Relative Structural Stabilities of β-Lactoglobulins A and B As Determined by Proteolytic Susceptibility and Differential Scanning Calorimetry†

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β-Lactoglobulin (β-Lg) genetic variants A and B were purified from milks of individual cows that were homozygous for each variant, and their structural stabilities were examined by susceptibility to proteolysis by immobilized trypsin and by differential scanning calorimetry (DSC). A 15% increase in the value for V_M/K_M was observed with proteolysis of β-Lg A as compared with β-Lg B. This observation is substantiated by a more rapid disappearance of intact β-Lg A during proteolysis of equimolar mixtures of the two variants. Lower structural stability of variant A is also indicated by a 5 °C lower thermal denaturation temperature observed for β-Lg A by DSC. Thus, the two amino acid residue change differentiating variants A and B (D64G and V118A) is manifested in a less stable structure for the A variant.

INTRODUCTION

Since identification of genetic variants of β-lactoglobulin (β-Lg) by Aschaffenburg and Drewry (1955), numerous studies of possible differences in the physicochemical properties of these two proteins have been reported. Comparison of β-Lg A and B variants reveals differences in solubility (Treece et al., 1964), thermal stability (Gough and Jenness, 1962; Imafidon et al., 1991), and structural stability in urea (Alexander and Pace, 1971). The primary structures differ only at two positions: A/B, D64G and V118A (Swaisgood, 1982). Circular dichroism (CD) studies of the secondary structure indicate no significant difference between variants A and B (Towend et al., 1967; Shimizu et al., 1985; Ebeler et al., 1990). The three-dimensional structures of crystalline forms of β-Lg A and B have been determined at 2.5-Å resolution (Monaco et al., 1987), using X-ray diffraction methods. The results show that the core of the β-Lg molecule consists of eight strands of antiparallel β-sheet which wrap round to form an antiparallel β-barrel with a short segment of α-helix on the surface. At this resolution, no significant differences in the structures of the two variants could be discerned; however, a conclusive comparison of the two structures must await higher resolution studies.

Immobilized proteinases have been successfully used to study protein structure (Church et al., 1981, 1982). The advantages of using immobilized proteinases to probe the structures of proteins are summarized by Swaisgood and Catignani (1987, 1991). In this study, the major objective was to investigate and compare the structural stability of β-Lg variants. Individual variants were isolated from the milk of cows that were homozygous for a particular β-Lg variant. The structural stabilities of both genetic variants were compared by measuring their susceptibility to proteolysis with immobilized trypsin. In addition, the thermal stabilities of β-Lg variants were determined by using differential scanning calorimetry.

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MATERIALS AND METHODS

All chemicals were of reagent grade, and distilled deionized water was used throughout.

Screening of β-Lg A and B. Milk samples from individual cows at the NCSU dairy farm were collected and cooled to 4 °C. The milk samples were immediately defatted by centrifugation at 10000*g* for 20 min at 4 °C. The casein was precipitated by adjusting the pH to 4.6 with a 10% acetic acid solution. The casein precipitate was removed by filtration through four layers of cheesecloth. The filtrate solutions were adjusted to 0.25 M sucrose and 0.025% bromophenol blue in 0.025 M Tris buffer, pH 6.8. Each sample from an individual cow was analyzed by nondenaturing polyacrylamide gradient gel electrophoresis to identify cows homozygous for the β-Lg A or B variant.

Electrophoresis. Polyacrylamide gradient gel electrophoresis was conducted according to the method of Laemmli (1970). For nondenaturing conditions, polyacrylamide gradient gel electrophoresis was performed without using sodium dodecyl sulfate (SDS) in the gels or buffers. A mixture of pure β-Lg A and B and BSA from Sigma Chemical Co. (St. Louis, MO) was used as a standard for identification of β-Lg A and B. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

Purification of β-Lg A and B. β-Lg A and B from milk of homozygous cows were purified according to the method of Fox et al. (1967). After removal of caseins, 3% (w/v) of trichloroacetic acid (TCA) was added to the whey protein solution to precipitate all proteins except β-Lg. The precipitate was separated by centrifugation at 12000*g* for 30 min at 4 °C. The supernatant was collected, adjusted to pH 2–3 with 1 M NaOH, concentrated, and dialyzed against distilled–deionized water until the pH of the solution was 6–7 using a Millipore dialysis system with a 10 000 molecular weight cutoff membrane. The concentrated solution was lyophilized, and the protein powder was collected and stored at −22 °C in a desiccator until use.

Circular Dichroism Spectra. CD spectra were measured with a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co. Ltd., Japan). Solutions (1.0 mg/mL) of β-Lg A and B were prepared by dissolving the lyophilized powder in 0.1 M NaCl adjusted to pH 4.0 or in 50 mM Tris-HCl buffer, pH 8.0, containing 0.02% NaN_3, and filtering through a 0.45-μm membrane filter to remove any particulate matter. Protein concentrations were determined spectrophotometrically using an absorptivity of 0.94 cm^2/mg (Swaisgood, 1982). CD spectra were obtained in 0.1-mm path length water-jacketed quartz cells maintained at 25 °C and represent an average of eight scans collected in 0.2-nm steps at a rate of 20 nm/min over the wavelength range 180–250 nm. CD spectra were baseline-corrected, and the data are presented as

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the mean residue ellipticites [θ], based on a mean amino acid residue weight of 110.

Immobilization of Trypsin. Controlled-pore glass (CPG) beads were cleaned and silanized following the procedures of Janolino and Swaisgood (1982). The resulting aminopropyl glass beads were succinylated by using the nonaqueous method of DuVal et al. (1984). Immobilization of trypsin (type XIII, TPCK treated from bovine pancreas, purchased from Sigma) on derivatized CPG beads was done by following the method of Janolino and Swaisgood (1982). Ten milliliters of trypsin solution (6.0 mg/mL in sodium phosphate buffer, pH 7.0, with 0.02% NaN_3) was added to succinamidopropyl glass beads that were preactivated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and enzyme solution was circulated at 4 °C for 24 h. Noncovalently bound trypsin was removed by washing the beads with 400 mL of 4 M urea in 0.05 M Tris-HCl, pH 7.5, followed by washing with 400 mL of 0.05 M Tris-HCl buffer, pH 7.5. The immobilized trypsin was stored in 0.05 M Tris-HCl, pH 7.5, containing 0.02% NaN_3, at 4 °C. Activity of immobilized trypsin was estimated according to the method of Taylor and Swaisgood (1980), using TAME as the substrate. An activity of about 37 μmol min^{-1} mL^{-1} glass bead was observed.

Tryptic Hydrolysis of β-Lg A and B. Susceptibility to proteolysis of the individual variants and mixtures in 50 mM Tris-HCl buffer, pH 8.0, at a concentration of 5 mg/mL was examined. Lyophilized β-Lg (40 mg of individual or mixtures of β-Lg A and B at a 1:1 molar ratio) was dissolved in 8.0 mL of 50 mM Tris-HCl, pH 8.0, with 0.02% NaN_3. After filtration with a 0.45-μm filter, 6 mL of the β-Lg solution was recirculated through a fluidized-bed bioreactor containing 2 mL of immobilized trypsin beads at 4 °C. Aliquots (300 μL) were withdrawn from the reaction mixture at various time intervals and centrifuged prior to HPLC analysis.

Kinetic constants, K_m and V_{max}, were determined for β-Lg A and B at substrate concentrations ranging from 0.5 to 8 mg/mL, using the above conditions. The reaction was terminated at 10 min and the disappearance of intact β-Lg was analyzed by HPLC. Values for K_m and V_{max} were estimated by the Direct Linear Plot method (Eisenthal and Cornish-Bowden, 1974).

FPLC Chromatography. The tryptic hydrolysate was analyzed by HPLC with a Mono Q HR 5/5 anion-exchange column (Pharmacia), which had been equilibrated with 20 mM piperazine, pH 6.0, to monitor the disappearance of intact β-Lg. The HPLC system consisted of a Model 510 pump, a Model UK6 injector, an automated gradient controller system, and a Model 990 photodiode array detector (Waters, Milford, MA). The detector was equipped with an APC IV series computer (NEC Information System, Inc., Foxborough, MA) for data acquisition and spectral analysis. Buffers were filtered through a 0.2-μm filter and degassed under vacuum with an ultrasonic cleaner (Branson 1200). Aliquots (100 μL) were injected and eluted at room temperature at a flow rate of 1.0 mL/min with a 20-min salt gradient of 0.0–0.3 M NaCl in 20 mM piperazine, pH 6.0. The absorbance of the eluate was monitored at 280 nm. Standard curves of peak areas vs protein concentration were constructed with pure β-Lg in 50 mM Tris-HCl buffer, pH 8.0, and analyzed by HPLC under the above conditions.

Analysis by Differential Scanning Calorimetry (DSC). The thermal denaturation of β-Lg A and B was studied with a Perkin-Elmer DSC System 4. Lyophilized samples were dissolved in 50 mM Tris-HCl buffer, pH 8.0, with 0.02% NaN_3 at a concentration of 20 mg/mL. Fifty microliters of each sample was taken and sealed in stainless steel capsules. As reference, a pan containing 50 μL of the Tris-HCl buffer was used. The scanning temperature was 30–96 °C at a heating rate of 15 °C/min. Indium standards were used for temperature and energy calibrations. T_d, T_m and ΔH were computed from the thermogram by the instrument data analyzer.

RESULTS

Purification of β-Lg Variants and Comparison of Their CD Spectra. Genetic variants of β-Lg A and B are small proteins, composed of 162 amino acids with variant differences at residues 64 and 118 (Swaisgood, 1982). In this study, milk from nine different randomly selected cows was used to screen for genetically homozygous individuals. The results of nondenaturing electrophoresis of individual cow's milk proteins after fat and caseins were removed are shown in Figure 1.

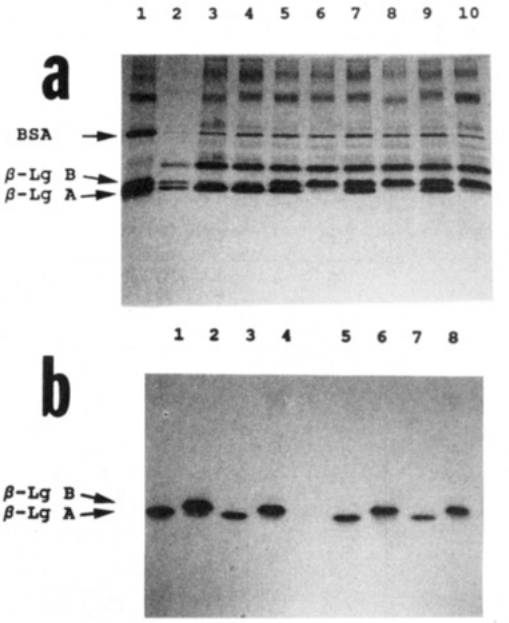

Figure 1. Nondenaturing polyacrylamide gel electrophoresis of milk proteins. (a) Whey protein from nine individual cows. Lane 1, standard protein, is a mixture of pure bovine serum albumin (BSA) and β-Lg A and B, each obtained from Sigma Chemical Co. (b) Purified β-Lg A and B from homozygous cow's milk. Lanes 1 (10 μg) and 5 (5 μg) are standard β-Lg A from Sigma; lanes 2 (10 μg) and 6 (5 μg) are standard β-Lg B from Sigma; lanes 3 (10 μg) and 7 (5 μg) are purified β-Lg A from homozygous cow's milk; lanes 4 (10 μg) and 8 (5 μg) are purified β-Lg B from homozygous cow's milk.

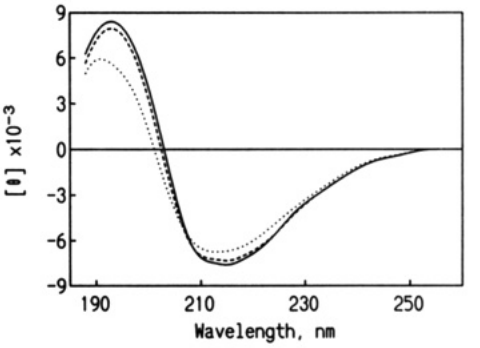

Figure 2. Circular dichroism spectra of β-lactoglobulin variants A and B at pH 4 and 8. Spectra were obtained in 0.1 mm path length cells at 25 °C. Solid and dashed lines represent β-Lg A and B, respectively, dissolved in 0.1 M NaCl at pH 4.0. The dotted line represents β-Lg B in 50 mM Tris buffer, containing 0.02% NaN_3, at pH 8.0.

β-Lg A and B were purified from the homozygous individual cow milks by the TCA precipitation method. As indicated in Figure 1, a single band was observed in each case, indicating that purified β-Lg A and B were obtained. The purified β-Lg A and B prepared in this study were comparable to or better than commercial preparations.

CD spectra for β-Lg variants A and B are compared in Figure 2. These spectra are identical at pH 4 and at pH 8, indicating no differences in the secondary structures of the preparations of these proteins used in this study. Furthermore, these spectra are identical to those previously reported for β-Lg variants under similar conditions (Townend et al., 1967; Ebeler et al., 1990; Foegeding et al.,

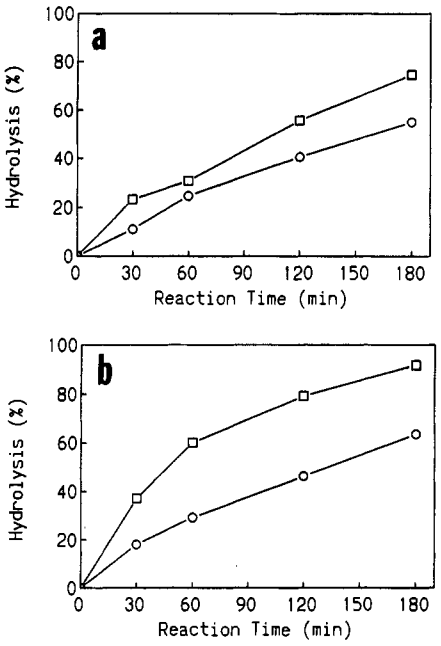

Figure 3. Hydrolysis time courses of β-Lg A and B obtained with immobilized trypsin. Reaction conditions were 50 mM Tris-HCl buffer (containing 0.02% NaN_3), pH 8.0, at 4 °C with a substrate concentration of 5 mg/mL and 2 mL of immobilized trypsin beads. (a) Hydrolysis of separate solutions of β-Lg A (□) and B (○); (b) hydrolysis of a mixture of β-Lg A and B, disappearance of β-Lg A (□) and B (○)

1992). The slight change in the CD spectra below 210 nm at pH 8 as compared to pH 4 may reflect a slight loosening of the structure or a subtle structural change. This suggestion is consistent with changes in other physicochemical parameters (Hambling et al., 1992).

Hydrolysis Rates. Structural stabilities of β-Lg A and B were compared by measuring their susceptibilities to proteolysis with immobilized trypsin. Hydrolysis occurs only at those susceptible bonds that are exposed and flexible and not part of some stable secondary structure (Swaisgood and Catignani, 1987). Susceptibility to proteolysis was determined at pH 8.0 in 50 mM Tris buffer containing 0.02% NaN_3 at 4 °C with a protein concentration of 5 mg/mL. The results in Figure 3a show that β-Lg A is more susceptible to trypsin than is β-Lg B. For example, at 30 min, 20% of the β-Lg A was hydrolyzed compared to about 10% for β-Lg B. With more extensive hydrolysis at 180 min, about 80% of β-Lg A was hydrolyzed but only 50% for β-Lg B. Similarly, in the mixture of both substrates at 1:1 molar ratio (2.5 mg/mL for each), β-Lg A was hydrolyzed more rapidly than β-Lg B (Figure 3b). In mixtures of equal concentrations, β-Lg A and B must have competitive access to trypsin; therefore, the faster rate of hydrolysis of the A variant must reflect a more flexible or less stable structure for β-Lg A as compared to β-Lg B.

Kinetic Constants. Initial velocity data were used to calculate the K_m and V_{max} of immobilized trypsin with respect to both β-Lg A and B. The results are presented in Table 1. Values for V_{max}/K_m indicate that β-Lg A is more susceptible to the proteinase than β-Lg B, thus supporting the results shown in Figure 3. It should be noted that these constants do not necessarily correspond to the hydrolysis of a specific peptide bond. Nevertheless, a single, large fragment appears to be formed from β-Lg A at early hydrolysis times (Huang, et al., 1994).

Differential Scanning Calorimetry. Since the thermal stability of a protein is related to its structure, DSC was used to examine the thermal stability of β-Lg A and B (Figure 4). The onset temperature (T_d), maximum temperature (T_m), and apparent enthalpy (ΔH) are presented in Table 1. The thermal transition temperature of β-Lg B is 5 °C higher than that of β-Lg A, determined under the same conditions (Figure 4; Table 1). However, the enthalpy values for both substrates are similar in magnitude. A similar result was reported by Imafidon et al. (1991), although different buffer systems, pH, and heating rates were used. Values obtained for T_m are in general agreement with those previously reported for mixtures of the two variants (de Wit and Swinkels, 1980; Park and Lund, 1984; Ma and Harwalkar, 1991; Foegeding et al., 1992) or for the individual variants (Imafidon et al., 1991; Griko and Privalov, 1992), considering that reported values for mixtures have varied roughly over a 10 °C range depending upon the buffer conditions.

Table 1. Apparent Kinetic Constants for Tryptic Proteolysis and Thermal Denaturation Parameters of β-Lg A and B

item	β-Lg A	β-Lg B
K_m[a] (mM)	0.44	0.22
V_{max}[a] (mmol/min)	1.8×10^{-5}	8.2×10^{-6}
V_{max}/K_m	4.2×10^{-5}	3.6×10^{-5}
T_d[b] (°C)	79.2	84.1
T_m[b] (°C)	82.1	87.3
ΔH[b] (cal/g)	0.88	0.91

[a] K_m and V_{max} values were determined at 4 °C in 50 mM Tris-HCl buffer, pH 8.0. The values were estimated from direct linear plots. Molecular mass: β-Lg A = 18 363, β-Lg B = 18 277. [b] Thermal denaturation temperatures of β-Lg A and B were determined by DSC from 30 to 95 °C at a heating rate of 15 °C/min. T_d, onset temperature; T_m, maximum temperature; ΔH, apparent enthalpy.

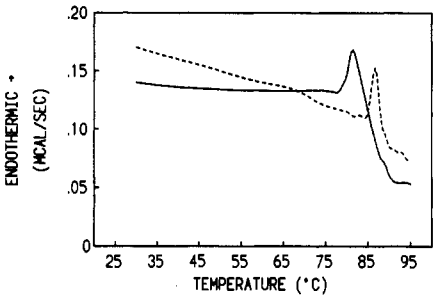

Figure 4. Denaturation thermograms of β-Lg A and B in 50 mM Tris-HCl buffer (containing 0.02% NaN_3), pH 8.0. Heating rate is 15 °C/min. (—) Thermogram obtained with 1.0 mg of β-Lg A; (- - -) thermogram obtained with 1.0 mg of β-Lg B.

DISCUSSION

The Asp/Gly change differentiating variants A and B at residue 64 appears to represent a surface location (Monaco et al., 1987) resulting in a larger electrophoretic mobility for β-Lg A during native gel electrophoresis due to the additional two negative charges on the protein dimer. This change, together with the Val/Ala difference at residue 118, results in rather dramatic differences in a number of physical and chemical properties (Ebeler et al., 1990; Shimizu et al., 1985; Swaisgood, 1982; Imafidon et al., 1981; Alexander and Pace, 1971; Townend et al., 1967; Treece et al., 1964; Gough and Jenness, 1962). The present results suggest that these changes are also manifested by differences in the structure or structural stability between these two variants.

The primary structures of β-Lg A and B (Swaisgood, 1982) indicate that both genetic variants contain the same 17 potential sites for cleavage by trypsin. However, they exhibit very different susceptibilities to proteolysis by this

enzyme. The differences are reflected both in the rates of hydrolysis as shown here and in the initial products formed (Huang et al., 1994). Such differences in hydrolysis rates could result from differences in the tertiary structure or from differences in structural stability. Since no significant differences in the crystal structures are apparent at 2.5-Å resolution (Monaco et al., 1987), an increased flexibility or decreased stability for the structure of β-Lg A as compared to that of β-Lg B is suggested.

A lower structural stability of β-Lg A is also supported by the results of DSC studies. The transition observed in the DSC experiments results from the thermally induced unfolding of the tertiary structure. Although the transition observed in the DSC profile is not reversible due to subsequent aggregation of the unfolded protein (de Wit and Swinkels, 1980), a thermodynamic analysis is meaningful since the association reactions are slower than the unfolding (de Wit and Swinkels, 1980; Privalov, 1982; Buchner et al., 1991). Thus, the unfolding transition for β-Lg A occurs at a temperature that is 5 °C lower than that for the B variant.

A similar conclusion regarding the relative stabilities of β-Lg A and B was reached by Imafidon et al. (1991) on the basis of DSC results. Values for the denaturation temperature do not appear to vary as greatly with pH (Park and Lund, 1984; Ma and Harwalkar, 1991) as they do with phosphate ion, especially at low pH (Griko and Privalov, 1992).

In a recent study of the rate of proteolysis of β-Lg A and B with papain at pH 8, Schmidt and Markwijk (1993) also observed more rapid hydrolysis of β-Lg A. These authors suggested that substitution of Asp for Gly at residue 64, producing a negative charge in the elongated loop, might explain the difference in hydrolysis rates. However, because a similar difference in proteolytic susceptibility was observed for both enzymes and because proteolytic susceptibility correlates with thermal stability, we suggest that flexibility (stability) of the structure is responsible for the difference in hydrolysis rates rather than the relationship of primary structure change and enzyme specificity. In fact, two major peptides isolated from limited proteolysis of β-Lg with immobilized trypsin (6200- and 6900-Da peptides) do not result from cleavage at residues Lys_{60} or Lys_{69} that are adjacent to residue 64 (Chen et al., 1993).

The conclusion that β-Lg A is less stable structurally than β-Lg B contrasts with that of Gough and Jenness (1962) and Sawyer (1967). However, these studies measured the amount of irreversible aggregation rather than the unfolding transition. On the other hand, both the susceptibility to proteolysis and the thermal transition detected by DSC reflect the unfolding transition. These observations suggest that the structures of the unfolded intermediates for β-Lg A are kinetically or thermodynamically less likely to proceed to an aggregated state.

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