and *β*-C160 and no evidence for *κ*-C11−*β*-C160 or *κ*-C88−*β*-C66. The *κ*-C88−*β*-C160 bond has been identified in heated goat's milk (*23*). The fact there were so few observations of these bonds is surprising, in light of the location of the *β*-C66−*β*-C160 bond on the surface of the native *β*-Lg. Even more unexpected is the fact *β*-C66−C66 and *β*-C160−C160 intermolecular bonds were not observed, although we have previously found them in heated (85 °C, 10 min) solutions of pure *β*-Lg (*26*). Interestingly, they were also apparently missing in mixtures of *β*-Lg and α-La heated for 10 min at 85 °C under low ionic strength conditions (*26*) similar to those in the present study. Possibly, the dynamics of SH−SS interchange, rather than simple accessibility, may be rendering these specific combinations less likely in the mixed systems studied. A better understanding of these dynamic and steric effects would require further study using complementary methods and possibly computer simulations.

Disulfide *κκ* bonds were also hardly observed in our study, and only single observations possibly identified as *κ*-C11−*κ*-C11 (in the glu-C digest) and *κ*-C11−*κ*-C88 (in the tryptic digest) were observed with no confirmation by other masses. This is expected, given the fact that the *κ*-CN used was monomeric and had been treated with mercaptoethanol, and future research is required to study whether the native oligomerization would change the results.

We can certainly conclude, however, that there are no molecular level steric factors preventing interaction of *β*-Lg with either of the two *κ*-CN cysteines, *κ*-C11 and *κ*-C88; thus, they

may both serve as anchoring positions for the aggregating whey proteins during heating of milk protein systems. This conclusion is partly based on the fact that the κ-CN concentration in the studied system was about 10-fold its cmc, so that most of it (∼90%) was presumably in micellar aggregates, in which its steric orientation should be similar to that in casein micelles (hydrophilic portion outward). This is also in agreement with Swaisgood (*16*), who stated that both cysteinyl residues of κ-CN are in the hydrophobic domain and occur in predicted loops or β-turns and thus should be readily accessible. Further studies, using casein micelles, would be warranted as the next step along this course of research.

ABBREVIATIONS USED

ACTH, adrenocorticotropic hormone; β-Lg or β, β-lactoglobulin; κ-CN or κ, κ-casein; MALDI-TOF-MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

We thank the following individuals from the University of Guelph: Elizabeth Parker and Edita Verespej from the Department of Food Science for isolating the proteins used in this study and Dr. Dyanne Brewer and Sibylle Heidelberger from the Department of Molecular Biology and Genetics for their invaluable professional assistance with MS.

LITERATURE CITED

(1) Livney, Y. D.; Corredig, M.; Dalgleish, D. G. Influence of thermal processing on the properties of dairy colloids. *Curr. Opin. Colloid Interface Sci.* **2003**, *8*, 359−364.
(2) Lucey, J. A.; Munro, P. A.; Singh, H. Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. *Int. Dairy J.* **1999**, *9*, 275−279.
(3) Guyomarc'h, F.; Queguiner, C.; Law, A. J. R.; Horne, D. S.; Dalgleish, D. G. Role of the soluble and micelle-bound heat-induced protein aggregates on network formation in acid skim milk gels. *J. Agric. Food Chem.* **2003**, *51*, 7743−7750.
(4) Van Hooydonk, A. C. M.; De Koster, P. G.; Boerrigter, I. J. The renneting properties of heated milk. *Neth. Milk Dairy J.* **1987**, *41*, 3−18.
(5) Hambling, S. G.; McAlpine, A. S.; Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry*−*1: Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 141−190.
(6) Brownlow, S.; Cabral, J. H. M.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution−still an enigmatic lipocalin. *Structure (London)* **1997**, *5*, 481−495.
(7) Jameson, G. B.; Adams, J. J.; Creamer, L. K. Flexibility, functionality and hydrophobicity of bovine β-lactoglobulin. *Int. Dairy J.* **2002**, *12*, 319−329.
(8) Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S. Y. β-Lactoglobulin: structural studies, biological clues. *Int. Dairy J.* **1998**, *8*, 65−72.
(9) Relkin, P. Thermal unfolding of β-lactoglobulin, α-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 565−601.
(10) Iametti, S.; Bonomi, F. Transient modification of the association equilibria in heated β-lactoglobulin. *Int. Dairy Fed. S. I.* **1996**, *9602*, 341−349.
(11) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced sggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303−4308.
(12) Roefs, S. P. F. M.; De Kruif, K. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.
(13) Hoffmann, M. A. M.; Van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* **1997**, *45*, 2942−2948.
(14) Le Bon, C.; Nicolai, T.; Durand, D. Growth and structure of aggregates of heat-denatured β-lactoglobulin. *Int. J. Food Sci. Technol.* **1999**, *34*, 451−465.
(15) 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.* **2000**, *9*, 791−800.
(16) Swaisgood, H. E. Chemistry of the caseins. In *Advanced Dairy Chemistry*−*1: Proteins*; Fox, P. F., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publishers: New York, 2003; Part A, 3, pp 139−202.
(17) DeKruif, C. G.; Holt, C. Casein micelle structure, functions and interactions. In *Advanced Dairy Chemistry*−*1: Proteins*; Fox, P. F., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publishers: New York, 2003; Part A, 3, pp 233−276.
(18) Dalgleish, D. G. The enzymatic coagulation of milk. In *Advanced Dairy Chemistry*−*1*: *Proteins*; Fox, P. F., Ed.; Elsevier Applied Science: London, U.K., 1992; pp 579−620.
(19) 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.
(20) 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.
(21) 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.
(22) 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.
(23) Henry, G.; Mollé, D.; Morgan, F.; Fauquant, J.; Bouhallab, S. Heat-induced covalent complex between casein micelles and beta-lactoglobulin from goat's milk: identification of an involved disulfide bond. *J. Agric. Food Chem.* **2002**, *50*, 185−191.
(24) Dalgleish, D. G. The sizes and conformations of the proteins in adsorbed layers of individual caseins on lattices and in oil-in-water emulsions. *Colloid Surf. B: Biointerfaces* **1993**, *1*, 1−8.
(25) Hollar, C. M.; Law, A. J. R.; Dalgleish, D. G.; Brown, R. J. Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* **1991**, *74*, 2403−2409.
(26) Livney, Y. D.; Verespej, E.; Dalgleish, D. G. Steric effects governing disulfide bond interchange during thermal aggregation in solutions of β-lactoglobulin B and α-lactalbumin. *J. Agric. Food Chem.* **2003**, *51*, 8098−8106.
(27) Swiss-Prot Protein knowledgebase. ExPASy Molecular Biology Server; Swiss Institute of Bioinformatics (SIB), 2003; http://www.expasy.org/sprot/.
(28) Baker, P.; Clauser, K. MS-Digest©. ProteinProspector 4.04, copyright 1995−2003; The Regents of the University of California, 2002; http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm.
(29) de la Fuente, M. A.; Singh, H.; Hemar, Y. Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends Food Sci. Technol.* **2002**, *13*, 262−274.
(30) 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.

Received for review January 8, 2004. Revised manuscript received June 9, 2004. Accepted June 23, 2004. The work was supported financially by the Ontario Dairy Council and the Natural Sciences and Engineering Research Council of Canada.

JF049955H