JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Heat-Induced Redistribution of Disulfide Bonds in Milk Proteins. 1. Bovine β-Lactoglobulin

LAWRENCE K. CREAMER,*,† ANNIE BIENVENUE,‡ HANNA NILSSON,§ MARIE PAULSSON,§ MIRIAM VAN WANROIJ,# EDWIN K. LOWE,† SKELTE G. ANEMA,† MICHAEL J. BOLAND,† AND RAFAEL JIMENEZ-FLORES‡

Fonterra Research Centre, Private Bag 11-029, Palmerston North, New Zealand; Food Engineering, Lund University, Box 117, SE-221 00 Lund, Sweden; Dairy Product Technology Center, California Polytechnic State University, San Luis Obispo, California 93407; and Department of Dairy Technology, Wageningen University and Research, Wageningen, The Netherlands

Changes in the structure and chemistry of β-lactoglobulin (β-LG) play an important role in the processing and functionality of milk products. In model β-LG systems, there is evidence that the aggregates of heated β-LG are held together by a mixture of intermolecular non-covalent association and heat-induced non-native disulfide bonds. Although a number of non-native disulfide bonds have been identified, little is known about the initial inter- and intramolecular disulfide bond rearrangements that occur as a result of heating. These interchange reactions were explored by examining the products of heat treatment to determine the novel disulfide bonds that form in the heated β-LG aggregates. The native protein and heat-induced aggregates were hydrolyzed by trypsin, and the resulting peptides, before and after reduction with dithiothreitol, were separated by high-performance liquid chromatography and their identities confirmed by electrospray ionization mass spectrometry. Comparisons of these peptide patterns showed that some of the Cys160 was in the reduced form in heated β-LG aggregates, indicating that the Cys160−Cys66 disulfide bond had been broken during heating. This finding suggests that disulfide bond interchange reactions between β-LG non-native monomers, or polymers, and other proteins could occur largely via Cys160.

KEYWORDS: **β-Lactoglobulin; disulfide bonding; heat-induced change; mass spectroscopy**

INTRODUCTION

The effect of heat on β-lactoglobulin (β-LG) and other milk proteins has received considerable attention, as heat is known to have an impact on dairy processing, such as fouling of equipment and prevention of milk coagulation during cheesemaking, because of the reaction of the denaturing whey protein with κ-casein on the casein micelle (*1*−*4*). In contrast, whey protein isolates and concentrates, with β-LG content of at least 50% of the protein, readily form heat- or pressure-induced gels and can be used as functional ingredients in food formulations.

It has long been recognized that the heat-induced aggregation of β-LG involves both disulfide bond interchange and modification to the hydrophobic interactions that are intramolecular in the native whey proteins and become intermolecular in the aggregates and gels (*5*, *6*). To study the disulfide bond interchange phenomena, it was necessary to work with more dilute solutions so that intermediate species could be identified and at lower temperatures to slow the reaction so that the intermediates in the pathway could be identified.

The structure of native β-LG is now well-known from both X-ray crystallographic studies (*7*−*9*) and high-resolution nuclear magnetic resonance studies (*10*−*12*). β-LG has nine β strands, eight of which fold into two β sheets that face each other. There is a three-turn α helix that sits above one of the β sheets. Three (Cys106, Cys119, and Cys121) of the five Cys residues sit within a very hydrophobic pocket between one side of the helix and segments of the G and H strands (**Figure 1**) (*8*). The Cys106−Cys119 disulfide bond is separated from Cys121 by the phenyl ring of Phe136.

It is generally recognized that the first effect of heat is the reversible dissociation of the native β-LG dimer into monomers (*5*, *13*). The second change is the partial unfolding of the β-LG monomer with a loss of helical structure (*14*, *15*), allowing the free sulfhydryl group on Cys121 to interact with the Cys106−Cys119 disulfide bond and, presumably, to reversibly create a Cys106−Cys121 disulfide bond and a free-thiol-containing Cys119 (*16*). This may be the "activated" monomer postulated (*5*, *13*) as the starting point for the aggregation reactions leading to larger polymers with other disulfide-bond-containing proteins, such as α-lactalbumin. It is at least equally possible that the

* Corresponding author (telephone 64-6-350-4649; fax 64-6-356-1476; e-mail lawrie.creamer@fonterra.com).
† Fonterra.
‡ California Polytechnic State University.
§ Lund University.
# Wageningen University and Research.

10.1021/jf049388y CCC: $27.50
Published on Web 00/00/0000 **PAGE EST: 8.2**

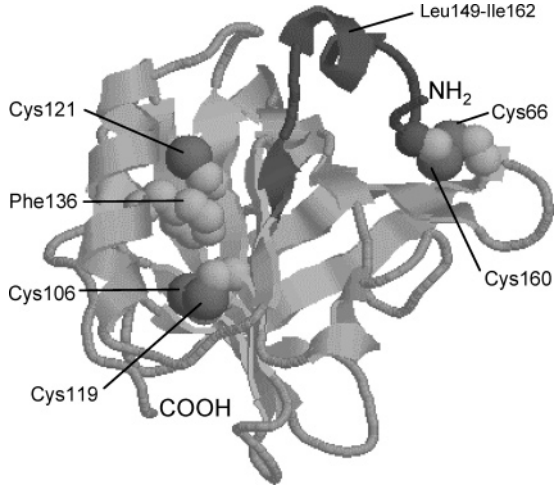

Figure 1. Diagram of the three-dimensional structure of β-LG that shows the relative positions of the five Cys residues, Phe136, and the C-terminal tryptic peptide, Leu149–Ile162. The diagram was drawn from the PDB file 1BEB using RASMOL ver. 2.6.

non-native protein with an exposed Cys121 (*16*) is the "activated" monomer postulated.

Both Surroca et al. (*17*) and Livney et al. (*18*) used high-resolution matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of peptide mixtures from tryptic digests of heated β-LG. Surroca et al. (*17*) analyzed the hydrolysates of the complete mixture and the polyacrylamide gel electrophoresis (PAGE)-separated monomeric, dimeric, and trimeric β-LG AB species, whereas Livney et al. (*18*) digested heat-treated β-LG B, separated the peptide mixture into fractions using high-performance liquid chromatography (HPLC), and analyzed selected HPLC fractions. Both groups identified that the peptide containing Cys106, Cys119, and Cys121 (Tyr102−Arg124) formed disulfide-bonded complexes with the peptides containing either Cys66 (Tyr61−Lys69 or Lys70) or Cys160 (Ala149−Ile162). Surroca et al. (*17*) also showed that the A variant of the Cys66 peptide could be bound to the B variant of the Tyr102−Arg124 peptide. Livney et al. (*18*) also reported peptide species that contained two or three of the Tyr102−Arg124 peptides linked by one or more disulfide bonds.

Manderson et al. (*19*) reported the presence of non-native monomers in heated β-LG solutions, supporting the size exclusion result of Iametti et al. (*20*) and confirmed by Schokker et al. (*21*). These were stable entities and did not appear to be the reactive intermediates postulated earlier (*5*, *13*). It has been suggested (*19*) that these species are in equilibrium with the native protein and with the non-native dimers (*22*), although others have considered them to be intermediates (*21*).

In this paper, we present the characterization of peptides from a series of tryptic hydrolysates of heat-denatured β-LG B and the identity of the peptides recovered by using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). Some of the preliminary material that formed a basis for this paper was presented as a poster (*23*) to show that the C-terminal peptides (Ala142−Arg148 and Ala149−Leu162, which contains Cys160 in the reduced form) form early in the reaction.

MATERIALS AND METHODS

Isolation of β-LG. β-LG was isolated from fresh milk of cows that were homozygous for β-LG A or B using the method described by Manderson et al. (*19*) and based on that of Mailliart and Ribadeau-Dumas (*24*). The β-LG solution (~50 mg/mL) was stored frozen. Aliquots containing 120 mg of β-LG were chromatographed at 4 °C on a column (50 × 600 mm) of Superdex 75 (Pharmacia, Uppsala, Sweden) in a dilute phosphate buffer at pH 6.0. After selection of the purest fractions, determined by native PAGE, these were bulked and dialyzed for 12 h.

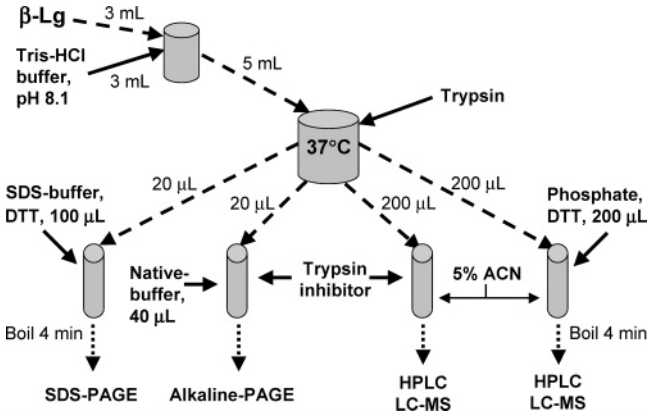

Figure 2. Schematic representation of the strategy used for sample preparation in this work. (See Materials and Methods for details.)

The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA). dl-Dithiothreitol (DTT), 2-mercaptoethanol, trypsin inhibitor (type I-S), TPCK-treated trypsin (catalog no. T 1426), and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis, MO). The HPLC solvents, which were of "far-UV" grade, and all other chemicals were obtained from BDH.

Heat Treatment. Three milliliter solutions of purified samples of β-LG A and B in a 26 mM sodium phosphate/68 mM NaCl buffer adjusted to pH 6.7, at a concentration of 1.15 mg/mL, were heated in a preheated test tube in a temperature-controlled water bath. The tubes were then cooled in ice water. The sample of β-LG B heated at 80 °C for 15 min was used for the data reported.

Hydrolysis. The overall scheme for β-LG treatment and hydrolysis is outlined in **Figure 2**. Three milliliters of a purified sample of β-LG B at a concentration of 7.00 ± 0.20 mg/mL was mixed with 3 mL of 100 mM Tris-HCl buffer, pH 8.1. After the protein had been added to the buffer, the pH was decreased to 7.85. Trypsin was dissolved in Milli-Q water to a concentration of 2.0 mg/mL. The protein/buffer solution was transferred to a plastic tube and warmed to 37 ± 0.1 °C in a water bath before 970 μL of the trypsin solution was added to give an enzyme/substrate (E:S) ratio of 1:10 (w/w) and a β-LG concentration of 2.097 mg/mL. Before the addition of enzyme and at various times after the addition of enzyme (0.5, 1, 2.5, 4, 6, 9, 13, 20, 30, 45, 65, 100, 150, 200, and 240 min), four samples were taken: 20 μL for sodium dodecyl sulfate (SDS)−PAGE, 20 μL for alkaline (native) PAGE, 200 μL for HPLC, and another 200 μL for HPLC, reduced with DTT. The samples for HPLC were also used for LC-ESI-MS (described below) to determine the masses of the peptides.

The samples for SDS-PAGE were transferred to plastic Eppendorf tubes, mixed with 100 μL of SDS-PAGE sample buffer containing 0.13 mg of DTT/mL, and boiled for 4 min.

The samples for alkaline (native) PAGE were transferred to plastic Eppendorf tubes and mixed with 29.7 μL of trypsin inhibitor solution (0.1 mg of trypsin inhibitor/mL) and 40 μL of alkaline PAGE sample buffer.

The samples for HPLC were transferred to plastic Eppendorf tubes and mixed with 200 μL of phosphate buffer (26 mM sodium phosphate/68 mM NaCl solution adjusted to pH 6.0) containing 6.54 mg of DTT/mL. The tubes were then put in boiling water for 4 min. After boiling, 400 μL of a 5% acetonitrile−0.1% TFA solution was added.

The second set of samples for HPLC was also transferred to plastic Eppendorf tubes and mixed with 297 μL of trypsin inhibitor (0.1 mg/mL). After mixing, 303 μL of a 5% acetonitrile−0.1% TFA solution was added.

All samples were stored at 4 °C until analyzed.

PAGE Analysis. Protein samples were separated by electrophoresis using Mini Protean II equipment (Bio-Rad Laboratories, Hercules, CA). One-dimensional alkaline (native) PAGE and SDS-PAGE were used as described by Manderson et al. (*19*). The Coomassie blue R250 stained gels were scanned using a Molecular Dynamics scanner (Molecular Dynamics Inc., Sunnyvale, CA). The gel scans were analyzed with ImageQuant 5.0 software to calculate the quantity of β-LG present [as a percentage of that in the control sample (not hydrolyzed)]. These results were then used to estimate the hydrolysis rate, which was done using Microsoft Excel 97.

Reversed-Phase (RP) HPLC Analysis. The hydrolysates were prepared in reducing sample buffer (molar ratio β-LG/DTT 1:1500) and nonreducing sample buffer so that the formation of new disulfide bonds could be followed. The samples for hydrolysis contained a final concentration of 1 mg/mL of β-LG and hydrolysis products, TFA 0.1% and acetonitrile 5%.

An Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany), which comprised an Agilent 1100 series quaternary pump and an Agilent 1100 series thermostated autosampler, was used. Two HPLC columns, Pharmacia μRPC C_2/C_{18} ST (column size = 4.6 mm $\times$ 100 mm) (Pharmacia Biotech, Uppsala, Sweden; code 17-5057-01), were connected in series. The temperature was controlled with an Agilent 1100 series thermostated column compartment and set to 25 °C. An aliquot of 100 μL of each sample and blank was injected into the system. The flow rate was 0.70 mL/min. The following elution protocol was used: (a) 5 min of 100% solvent A, (b) 0−100% solvent B over 70 min, (c) 5 min of 100% solvent B, and (d) 15 min of 100% solvent A. Solvent A was an aqueous solution containing 5% acetonitrile and 0.1% TFA. Solvent B was an aqueous solution containing 60% acetonitrile and 0.1% TFA. Absorbance data were collected at 1 nm intervals using a Hewlett-Packard 1040A multiwavelength diode array detection system (Hewlett-Packard Co., Camas, WA). The signal was monitored at 205, 210, 220, 280, and 295 nm. The data were transferred and further processed using the HP ChemStation software (1990−1998; rev. A. 06.03.509).

LC-ESI-MS. The peptides were separated by RP HPLC on a Waters HPLC system (Waters Associates, Millipore Corp., Waters Chromatography Division, Milford, MA) Alliance 2690 separations module, which comprised a Waters 486 MS tunable absorbance detector and a Waters 996 photodiode array detector. Signals at 205 and 280 nm were monitored. The same HPLC columns and method previously described were used in the Waters system. The software used was Waters Millennium version 3.05.01 within a Windows operating system.

The eluting peptides were characterized by mass spectrometry using a Perkin-Elmer Sciex (Thornhill, ON, Canada) triple-quadrapole API 300 LC/MS/MS system. The eluent stream from the Waters HPLC system was split such that ~10−15 μL/min was introduced into the mass spectrometer using an electrospray Perkin-Elmer Sciex API ion spray inlet interface. Ions were generated and focused using a positive ion spray voltage of 5600 V with detection between *m*/*z* values of 200 and 3000 amu. Parameters for operation of the mass spectrometer were as follows: step size, 0.2 amu; dwell time, 0.5 ms; scan time, 7 s; and 25 V. The RNG and IQ2 voltages were ramped from 140 to 280 V and from −15 to −90 V, respectively. The mass spectrometer was calibrated using a polypropylene glycol standard as outlined in the Perkin-Elmer Sciex API 300 manual, using MassChrom 1.0 software. The data were analyzed using BioMultiView 1.3.1 (Perkin-Elmer Sciex), and the reference mass library used was Peptide Map 2.2 (Perkin-Elmer Sciex).

RESULTS

Preliminary results showed that, whereas the trypsin treatment of native protein apparently hydrolyzed a large number of bonds essentially simultaneously, hydrolysis of the heat-modified proteins rapidly produced a number of peptides followed by a slower release of further peptides. Comparison of the hydrolysis of the A and B variant proteins showed that there was a greater difference between the initial hydrolysis rates of the native protein and the heated protein for β-LG B. These experiments also showed that, to encompass a wide range of rates of release of the tryptic peptides, the time intervals needed to be closer together early in the reaction and more widely spaced later in the reaction.

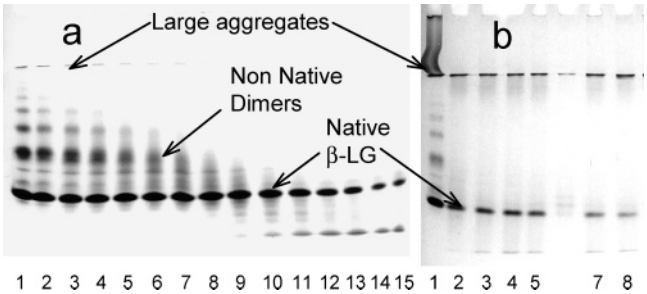

Figure 3. Alkaline-PAGE patterns of heated β-LG B (80 °C, 15 min): (**a**) hydrolyzed for 0, 1, 2.5, 4, 6, 9, 13, 20, 30, 45, 65, 100, 150, 200, and 240 min (lanes 1–15) at 37 °C, pH 7.85, and an E:S ratio of 1:30; (**b**) hydrolyzed for 0, 1, 2.5, 4, 6, (blank), 65, and 100 min (lanes 1–8) at 37 °C, pH 7.85, and an E:S ratio of 1:10.

Hydrolysis of Heated β-LG B. The alkaline PAGE pattern of β-LG B heated at 80 °C for 15 min in a phosphate buffer is shown in the zero-time lane of **Figure 3**. **Figure 3a** shows the pattern obtained at an E:S ratio of 1:30, and **Figure 3b** shows the pattern for the samples from heated β-LG B treated at an E:S ratio of 1:10, which were analyzed by HPLC. There were a number of protein bands of lower mobility than the native β-LG B, which were identified as the non-native monomers, dimers, trimers, etc. by comparison with earlier results (*19*). There was also material caught at the top of the stacking gel, within the stacking gel, and at the interface between the stacking and resolving gels. About half of the original protein remained in the native state.

After 1 min of hydrolysis at the 1:10 ratio, almost all of the smaller non-native β-LG aggregates were absent from the PAGE pattern (**Figure 3b**, lane 2), and, after 65 min, the native protein was no longer visible (**Figure 3b**, lane 7). Thus, much of the heat-modified β-LG B was hydrolyzed very early in the hydrolysis reaction, whereas virtually all of the native β-LG B was hydrolyzed in the first 60 min.

Identification of the Peptides. The HPLC traces obtained from a native β-LG B hydrolysate were comparable with those obtained earlier (*25*) or previously published (*26*−*29*), and preliminary assignments could be made. The use of 205 nm absorbance in the present study meant that the method was more sensitive to peptide bonds and relatively less sensitive to some side chains than measurements made at 214 or 220 nm. The simultaneous absorbance measurements made at 280 and 295 nm allowed the identification of the peptides containing Tyr (e.g., Val41−Lys60), Trp (e.g., Trp61−Lys70), and both (e.g., Val15−Tyr20) (**Table 1**). Analysis of some mixtures with an LC-ESI-MS instrument confirmed the identity of many of the peptides in the HPLC peaks produced from the hydrolysis mixtures (**Table 1**). Reduction of the hydrolysate with DTT prior to HPLC separation allowed the identification of disulfide-bonded peptides by the changes in peak intensity, for example, peptides 2 (Trp61−Lys69), 14 (Trp61−Lys70:Leu149−Ile162), and 17 (Leu149−Ile162). **Figure 4** shows a typical chromatogram of a hydrolysate of a heated sample of β-LG B, and **Table 1** shows the probable identity of each of the peptides. The numbering was based on the elution order found earlier (*25*), but the major peptides in peaks 10 and 11 were eluted in reverse order in this study (**Figure 4**), that is, Thr76−Lys83 (peptide/peak 11) eluted before Val15−Tyr20 (peptide/peak 10).

Table 1. Identity and Approximate HPLC Elution Times of the Major Tryptic Peptides of β-LG B

peptide/ peak no.	elution time[a] (min)	peptide
1	15.6	Ile71–Lys75
2	19.9	Trp61–Lys70
3	20.4	Trp61–Lys69
4	21.2	Ile84–Lys91
5	23.3	Gly9–Lys14
6	25.8	Thr125–Lys135
7	28.0	Leu1–Lys8
8	30.6	Ala142–Arg148
9	31.6	Val92–Lys101
10	32.6	Val15–Tyr20
11	32.3	Thr76–Lys83
12	33.5	Val92–Lys100
13	33.9	Thr125–Lys138
14	37.7	Trp61–Lys70:Leu149–Ile162
15	38.4	Trp61–Lys69:Leu149–Ile162
16	43.85	Ser21–Arg40
17	44.2	Leu149–Ile162
18	46.1	Val41–Lys60
19		Lys101-Arg124
20	49.4	Tyr102–Arg124
25	55.5	Val15–Arg40
26	65.0	intact β-LG

[a] Times taken from the data used in **Figure 4.** Peak 19 normally elutes 0.5 min prior to peak 20. It is commonly seen when native β-LG A is hydrolyzed but is at lower concentrations in β-LG B hydrolysates (*33*).

Relative Release Rates of the Peptides. HPLC analysis of the 15 hydrolysates per hydrolysis run of the native β-LG B showed that a number of peptide peaks appeared to be initially released at the same rate and that a number appeared more slowly. A selection of chromatograms is shown in **Figure 5**. The zero-time chromatograms of native β-LG B (**Figure 5a**,**c**) showed very little absorbance between 15 and 50 min and a peak at ~67 min. The heated samples (**Figure 5b**,**d**) had a peak broadly centered on ~66 min (not shown), corresponding to the whole protein. These β-LG B peaks were essentially absent after 30 min of hydrolysis.

Hydrolysis Patterns of Native β-LG. Figure 5a shows chromatograms of the zero-time sample and the 1, 2.5, and 13 min hydrolysates of native β-LG B. Clearly the patterns were very similar qualitatively, but the sizes of some of the peaks increased more rapidly than others with hydrolysis time. Peaks of peptides 2 (Trp61−Lys70), 3 (Trp61−Lys69), and 17 (Leu149−Ile162) were not apparent, but peptides 14 (Trp61−Lys70:Leu149−Ile162) and 15 (Trp61−Lys69:Leu149−Ile162) were prominent and increased with time, showing that the disulfide bond between Cys66 and Cys160 (**Figures 1** and **6**) remained intact during the hydrolysis and the hydrolysate analysis. Reduction of the hydrolysates prior to HPLC analysis changed the chromatographic pattern (**Figure 5c**) so that peptides 2, 3, and 17 were present and peptides 14 and 15 were not present, showing that DTT treatment of the hydrolysates reduced peptides 14 (Trp61−Lys70:Leu149−Ile162) and 15 (Trp61−Lys69:Leu149−Ile162) to peptides 2 (Trp61−Lys70) plus 17 (Leu149−Ile162) and 3 (Trp61−Lys69) plus 17 (Leu149−Ile162), respectively. This comparison of native β-LG B hydrolysates is shown in **Figure 7** as chromatograms **a** and **c** with the chromatographic peaks labeled appropriately.

Hydrolysis Patterns of Heated β-LG B. When the heated β-LG B was hydrolyzed and analyzed by HPLC, peptides 2, 3, 14, 15, and 17 were present (**Figures 4** and **5b**). Peptides 8 and 17 gave the largest peaks in the 1-min chromatogram and, in contrast to peptides 2, 3, 14, and 15, the peak from peptide 17 did not increase in size with hydrolysis time. This showed that the 148−149 peptide bond was very labile and that a proportion of the Cys160 of peptide 17 was not disulfide bonded to Cys66, or any other Cys residue, in the heated β-LG B. Analysis of the reduced hydrolysate (**Figure 5d**) showed that a considerable quantity of peptide 17 was released after 1 min of hydrolysis and that its concentration did not increase after 2.5 min of hydrolysis. In contrast, the peaks from peptides 4 (Ile84−Lys91) and 18 (Val41−Lys60) did increase (**Figure 5b**,**d**). After 13 min of hydrolysis (**Figure 5d**), peak 17 increased, probably because the native β-LG B was being hydrolyzed. This comparison of heated β-LG B hydrolysates is also shown in **Figure 7** as chromatograms **b** and **d**.

Disulfide Bonding of Cys160 after Heat Treatment of β-LG B. Comparison of patterns obtained using heated (**Figure 5b**) instead of native (**Figure 5a**) β-LG B showed that the 1-min hydrolysate from heated β-LG B contained two significant peaks, namely, peaks 8 (Ala142−Arg148) and 17 (Leu149−Ile162); these peptides are adjacent in the β-LG B sequence (**Figures 6** and **8**). However, these peptides appeared to be less significant in the hydrolysates of native β-LG B. Consequently, a further comparison was made to show the significant effect of heat treatment on the release of the C-terminal peptides from native and heat-treated β-LG. In this instance, the ratios of the integrated areas of peak 17 to peak 8 were calculated and plotted against hydrolysis time (**Figure 9**). Thus, peptide 8 acted as an "internal standard" for the quantitative appearance of peptide 17, which was dictated by the existence of disulfide bonding of Cys160 to other Cys residues.

The top two lines of **Figure 9** show that the peak area ratios of peptides 17 to 8 were between 1.7 and 2.0 for the reduced hydrolysates for the first 65 min of hydrolysis. (After that time, nonspecific hydrolysis diminished the quantity of peptide 8 in the mixture.) The lower two lines had a much lower ratio of peptide 17 to peptide 8, showing that a considerable proportion of peptide 17 was disulfide bonded to another peptide. For the native protein this would have been peptides 2 and 3 to give peptides 14 and 15, respectively [cf. **Figure 7a** (native) and **Figure 7b**]. Comparison of the peptide ratios (**Figure 9**) of the nonreduced samples during hydrolysis showed a high proportion (~35%) of the available peptide 17 was released initially. The reduction in this ratio during the first 65 min suggested that some disulfide shuffling could have occurred.

Figure 8 summarizes our results of the tryptic hydrolysis of heated β-LG. The top bar of the diagram represents the linear backbone of β-LG and would be identified as peak 26 in our chromatograms. The short vertical arrows above the bar show the very fastest cleavages that give rise to peptides 7, 8, and 17, which are shown in the second bar of the diagram. These peptides are released during the digestion and are detected at the earliest possible time points of analysis, before 1 min of reaction, and are therefore shaded as "very fast". Subsequent quantitative detection of the peaks during the course of the hydrolysis allowed us to classify them subjectively on their order of appearance.

The arrows throughout the diagram also show the tryptic sites in the protein and intermediate peptides, which are labeled, that give rise to the final peptide mix. In all of the representations, the disulfide sites have been left as found in the native protein.

DISCUSSION

The present study on the release of tryptic peptides from native and heat-treated β-LG has shown that the two C-terminal

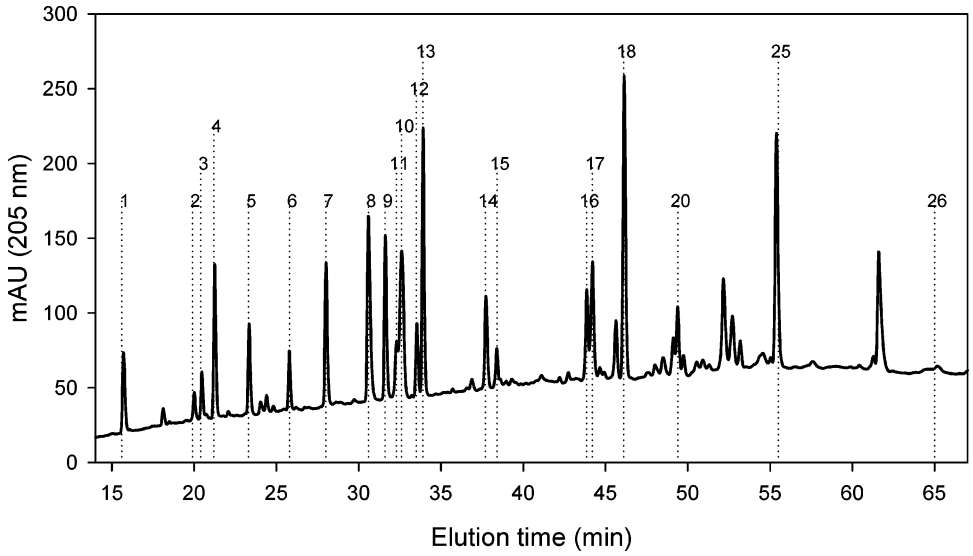

Figure 4. HPLC chromatogram (205 nm) of a 13-min tryptic hydrolysate of heated β-LG (80 °C, 15 min, at pH 7.7 and an E:S ratio of 1:10), showing the elution time and position of the identified β-LG tryptic peptides listed in **Table 1**. Peptide 19 was not clearly seen in this sample (see **Table 1**).

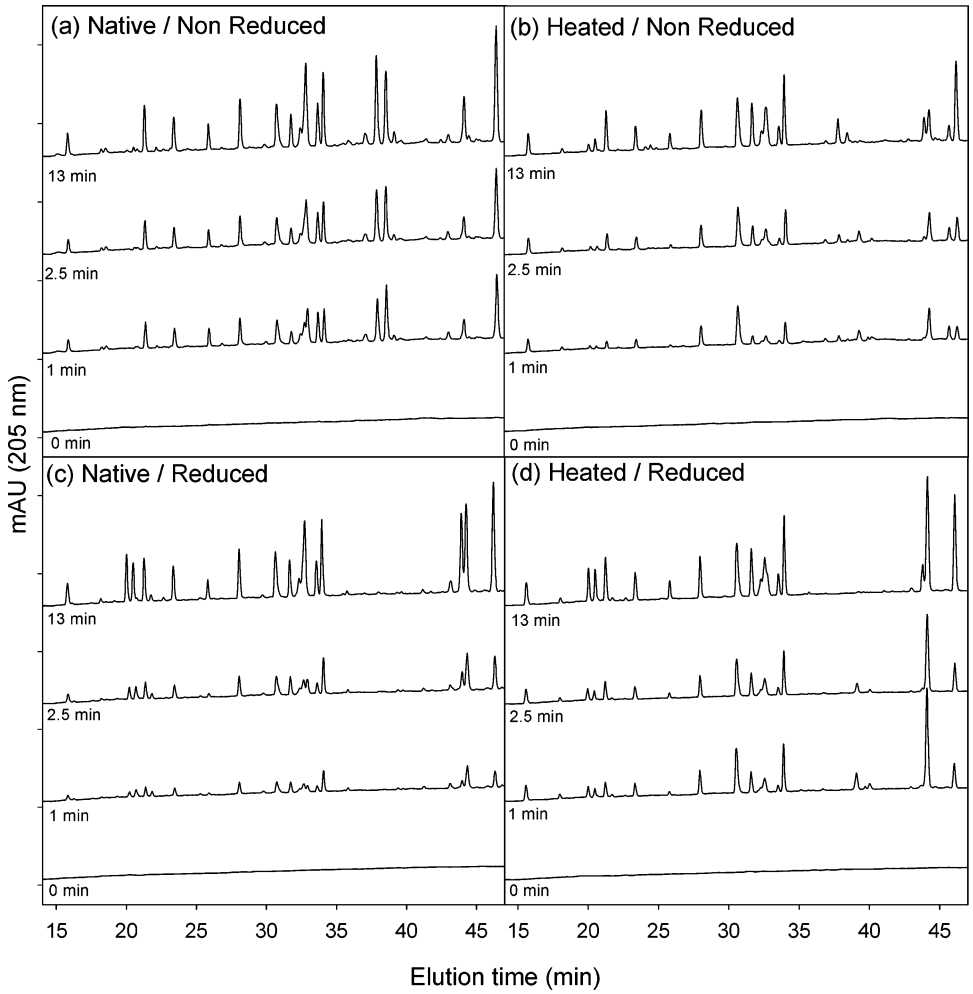

Figure 5. HPLC chromatograms showing the effects of hydrolysis time for native and heated (80 °C, 15 min) β-LG B: (**a**) native protein and nonreduced peptides; (**b**) heated protein and nonreduced peptides; (**c**) native protein and reduced peptides; (**d**) heated protein and reduced peptides.

tryptic peptides, Leu149−Ile162 and Ala142−Arg148 (**Figure 8**), are released more rapidly from heat-treated β-LG than from the native protein. This indicates that bonds Lys141−Ala142 and Arg148−Leu149 in the C-terminal region are more accessible to the active site of trypsin in the heat-treated protein, which suggests that the 25-residue C-terminal region of the native protein is unlikely to be involved in any formal structure, such as tight turns, β sheet, or α helix.

It was also shown that ~35% of the Cys160 residues were not part of a disulfide bond, whereas all of the Cys160 residues are disulfide bonded to Cys66 in the native protein (**Figures 1** and **6**). β-LG has five Cys residues; if the distribution of

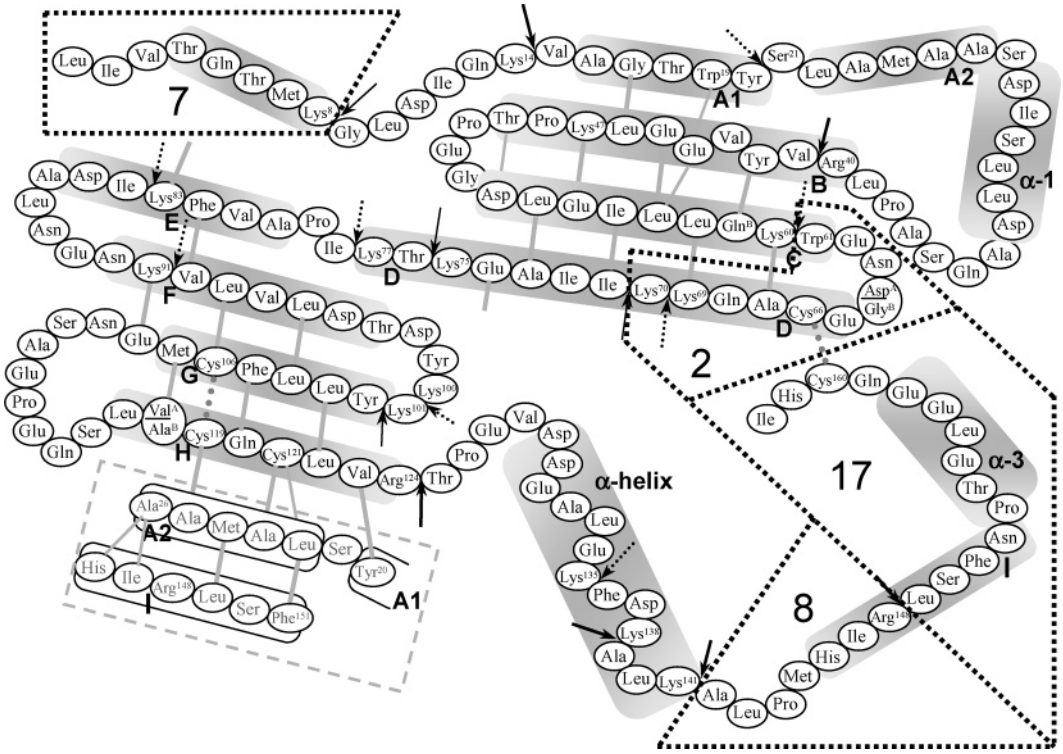

Figure 6. Schematic representation of the domains in β-LG affected by trypsin hydrolysis. The shaded regions show the amino acids that have H-bonds representative of β-sheet or α-helix. Labels A–I indicate the identified β-strands that make up the two sheets (**Figure 1**). The helical regions are identified as α-1, α-helix, and α-3. The boxed regions show the rapidly released peptides from heated β-LG (7, 8, and 17), and the peptide that is disulfide-bonded to 17 in native β-LG is shown as 2.

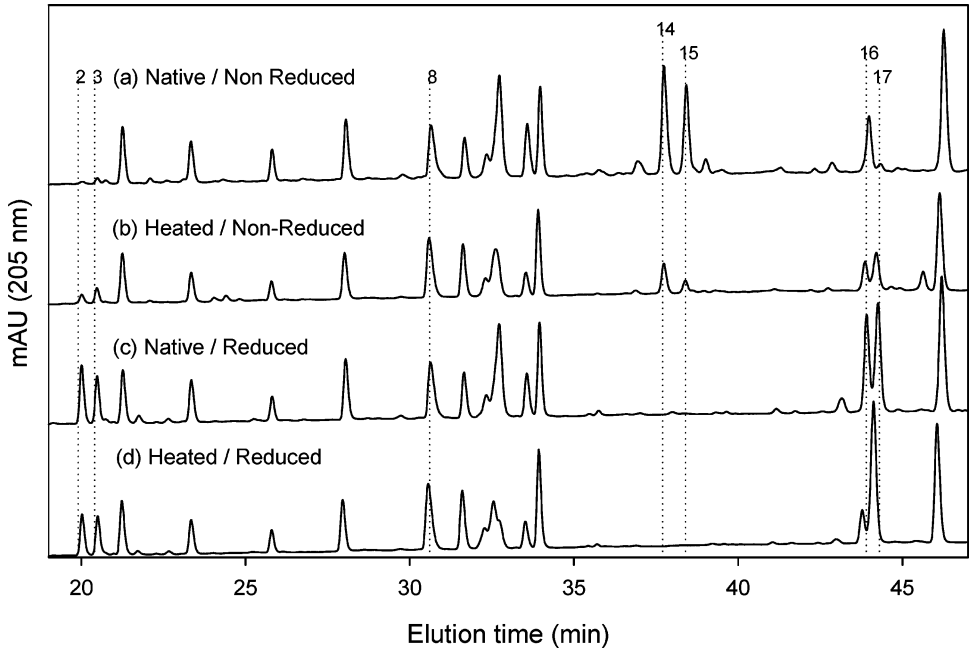

Figure 7. Comparison of the resulting peptides after 13 min of tryptic digestion of heated β-LG (80 °C, 15 min) and native β-LG B: (**a**) native protein and nonreduced peptides; (**b**) heated protein and nonreduced peptides; (**c**) native protein and reduced peptides; (**d**) heated protein and reduced peptides.

unbonded Cys residues (CysH) was evenly divided among these sites, then it might be expected that 20% of Cys160 would be unbonded. The data in **Figures 7** and **9** suggest that the initial proportion is close to 35%, showing that about one-third of the Cys160 residues could be available to interchange with a disulfide bond in another β-LG molecule or in some other protein. The gradual decrease of CysH160 during tryptic hydrolysis (**Figure 9**) could have occurred because of disulfide bond shuffling at pH 7.85 or a gradual oxidation of all the thiols. Nevertheless, and regardless of subsequent reactions, there is an initial burst of free CysH160 in heat-treated β-LG. It seems likely that this reaction occurs simultaneously with, or shortly after, the CysH121−CysH119 interchange (*16*).

The patterns in **Figures 5** and **7** show that there are bumps eluting where peaks 3 (Trp61−Lys69) and 17 (Leu149−Ile162) normally elute. (The identity of the peptides in these peaks was

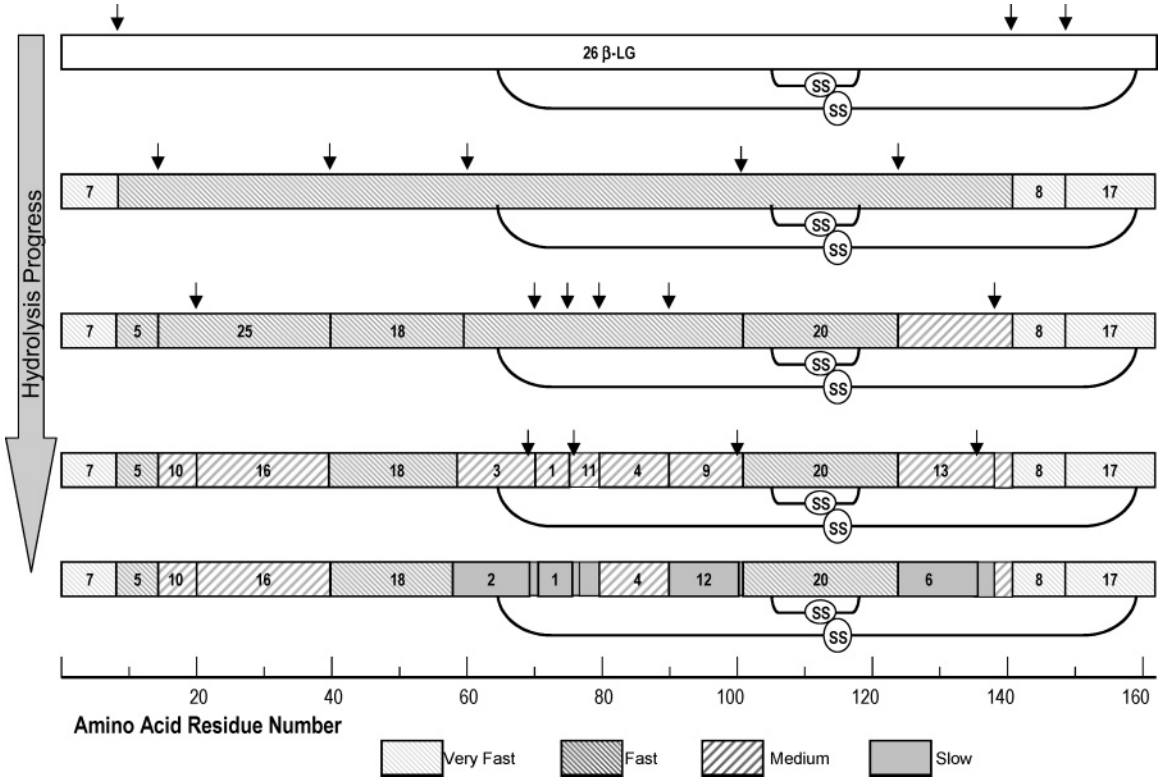

Figure 8. Line representation of peptide generation during tryptic hydrolysis of β-LG. Relative rates of appearance are represented by the different shadings of the peptide segments. The scale represents the amino acid residue number, and the arrows indicate the hydrolysis sites for trypsin (Lys, Arg, and Tyr20–Ser21).

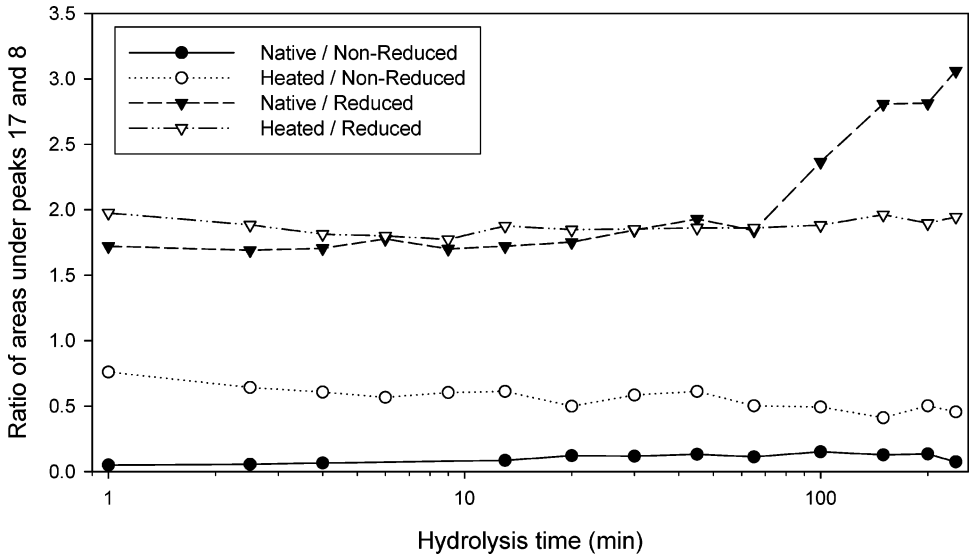

Figure 9. Effect of hydrolysis time on the ratios of the peak areas of peptides 17 (Leu149–Ile162) and 8 (Ala142–Arg148) from native and heated β-LG (80 °C, 15 min), before and after hydrolysate reduction with DTT.

not determined in these samples.) These bumps are small for the native, nonreduced sample (a) and, consequently, some shuffling probably occurs but it is minor.

Examination of the hydrolysate of heated β-LG (**Figure 5b**) shows that there is an early release of Trp61−Lys70, Trp61−Lys70:Leu149−Ile162, and Leu149−Ile162, possibly indicating that a small proportion of Cys66 is also not incorporated into a disulfide bond. The techniques used in the present study could not distinguish between Cys106, Cys119, and Cys121 to identify the proportions of these residues that were or were not involved in disulfide bonds. However, the presence of unbonded Cys119 in heated β-LG was shown by Croguennec et al. (*16*), and Surroca et al. (*17*) showed that peptide Tyr102−Arg124 could be disulfide linked to Leu149−Ile162 or another Tyr102−Arg124 peptide. This evidence supports the possibility put forward by Manderson et al. (*19*) that the native protein is one of 30 theoretically possible covalent structures with a polypeptide chain, two disulfide bonds, and a free Cys residue. Thus there are 29 possible non-native primary structures and maybe even more tertiary structures. At higher temperatures, it might be expected that the lowest energy conformation is not the native structure and that, once the system is cooled, some of these non-native species would be trapped. The present result, namely, that a significant proportion of the non-native β-LG has Cys160

not in a disulfide bond, could be a consequence of an overall energy gain at high temperature by the loosening of the C-terminal 25 amino acid sequence. This possibility is supported to some extent by the H/D exchange experiments of Belloque and Smith (*30*) and Edwards et al. (*31*) and the refolding experiments of Yagi et al. (*32*).

In conclusion, the present results demonstrate that trypsin cleaves heat-treated β-LG at the same sites as in the native protein but that the release of the C-terminal peptides is faster from the heat-treated protein than from the native protein. The peptide Leu149−Ile162:SS:Trp61−Lys70 predominates in the hydrolysate of native β-LG, whereas a proportion of the Leu149−Ile162 peptide is found in the hydrolysate of the heated protein, demonstrating that a significant proportion of Cys160 has become one of the Cys residues that is not involved in a disulfide bond. Consequently, Cys160 is likely to be a major player in the inter-protein disulfide bonding responsible for the covalent cross-linking in heat-induced whey protein aggregates.

ABBREVIATIONS USED

ESI, electrospray ionization; 2D, two-dimensional; κ-CN, κ-casein; 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; DTT, DL-dithiothreitol; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization−time of flight.

ACKNOWLEDGMENT

We thank Don Otter, Christina Coker, and Carmen Norris for help with HPLC and mass spectrometry and Geoff Jameson, Pat Edwards, and Lindsay Sawyer for valuable discussions. We give particular thanks to a referee who suggested that disulfide bond shuffling during hydrolysis could be significant.

LITERATURE CITED

(1) Sawyer, W. H. Complex between β-lactoglobulin and κ-casein. A review. *J. Dairy Sci.* **1969**, *52*, 1347−1355.

(2) Elfagm, A. A.; Wheelock, J. V. Interaction of bovine α-lactalbumin and β-lactoglobulin in heated milk. *J. Dairy Sci.* **1978**, *61*, 28−32.

(3) Lalande, M.; Tissier, J. P.; Corrieu, G. Fouling of a plate heat exchanger used in ultra-high-temperature sterilization of milk. *J. Dairy Res.* **1984**, *51*, 557−568.

(4) 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.

(5) McKenzie, H. A. β-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1971; pp 257−330.

(6) Havea, P.; Carr, A. J.; Creamer, L. K. The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels. *J. Dairy Res.* **2004**, *71*, 330−339.

(7) Creamer, L. K.; Sawyer, L. β-Lactoglobulin. In *Encyclopedia of Dairy Sciences*; Roginski, H., Fuquay, J. W., Fox, P. F., Eds.; Academic Press: San Diego, CA, 2003; pp 1932−1939.

(8) 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.

(9) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, E. N.; Jameson, G. B. Functional implications of structural differences between variants A and B of bovine β-lactoglobulin. *Protein Sci.* **1999**, *8*, 75−83.

(10) Uhrinova, S.; Uhrin, D.; Denton, H.; Smith, M.; Sawyer, L.; Barlow, P. N. Complete assignment of ^{1}H, ^{13}C and ^{15}N chemical shifts for bovine β-lactoglobulin: secondary structure and topology of the native state is retained in a partially unfolded form. *J. Biomol. NMR* **1998**, *12*, 89−107.

(11) Kuwata, K.; Hoshino, M.; Forge, V.; Era, S.; Batt, C. A.; Goto, Y. Solution structure and dynamics of bovine β-lactoglobulin A. *Protein Sci.* **1999**, *8*, 2541−2545.

(12) Fogolari, F.; Ragona, L.; Zetta, L.; Romagnoli, S.; De Kruif, K. G.; Molinari, H. Monomeric bovine β-lactoglobulin adopts a β-barrel fold at pH 2. *FEBS Lett.* **1998**, *436*, 149−154.

(13) Mulvihill, D. M.; Donovan, M. Whey proteins and their thermal denaturation−a review. *Irish J. Food Sci. Technol.* **1987**, *11*, 43−75.

(14) 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.

(15) 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.

(16) 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.

(17) Surroca, Y.; Haverkamp, J.; Heck, A. J. R. Towards the understanding of molecular mechanisms in the early stages of heat-induced aggregation of β-lactoglobulin AB. *J. Chromatogr. A* **2002**, *970*, 275−285.

(18) 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.

(19) 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.

(20) 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.

(21) 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.

(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) Bienvenue, A.; Norris, C. S.; Boland, M. J.; Creamer, L. K.; Jimenez-Flores, R. Disulfide bonding patterns between β-lactoglobulin and κ-casein in a heated and spray-dried milk model. *J. Dairy Sci.* **2002**, *85* (Suppl. 1), 340.

(24) 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*, 743−747.

(25) Nilsson, H. Hydrolysis of β-lactoglobulin A, B and C with trypsin. Master's thesis, Lund University, 2000.

(26) Dalgalarrondo, M.; Chobert, J. M.; Dufour, E.; Bertrandharb, 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.

(27) 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.

(28) Morgan, F.; Léonil, J.; Mollé, D.; Bouhallab, S. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413−417.

(29) Dufour, E.; Dalgalarrondo, M.; Adam, L. Conformation of β-lactoglobulin at an oil/water interface as determined from proteolysis and spectroscopic methods. *J. Colloid Interface Sci.* **1998**, *207*, 264−272.

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

(31) 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.

(32) Yagi, M.; Sakurai, K.; Kalidas, C.; Batt, C. A.; Goto, Y. Reversible unfolding of bovine β-lactoglobulin mutants without a free thiol group. *J. Biol. Chem.* **2003**, *278*, 47009−47015.

(33) Creamer, L. K.; Nilsson, H. C.; Paulsson, M. A.; Coker, C. J.; Hill, J. P.; Jiménez-Flores, R.. Effect of genetic variation on the tryptic hydrolysis of bovine β-lactoglobulin A, B, and C. *J. Dairy Sci.* **2004**, *87*, in press.

Received for review April 15, 2004. Revised manuscript received September 15, 2004. Accepted September 21, 2004. We acknowledge financial support for this work from the New Zealand Foundation for Research, Science and Technology, Contracts DRIX0001 and DRIX0201, the California Dairy Research Foundation, the CSU−Agricultural Research Initiative, Anna Whitlock's Foundation, Makarna Lindeqvist Foundation, and Svenska Livsmedelstekniska föreningen, Lund University.

JF049388Y