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Heat-Induced Covalent Complex between Casein Micelles and β-Lactoglobulin from Goat's Milk: Identification of an Involved Disulfide Bond

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Goat milk is characterized by a very low heat stability that could be attributed, in part, to the covalent interaction between whey proteins and casein micelles. However, the formation of such a complex in goat milk has never been evidenced. This study was designed to assess whether heat-induced covalent interaction occurs between purified casein micelles and β-lactoglobulin. We used a multiple approach of ultracentrifugation of heated mixture, chromatographic fractionation of resuspended pellets, sequential enzyme digestion of disulfide-linked oligomers, and identification of disulfide-linked peptides by on-line liquid chromatography−electrospray ionization mass spectrometry (LC−ESI/MS), and tandem MS. We identified three different types of disulfide links: (1) expected intermolecular bridges between β-Lg molecules; (2) disulfide bond involving two κ-casein molecules; and (3) a disulfide bond between two peptides, one from β-Lg and the other from κ-casein. The involved sites in this last bond were Cys^{160} of β-Lg and Cys^{88} of κ-casein. Although the identified heterolinkage is possibly only one of several different types, the results of this study constitute the first direct evidence of the formation of a covalent complex between casein micelles and β-lactoglobulin derived from goat milk.

KEYWORDS: Goat milk; β-lactoglobulin/κ-casein complex; enzyme digestion; disulfide bond; mass spectrometry

INTRODUCTION

Production of goat's milk, its processing, and its use in the cheese industry have considerably increased during the last 10 years. It is industrially processed into a variety of dairy products including UHT-treated milk. However, because of its relatively low economic importance, there have been only a few investigations on the changes of its physicochemical properties during processing and storage.

The major problem encountered is the lower heat stability of caprine milk at its natural pH as compared to that of bovine milk (*1*). Indeed, the heat stability of caprine milk exhibits a complex dependence on pH values (*2*). The typical heat stability versus pH profiles obtained for goat milk showed a maximum stability at pH 6.9−7, which is higher than its natural pH (6.5−6.6) (*2*, *3*). As far as bovine milk is concerned, it is now generally accepted that β-lactoglobulin (β-Lg) and κ-casein (κ-CN) interaction is an important parameter involved in the pH-dependent heat stability (*4*).

In fact, the formation of such a complex has been extensively studied and well described in milk system (*5*), in model micelle systems (*6*), and in solution of pure β-Lg and κ-CN (*7*). From these studies, the main conclusion is that noncovalent complexes form prior to intermolecular disulfide bond formation that is detectable after heating at a temperature higher than 75−80 °C. The main information about the interaction between whey proteins and caseins was deduced after ultracentrifugation of heated samples and analysis of residual material in the supernatant; i.e., residual β-Lg and individual casein dissociated from the micelles (*2*, *8*). In these works, it is assumed that whey proteins, which co-sediment with the micellar pellets, are bound to the micelles, although the large aggregates from self-association of β-Lg could also sediment with the micelles (*9*).

Unlike the detailed studies on bovine milk, there is no evidence regarding the formation and the nature of such complexes between caseins and β-Lg from goat's milk, except the work of Wallander et al. (*10*) who suggested the formation of a heat-induced complex between purified β-Lg and κ-CN. Recently, two studies suggested a possible formation of such complexes to explain the effect of pH on the heat stability of goat's milk. Anema and Stanley (*2*) suggested that the poor heat stability of goat milk at its natural pH could be related to

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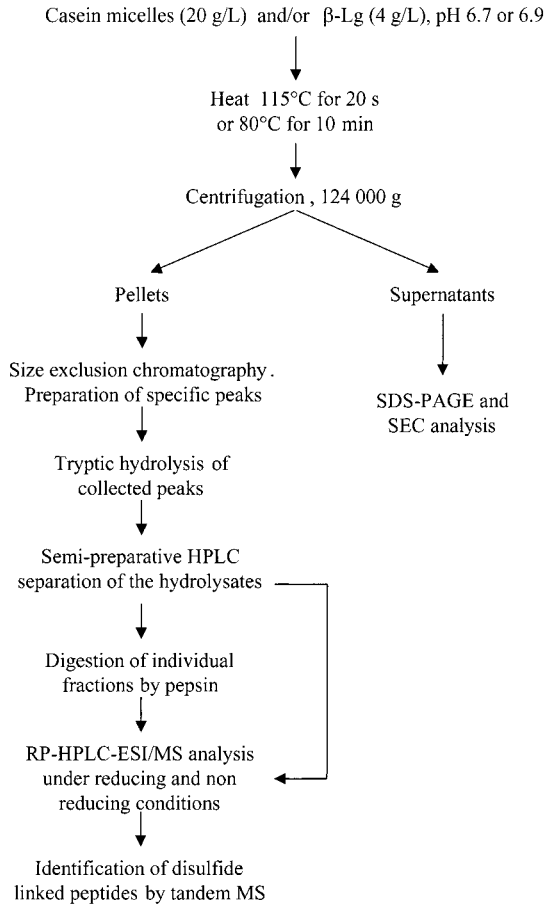

Figure 1. Protocol for the isolation and identification of heat-induced β-lactoglobulin/casein micelles complex.

a low occurrence of the interaction between whey proteins and caseins. Morgan et al. (*11*) hypothesized that the formation of a complex could explain the observed improvement of the heat stability at pH 6.9.

As part of a larger study on the origin of the heat instability of goat's milk, the present investigation aimed to obtain unequivocal evidence of the formation of a covalent complex between β-Lg and caseins from goat's milk through direct identification of at least one of the involved sites. For this purpose our research approach was the following: heat treatment of a mixture of purified β-Lg and casein micelles, ultracentrifugation, chromatographic analysis and fractionation, enzymatic hydrolysis, search for and identification of heat-induced disulfide bond(s) by on-line liquid chromatography−electrospray ionization mass spectrometry (LC−ESI/MS) and tandem MS. The obtained results clearly demonstrate the occurrence of a covalent linkage between β-Lg and κ-CN after heating.

MATERIALS AND METHODS

Materials. Raw milk was obtained from individual goats, homozygous at the α_{s1}-casein locus EE, at a local dairy farm. The milk was first skimmed by centrifugation at 3000*g* for 20 min at 30 °C. Whole native casein and whey proteins were separated by membrane technology as previously described by Pierre et al. (*12*). Briefly, casein micelles were extracted from skimmed and debacterized milk by microfiltration on a 0.1-μm membrane. The microfiltrate was then concentrated and diafiltered on a 5-kDa ultrafiltration membrane to prepare whey protein isolate (WPI). The resulting permeate (i.e., milk ultrafiltrate) was used for the heat-treatment experiments described below. Casein micelles and whey protein fractions were lyophilized before use. Pure goat's β-Lg was purified from WPI by preparative cation-exchange chromatography according to the method described by Andrews et al. (*13*) but using a SOURCE 15Q column (Amersham Pharmacia Biotech, Uppsala, Sweden). The purified protein was dialyzed extensively against Milli-Q purified water and lyophilized.

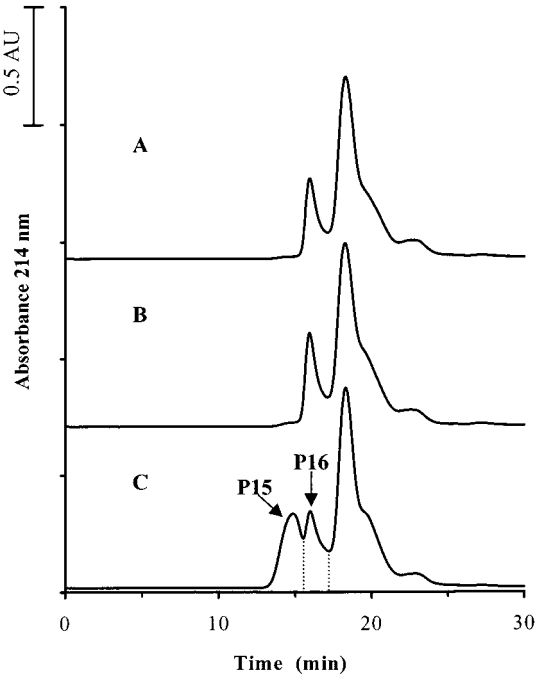

Figure 2. Elution profiles of ultracentifugation pellets of unheated and heated samples obtained by size-exclusion chromatography on a superdex 75 column. (A) unheated β-Lg/casein mixture; (B) heated casein micelles; (C) heated β-Lg/casein mixture. Heating conditions were 115 °C, 20 s at pH 6.7. P15: peak eluted at 15 min; P16: peak eluted at 16 min.

Tosyl phenylalanine−chloromethyl ketone treated trypsin (EC 3.4.21.4) and porcine pepsin (EC 3.4.21.1) were obtained from Sigma Chemical (St. Louis, MO). All other reagents were of analytical grade.

Heat Treatment Experiments. The casein micelles and β-Lg were resuspended in the milk ultrafiltrate, and the pH was adjusted to 6.7 or 6.9 with 2.5% NH_4OH. For each pH value, three sample tubes were prepared: casein micelles (20 mg/L), β-Lg (4 mg/mL), and a mixture of casein micelles (20 mg/L) and β-Lg (4 mg/mL). The samples were heated in a thermostatically controlled oil bath, in glass test tubes, either for 10 min at 80 °C or for 20 s at 115 °C. After the samples were heated, they were cooled by immersion in iced water. Samples were then ultracentrifuged at 124,000*g* for 45 min at 20 °C. Resulting supernatants were analyzed by SDS−PAGE and size-exclusion chromatography. The pellets were resolubilized in 0.05 M Tris/HCl buffer pH 8, containing 0.075 M NaCl and 4.4 M urea. The various steps followed for the isolation and identification of β-Lg/casein micelles complex are summarized in Figure 1.

Size-Exclusion Chromatography (SEC). *Analytical SEC.* Supernatants and solubilized pellets were analyzed by SEC on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with 0.1 M Tris/HCl buffer and 0.15 M NaCl, pH 8. Samples were diluted, and 200 μL was injected and eluted at a flow rate of 0.5 mL/min. The apparatus used was the Pharmacia fast protein liquid chromatography system equipped with a LCC-500 system controller, two P500 pumps, and an UV detector. The absorbance was monitored at 214 nm.

Preparative SEC. Heat-induced polymers were prepared using a Pharmacia BioPilot system fitted with a preparative column (HiLoad 26/60 Superdex 75 column) equilibrated with the same buffer as above and elution performed at 1 mL/min. The chromatographic peaks of interest were collected, and fractions were extensively dialyzed against Milli-Q purified water before lyophilization.

SDS−PAGE. SDS−PAGE of the polymeric material eluted from SEC (i.e., peaks 15 and 16 min) was performed under reducing and nonreducing conditions using the method described by Anema and Stanley (*2*).

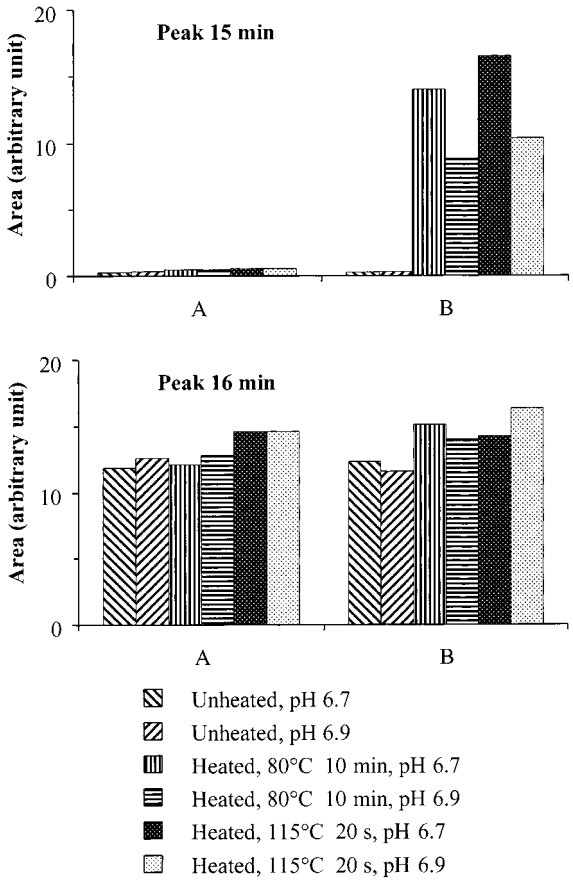

Figure 3. Evolution of peaks eluted at 15 and 16 min as a function of pH and heating temperature. (A) casein micelles alone; (B) β-Lg/casein micelles mixture.

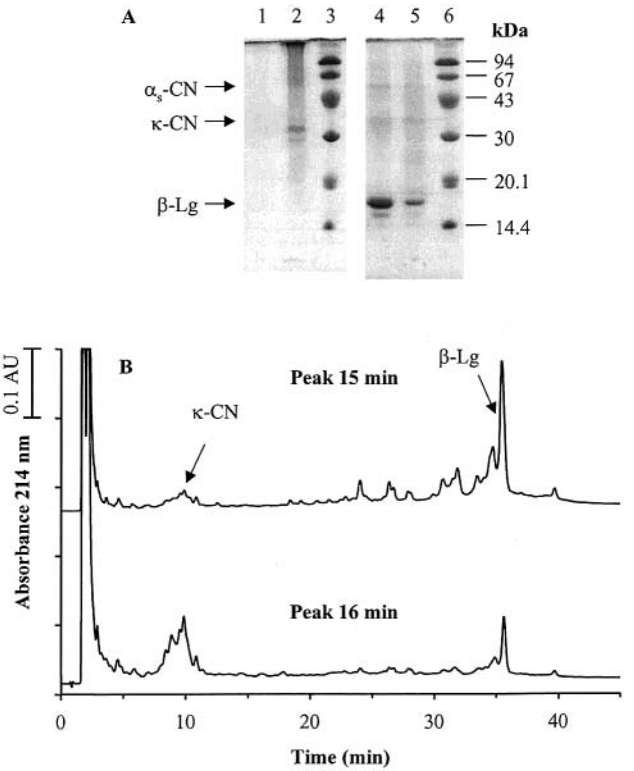

Figure 4. Characterization of protein material contained in peaks 15 and 16 min. (A) SDS−PAGE before (1, 2) and after (4, 5) disulfide bond reduction. Samples: 1 and 4, material from peak 15 min; 2 and 5, material from peak 16 min; 3 and 6, molecular weight standards. (B) RP-HPLC analysis of samples reduced with DTT.

RP-HPLC. Material in peaks collected from SEC was analyzed by reversed-phase HPLC on a Vydac C4 column (4.6 mm i.d. × 150 mm). The column was equilibrated in 62% solvent A (0.106% trifluoroacetic acid [TFA] in water) and 38% solvent B (0.1% TFA in 80% aqueous acetonitrile). Elution was performed at a flow rate of 1 mL/min with linear gradient up to 57% solvent B in 45 min. The column temperature was 40 °C, and peak detection was monitored at 214 nm. The eluted proteins were identified by electrospray mass spectrometry performed on collected individual peaks.

Enzyme Digestion and RP-HPLC Fractionation of the Hydrolysates. The polymeric material collected from SEC was first submitted to the hydrolysis by trypsin at 40 °C in 7.5 mM phosphate buffer with 2.2 M urea, pH 6.5, for 150 min at an enzyme/substrate ratio of 1:250 (w/w). The resulting hydrolysate was fractionated by semipreparative RP-HPLC using a stepwise gradient of solvent B (0.1% TFA in 80% aqueous acetonitrile) on a RESOURCE RPC 3-mL column (6.4 mm i.d. × 100 mm, Amersham Pharmacia) equilibrated in solvent A (0.106% TFA in water) at a flow rate of 2 mL/min. The collected fractions were freeze-dried and each fraction was further hydrolyzed by pepsin. The peptic hydrolysis was performed in 20 mM KCl/HCl buffer pH 2.1 at an enzyme/substrate ratio of 1:250 (w/w). Digestion was performed at 37 °C during 16 h.

These samples were further analyzed before and after reduction with dithiothreitol (DTT). For reduction experiments, samples were treated overnight at ambient temperature with 10 mM DTT in 5 mM Tris/HCl buffer, pH 8.

Liquid Chromatography−Electrospray Mass Spectrometry (LC−ESI/MS) and Tandem MS. Mass spectra were recorded on a PE-Sciex API III Plus mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada). Tryptic or tryptic/peptic peptides were separated by RP-HPLC on a Symmetry C18 column (2.1 mm i.d. × 150 mm, Waters, Milford, MA) directly interfaced with the mass spectrometer. Elution was performed at a flow rate of 0.25 mL/min (40 °C) using solvent A (0.106% TFA in water) and solvent B (0.1% TFA in 80% aqueous acetonitrile). A postcolumn flow splitter was used to introduce 1/10 of the HPLC eluate into the mass spectrometer. The ion source voltage and the orifice voltage were set at 4.8 kV and 70/90 V, respectively. The mass spectrometer was operated in positive ion mode and was scanned over a *m*/*z* range of 500−2400 with a step size of 0.3 Da and a dwell time of 1 ms per step. Molecular masses were determined from the multiple charge ions using BioMultiView Software 1.3.1 (PE Sciex).

Peptides of interest detected during LC−ESI/MS were collected, freeze-dried, and submitted to tandem MS. Samples were diluted in 49:49:2 water/acetonitrile/formic acid (v/v/v) and were infused in the mass spectrometer at 3 μL/min. The collision energy, chosen as a function of *m*/*z* of the parent ion, was in the range of 25 to 50 eV.

RESULTS

To assess the occurrence of a covalent β-Lg/caseins complex, a protocol for the isolation of involved links was devised (Figure 1). The first step was to determine the effect of incubation pH and heating temperature and the distribution of heat-induced specific oligomers after ultracentrifugation. Casein micelles, β-Lg, or a mixture of both were heated either at 115 °C or 80 °C at pH 6.7 and 6.9. Comparison of SEC profiles of heat-treated samples constituted the first criteria in the search for a heat-induced complex between caseins and β-Lg. SEC profiles of supernatants, as well as their SDS−PAGE pattern, do not show the presence of new generated polymers. Less than 10% of individual proteins was recovered in the supernatant of the heated mixture (results not shown). These results are in agreement with those recently reported by Anema and Stanley (*2*) who showed that less than 20−30% of individual proteins were recovered in the supernatant after centrifugation of heated milk at 65,000*g*.

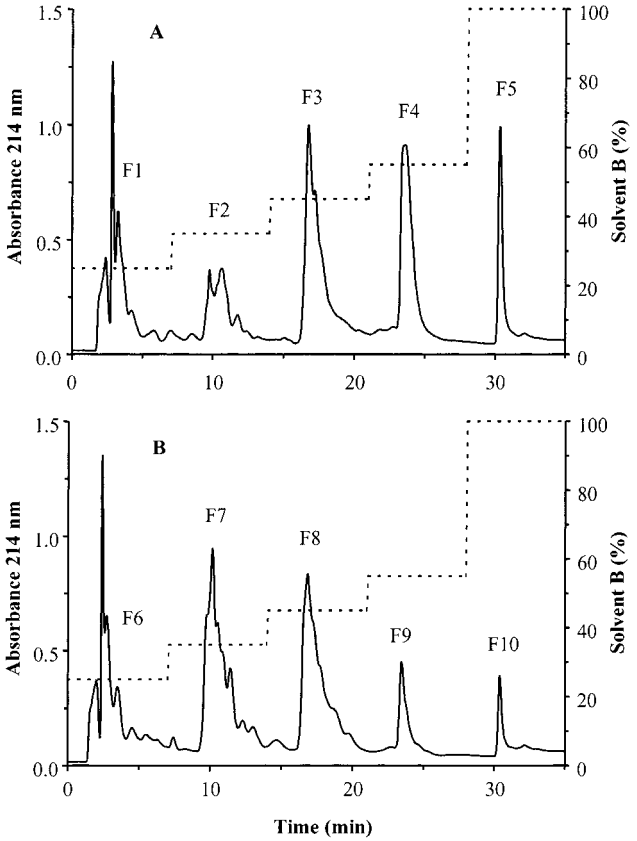

Figure 5. Semipreparative RP-HPLC fractionation of tryptic digest of the high molecular weight material eluted in peak 15 min (A) and peak 16 min (B) from size-exclusion chromatography. Separation was performed with a stepwise gradient (dashed line) on a RESOURCE RPC column (6.4 mm i.d. × 100 mm). Fractions were collected manually at a flow rate of 2 mL/min.

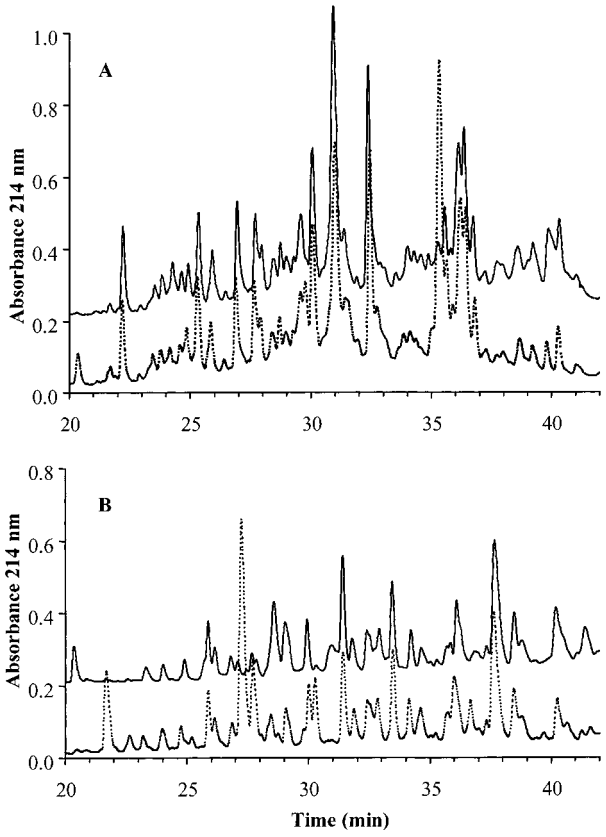

Figure 6. Typical RP-HPLC chromatograms of peptic digest of fractions F2 (A) and F8 (B) before (top profile) and after (bottom) disulfide bond reduction with dithiothreitol. Reduction was performed with 10 mM DTT in 5 mM Tris/HCl buffer pH 8.

Table 1. LC–ESI/MS Results for the DTT-Sensitive Peptides of Fractions F2, F3, F4, F7, F8, and F9 Submitted to Peptic Digestion and Analyzed under Reducing and Non-Reducing Conditions

sample	measured masses
nonreduced	977.3; 1340.7; 2231.4; 2314.2; 2670.8; 2896.5; 3117.6; 3121.4; 4450.9
reduced	649.4; 671.3; 692.9; 791.6; 826.4; 929.3; 939.5; 986.6; 991.4; 1053.5; 1192.0; 1370.6; 1399.4; 1433.2; 1483.7; 1556.0; 1561.5; 1858.5; 1960.0; 2003.9; 2039.8; 2106.7; 2227.0

Table 2. Identification by Tandem Mass Spectrometry of Peptides Generated after Reduction

measured mass	amino acid sequence	protein precursor	theoretical mass
649.4	[f 117–122]	β-Lg	649.3
671.3	[f 157–162]	β-Lg	671.2
991.4	[f 87–95]	κ-CN	991.4
1192.0	[f 61–70]	β-Lg	1191.5
1433.2	[f 58–69]	β-Lg	1432.7
1556.0	[f 149–162]	β-Lg	1556.8
1561.5	[f 58–70]	β-Lg	1561.8
1960.0	[f 1–16]	κ-CN	1960.1
2003.9	[f 105–122]	β-Lg	2003.3
2106.7	[f 1–17]	κ-CN	2107.3
2227.0	[f 76–95]	κ-CN	2227.5

Consequently, we do not consider the supernatant material any further in this paper, and further characterization was focused on the material that was recovered in the ultracentrifugation pellets.

Effect of pH and Heating Conditions on SEC Profiles. SEC profiles of pellets from heated caseins/β-Lg mixture (Figure 2C) showed the appearance of a peak eluted at 15 min (apparent molecular weight ≥ 100 kDa) which was not detected in the unheated mixture nor in the control sample (i.e., heated casein micelles) (Figure 2A, B). Also, the peak eluted at 16 min (apparent molecular weight between 70 and 100 kDa) was considered even if it was present in the unheated sample, because its intensity slightly increased after heating (see below). It should be noted that the pellet obtained for heated β-Lg alone was insoluble even in the presence of urea 8 M. We were then unable to determine the protein material of this pellet, which explains the absence of a chromatographic peak. The effects of pH and heating temperature on the intensity of these two peaks are shown in Figure 3. The material eluted at peak 16 min was already present in the control sample, whatever the experimental conditions, with an overall intensity similar to that found for β-Lg/casein micelles mixture. However, its intensity slightly increased after heating at 80 °C, as well as at 115 °C, in both samples i.e., casein micelles alone and β-Lg/casein micelle mixture. In contrast, the protein material eluted at 15 min appeared only after heating the casein micelles/β-Lg mixture. Its intensity was higher after heating at pH 6.7 than at pH 6.9 for both temperatures studied (Figure 3). Also, its abundance was higher after heating at 115 °C. Therefore, heat treatment of a mixture of casein micelles and β-Lg at 115 °C, pH 6.7, promoted the formation of the material present in peak 15 min.

Table 3. Identification and Assignment of Interchain Disulfide-Linked Peptides by LC–MS/MS and Their Localization in the Primary Sequences of Protein Precursors

protein precursors	measured mass nonreduced sample	constituted masses reduced sample	identified link[c]	theoretical mass
β-Lg + κ-CN	2896.5[a]	671.3 + 2227.0	β-Lg[f 157–162]–S–S–κ-CN[f 76–95]	2896.3
β-Lg	1340.7[a]	671.3 + 671.3	β-Lg[f 157–162]–S–S–β-Lg[f 157–162]	1340.6
	2231.4[a]	671.3 + 1561.5	β-Lg[f 157–162]–S–S–β-Lg[f 58–70]	2230.8
	2670.8[b]	671.3 + 2003.9	β-Lg[f 157–162]–S–S–β-Lg[f 105–122]	2671.2
	3117.6[a]	1556.0 + 1561.5	β-Lg[f 149–162]–S–S–β-Lg[f 58–70]	3115.5
	3121.4[a]	1561.5 + 1561.5	β-Lg[f 58–70]–S–S–β-Lg[f 58–70]	3121.0
κ-CN	4450.9[a]	2227.0 + 2227.0	κ-CN[f 76–95]–S–S–κ-CN[f 76–95]	4452.0

[a] Measured mass in nonreduced sample = combination of masses in reduced sample – (2 protons) added during the reduction step. [b] Measured mass in nonreduced sample = combination of masses in reduced sample – (4 protons) added during the reduction step (this peptide contains an intra disulfide bond Cys^{106}–Cys^{119} of β-Lg. [c] Amino acid sequences identified by tandem mass spectrometry after purification and reduction of disulfide linked peptides.

Consequently, these pH and temperature values were selected for the next purification and characterization steps.

Characterization of the Material Eluted in Peaks 15 and 16 Min. A sufficient amount of the high molecular weight material (HMWM) eluted in peaks 15 and 16 min was purified on preparative size-exclusion chromatography. About 10 mg of the material present in each peak was then purified for further characterization.

SDS−PAGE and RP-HPLC of HMWM. To determine the nature of proteins involved in the formation of the HMWM, SDS−PAGE analysis was performed with and without disulfide bond reduction. Results of both samples are presented in Figure 4A. For the material from peak 15 min, no stained band was detected under nonreducing conditions, suggesting that the molecular weight of the involved species was too high to enter the gel. The electrophoretic pattern of unreduced material of peak 16 min showed the presence of monomeric κ-casein and several unresolved bands with molecular weights higher than 60 kDa. SDS−PAGE of reduced samples revealed clearly that the HMWM of both peaks contained a net band corresponding to the monomeric form of β-Lg and a faint band corresponding to κ-casein. β-Lg was more abundant in the material of peak 15 than that of peak 16. These results suggest an important role of disulfide bonds in the formation of this heat-induced material. As shown in Figure 4B, the presence of β-Lg and κ-casein in these samples was confirmed by RP-HPLC analysis under reducing conditions. Traces of α_{s1}- and α_{s2}-casein were also detected in the material originating from peak 16 min.

Enzyme Digestion of HMWM and Fractionation. HMWM of peaks 15 and 16 min were submitted to tryptic digestion, and the hydrolysates were then fractionated by semipreparative RP-HPLC. The collected fractions from each sample are indicated in Figure 5. LC−ESI/MS analysis of each fraction, with and without reducing agent, indicated that fractions F2, F3, and F4, as well as F7, F8, and F9, contained disulfide-linked peptides (results not shown).

Peptic Digestion and Localization of Disulfide Bond Peptides. To identify the nature of disulfide-linked peptides, the material of the six fractions indicated above was further digested by pepsin, and the resulting peptides were analyzed by LC−ESI/MS under native and reducing conditions. Figure 6 shows typical chromatographic profiles of digested fractions F2 and F8 with and without DTT. The molecular masses of peptides that disappeared after addition of DTT and of the resulting new peptides were considered. Table 1 summarizes all the molecular masses detected in the six fractions. Nine molecular masses from M_r = 977.3 to 4450.9 which corresponded to DTT-sensitive peptides were detected. In the same time, more than 20 molecular masses were generated under reducing conditions. The first identification step of these reduced peptides started with the masses determination in combination with the known primary amino acid sequences of goat milk proteins (α_{s2}-casein, κ-casein, and β-Lg) including post-transductional modifications and the presence of at least one cysteine residue. All the searched masses were found in the primary sequence of either β-Lg or κ-casein, and none were found in α_{s2}-casein sequence. The assignment of some of these sequences was performed by sequence analysis of the peptides using tandem MS. The corresponding results are summarized in Table 2. Among the identified sequences, seven that originated from β-Lg and five that originated from κ-casein were found. Different theoretical possibilities of disulfide-linked peptides were devised by combination of identified peptides (Table 3). The results show that four peptides are fragments linked by newly formed disulfide bonds between two β-Lg molecules (i.e., β-Lg[f 157−162]−S−S−β-Lg[f 157−162]; β-Lg[f 157−162]−S−S−β-Lg-[f 105−122]; β-Lg[f 58−70]−S−S−β-Lg[f 58−70]) or between two κ-casein molecules (i.e., κ-CN[f 76−95]−S−S−κ-CN[f 76−95]), and only two peptides are linked by the original disulfide bonds of β-Lg (i.e., β-Lg[f 157−162]−S−S−β-Lg[f 58−70] and β-Lg[f 149−162]−S−S−β-Lg[f 58−70]). For the remaining peptide, with M_r = 2896.5, which was mainly detected in fraction F2, it appeared that it could involve a disulfide bond between cysteinyl residue 160 of β−Lg fragment [f 157−162] (M_r = 671.3) and cysteinyl residue 88 of κ-CN fragment [f 76−95] (M_r = 2227.0). To confirm this assumption, the peak containing the peptide with M_r = 2896.5 was collected and reanalyzed by RP-HPLC before and after reduction with DTT. As shown in Figure 7, the results clearly established that the molecular mass 2896.5 was a combination between M_r = 671.3 and 2227.0. The corresponding amino acid sequences, determined by tandem MS (Figure 8), unambiguously confirmed the identities of these peptides which were [β-Lg, f 157−162] (Figure 8A) and [κ-CN, f 76−95] (Figure 8B), respectively.

DISCUSSION

Unlike the detailed studies on the formation and technological consequences of heat-induced casein micelles−β-Lg complex in bovine milk (*4*), there have been no investigations concerning the occurrence of such a complex between proteins from goat milk. The potentially reactive sites in goat proteins were Cys^{66}, Cys^{106}, Cys^{119}, Cys^{121}, and Cys^{160} of β-Lg; Cys^{10}, Cys^{11}, and Cys^{88} of κ-CN; and Cys^{37} and Cys^{41} of α_{s2}-CN. In this study, we have developed a research approach which allowed direct evidence of the formation of heat-induced covalent linkage between β-Lg and casein micelles from goat milk. This complex

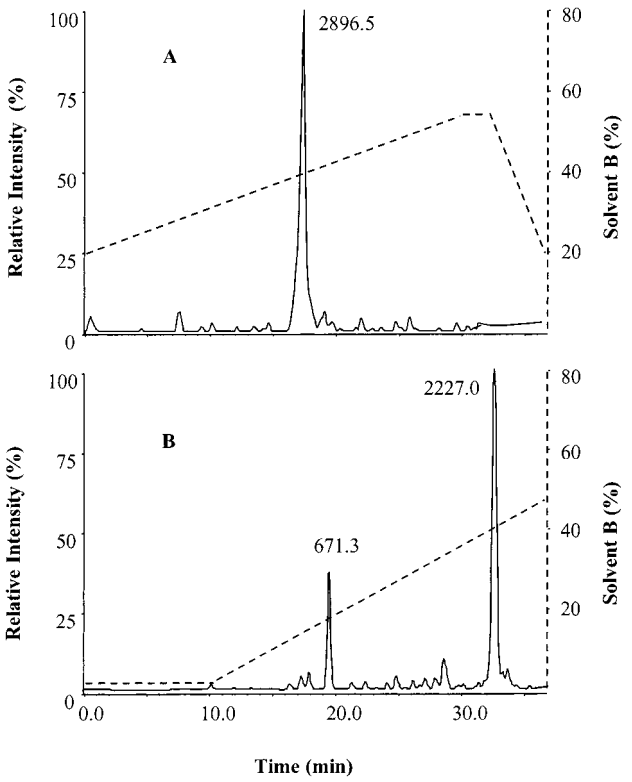

Figure 7. Total ion current (TIC) of the LC–ESI/MS run of the peptide M_r = 2896.5 purified from peptic digest of fraction F2 from Figure 5. TIC before (A) and after (B) sample reduction with DTT. Peptides were eluted with adapted linear gradients of solvent B (dashed lines).

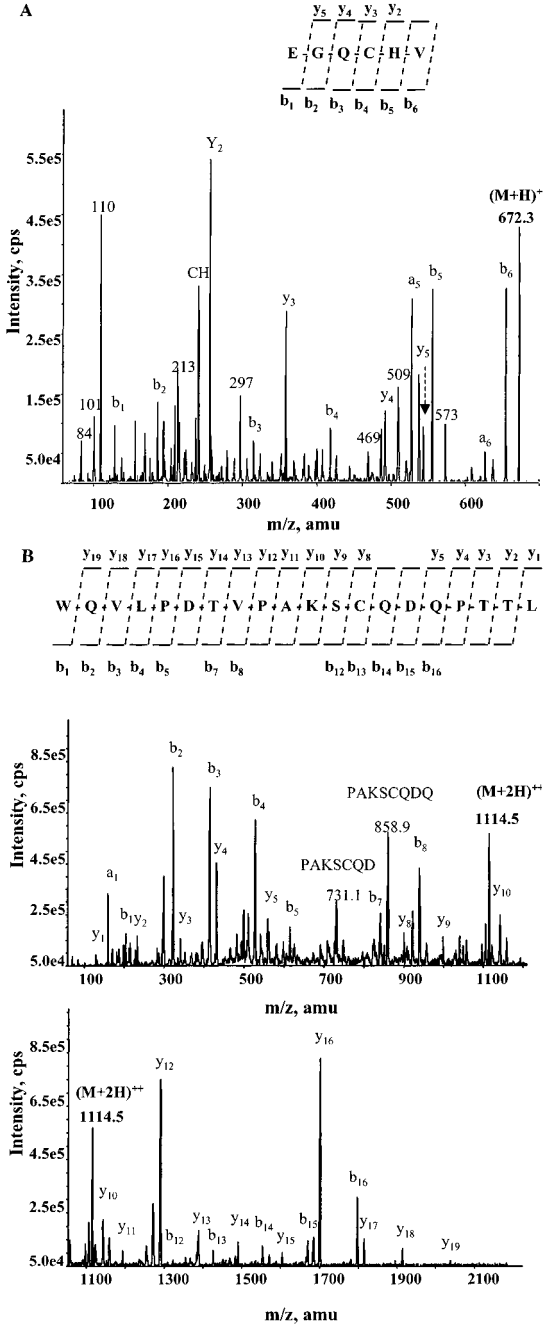

Figure 8. Tandem mass spectra of the ions 671.3 (A) and 2227.0 (B) obtained from reduced peptide with M_r = 2896.5 (illustrated in Figure 7). Collision was performed on the single-protonated precursor ion $(M + H)^+$ (*m/z* 672.3) for (A) and on the double-protonated precursor ion $(M + H)^{2+}$ (*m/z* 1114.8) for (B). The corresponding amino acid sequences are indicated (insert). Nomenclature: *y* and *b* denote C-terminus and N-terminus parts of fragmented ions, respectively (*14*).

was identified in the ultracentrifugation pellet of heated sample which was expected to contain, among others, high amounts of covalent oligomers of denatured β-Lg and covalent oligomers of κ- and α_{s2}-casein (*9, 15*).

The covalent complex was qualitatively identified in HMWM eluted from SEC, which also contained polymers formed by autopolymerization of κ-casein and β-Lg. The percentage of each polymeric species is still unknown. The presence of these two homopolymers was supported by the identification of disulfide-linked peptides such as β-Lg[f 157−162]−S−S−β-Lg[f 157−162] or κ-CN[f 76−96]−S−S− κ-CN[f 76−96] which were also reported to be induced during heating of pure β-Lg or caseins (*15*−*17*).

In agreement with the results published for bovine β-Lg (*16*), the identified disulfide-linked peptides never consisted of more than two amino acid chains. Although the formation of disulfide links involving three or more fragments is theoretically possible, it is probable that our heat treatment condition (115 °C, 20 s) limited the formation of such peptidic aggregates and so subsequent polymerization processes occurred mainly throughout noncovalent interactions. Another explanation would be that the concentration of these molecular species was too low to be detected under our experimental conditions.

Besides the formation of these expected interlinks between homoproteins, i.e., κ-CN/κ-CN and β-Lg/β-Lg, an interchain disulfide bond involving β-Lg and κ-casein was identified. The involved sites were found to be Cys^{160} of β-Lg and Cys^{88} of κ-CN. While Cys^{88} of κ-CN exists as free thiol in native protein, the cysteinyl residue of β-Lg is known to be involved in a native intrachain disulfide bond of native protein (Cys^{66}−S−S−Cys^{160}) but also, at least in the case of bovine β-Lg, to be highly reactive in forming new disulfide links throughout SH/SS interchange reactions (*16*−*18*). The reactivity of this original bond, favored either by heating (*18*), or during incubation at alkaline pH (*16*), was attributed to its high accessibility at the surface of the native protein (*19*).

The formation of a complex between β-Lg and casein micelles at pH 6.9 was recently hypothesized to explain the behavior and the heat stability of goat's milk *(11)*. The authors based their assumption on the fact that a higher percentage of nonsoluble β-Lg was found in milk heated at pH 6.9 compared to that heated at pH 6.7. In our study, the covalent bond between β-Lg and κ-CN was evidenced at pH 6.7, the natural pH of

caprine milk. This does not rule out, obviously, its occurrence at higher pH values.

The results of the present study lead us to conclude that a covalent complex between goat's milk β-lactoglobulin and κ-casein is formed during heating. We should note that the aim of the present work was to identify at least one disulfide bond involving β-Lg and one of molecule from casein micelle. Consequently, the occurrence of β-Lg/κ-CN complexes involving other sulfidryl groups, as well as other complexes such as between β-Lg and α_{s2}-casein, is not ruled out. In any case, if these other complexes exist, they are probably less abundant than the identified one.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; κ-CN, κ-casein; HMWM, high molecular weight material; DTT, dithiothreitol; LC−ESI/MS, liquid chromatography−electrospray ionization mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; SEC, size-exclusion chromatography.

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