Thermal Stabilization of β-Lactoglobulin by Whey Peptide Fractions

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Tryptic hydrolysate of whey protein isolate (WPI) was fractionated by anion-exchange chromatography (AEC) and hydrophobic interaction chromatography (HIC). Individual β-lactoglobulin (β-lg), a mixture of β-lg and nonfractionated hydrolysate, and β-lg:peptide mixtures (3:1 weight ratio) were solubilized in acetate or phosphate buffers, and their heat denaturation profiles between 25 and 100 °C were studied over the pH range of 4.6−8.0. Thermal denaturation of individual β-lg was greatly influenced by pH, its denaturation temperature (T_D) decreasing from 77.4 to 66.9 °C for pH 4.6−8.0, respectively. The addition of nonfractionated hydrolysate to β-lg accentuated this effect, whereas T_D and heat enthalpy (ΔH_D) were increased in the presence of the peptide fractions. Fractions obtained by AEC (F_2−F_8) thermally stabilized β-lg as a function of their increasing ionic charge, and this effect became more important as the pH was raised from 4.6 to 8.0. The results obtained with HIC fractions (F_A, F_B, and F_D) showed a T_D of 78−80 °C over the pH range under study. The binding of peptides to β-lg, possibly via ionic or hydrophobic interactions, may stabilize β-lg structure against heat treatment.

Keywords: *β-Lactoglobulin; whey peptides; thermal denaturation; differential scanning calorimetry*

INTRODUCTION

β-Lactoglobulin (β-lg) is the major whey protein (50−55%) in bovine milk (Swaisgood, 1982; Harwalkar, 1985; Gu and Brady, 1992). At the physiological pH of bovine milk, β-lg exists as a dimer with a molecular weight of ∼36 000 Da (Newcomer et al., 1984; Godovac-Zimmerman and Brautnitzer, 1987; Hambling et al., 1992). The monomer of bovine β-lg is composed of 162 amino acid residues and possesses a free thiol group, and its globular structure is stabilized by two intrachain disulfide bridges (Papiz et al., 1986; Monaco et al., 1987). Reduction of these two disulfide bridges destabilizes the conformation of the protein when it is subjected to various denaturation treatments (Reddy et al., 1988). Heat treatments, such as preheating, pasteurization, and sterilization, decrease the stability of β-lg and produce a detrimental effect on whole milk and whey products (de Wit and Klarenbeek, 1984). The functional properties of dairy ingredients are mainly related to the structural and physicochemical characteristics of protein components, so the heat sensitivity of β-lg causes important practical problems for the food industry.

The thermal behavior of β-lg has been extensively studied by techniques such as optical rotatory dispersion (Timasheff et al., 1966; Sawyer et al., 1971), circular dichroism (Townend et al., 1967; Lapanje and Poklar, 1989), solubility loss (Hillier and Lyster, 1979), infrared spectroscopy (Rüegg et al., 1975; Casal et al., 1988), and differential scanning calorimetry (DSC; de Wit and Klarenbeek, 1981; Park and Lund, 1984). When the temperature is increased from 30 to 55 °C, the dimeric form of β-lg dissociates into monomers (Dupont, 1965; Sawyer, 1969). At higher temperatures, unfolding of the molecule occurs concomitantly with increased activity and oxidation of the thiol group. This is followed by irreversible polymerization of denatured proteins, which forms aggregates (McSwiney et al., 1994, de Wit, 1981; Hambling et al., 1992). The thermal denaturation of β-lg is related to the presence and the reactivity of cysteine and cysteine residues and is influenced by many factors, such as protein concentration, pH, and presence of salts or other molecules (Relkin, 1994). These factors contribute to the modification or alteration of the structural conformation and the interaction of β-lg with other components (Relkin and Launay, 1990; Laligant et al., 1991). A well-known interaction is the formation of the κ-casein:β-lg complex during heat treatments of milk, which leads to lower susceptibility of κ-casein to chymosin and limits its potential use in rennetted cheese products (Sawyer, 1968; McKenzie, 1971; Singh and Creamer, 1993). This heat-induced interaction between β-lg and casein micelles can also improve the texture of other dairy products, such as yogurt (de Wit, 1981). Changes in thermal stability of β-lg by interaction with κ-casein have been ascribed to intermolecular disufide bonding between both proteins (Jang and Swaisgood, 1990) and to noncovalent hydrophobic interactions in the initial stages of the interactions (Haque et al., 1987; Haque and Kinsella, 1988; Jang and Swaisgood, 1990).

However, the binding of some ligands to different proteins has been reported to stabilize their structure and increase their resistance to heat denaturation. For example, it has been shown that fatty acids stabilize bovine serum albumin (BSA) and β-lg against heat and calcium (Ca^{2+}) binding to α-lactalbumin (α-la) or iron (Fe^{3+}) binding to lactoferrin and also improves the heat stabilities of α-la and lactoferrin (Gumpen et al., 1979; Relkin et al., 1993). According to Puyol et al. (1994), β-lg possesses specific binding sites for fatty acids and retinol, which thermally stabilize the protein. No reports have been yet published on the effect of peptides on the thermal stability of β-lg, so the present study focuses on the thermal behavior of β-lg in the presence of peptide fractions at different pH values as determined by DSC.

MATERIALS AND METHODS

Materials. Commercial whey protein isolate (WPI; 95% w/w protein) was obtained from Le Sueur Isolates (Le Sueur,

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MN). β-Lg (bovine milk, 3× crystallized and lyophilized, variant A), trypsin (bovine pancreas, type XIII) treated with tosyl phenylalanine chloromethyl ketone (TPCK), sodium benzoyl-L-arginine ethyl ester (BAEE), BSA, ovalbumin, adrenocorticotropic hormone (ACTH), bacitracin, L-leucyl-L-leucyl-L-leucine, and L-leucine were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were analytical grade. Buffers and mobile phases were prepared with HPLC grade water (Modulab, Fisher Scientific, Canada) and filtered through Millipore 0.22-μm filters (Millipore, Bedford, MA).

Specific Activity of Trypsin-TPCK. Enzyme activity of trypsin-TPCK was determined by spectrophotometry according to the method of Schwert and Takenaka (1955) with BAEE as substrate. Trypsin has 11 532 BAEE units/mg protein.

Preparation of Tryptic Hydrolysate (TH). The WPI was rehydrated in water (3.5% w/v protein) in a stirred fermentor (New Brunswick Scientific Company, Edison, NJ), and the solution was adjusted to pH 8.0 with 2 N NaOH. The mixture was heated to 42 ± 1 °C, and the trypsin solution (0.5% w/v in 0.001 N HCl) was added (enzyme:substrate ratio, 1:200). During hydrolysis, the reaction mixture was maintained at pH 8.0 by addition of 2 N NaOH according to the pH-stat technique of Adler-Nissen (1977). After 45 min of hydrolysis, the proteolytic products were removed continuously by ultrafiltration with a regenerated cellulose acetate spiral wound membrane with a nominal molecular weight cutoff of 30 000 (S1-Y30, 0.1 m^2, Amicon, Danvers, MA). The reaction mixture was concentrated three times at 20 °C under a transmembrane pressure of 149 kPa. The permeate, designated as tryptic hydrolysate (TH), was lyophilized and stored at 0 °C until further analysis and chromatographic fractionation.

Fractionation of TH by Anion-Exchange Chromatography (AEC). The TH (165 mg) was fractionated on an anion-exchange column (TSK gel DEAE-5PW, 150 × 21.5 mm i.d., Beckman) by semipreparative HPLC (Beckman Instruments Inc., Mississauga, Canada) with a chromatographic apparatus equipped with two pumps (model 126) and a variable UV detector (model 166) operating at 214 nm. The chromatographic apparatus was fitted with Gold Prep analytical software (Version 7.1). The column was equilibrated with tris buffer (20 mM, pH 8.0) at a flow rate of 6 mL/min. Elution was performed with a linear gradient of 0.5 M NaCl (0−65%) in the same buffer over a period of 120 min. Eight fractions (F_1−F_8) were collected, dialyzed with a 100-Da molecular weight cutoff tubing (Spectra Por; Spectrum Medical Industries Inc.), and lyophilized.

Fractionation of TH by Hydrophobic Interaction Chromatography (HIC). The TH (600 mg) was first fractionated with a preparative Michel-Miller column (300 × 24.5 mm i.d.) filled with Vydac C_{18} resin (15−20 mm, Waters, Canada). The mobile phase was a 5−45% gradient of acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid (TFA). The flow rate was 6 mL/min, and the TH was eluted with 800 mL of mobile phase. Four fractions (F_A−F_D) were collected after 200, 240, 400, and 480 mL; of mobile phase, and then evaporated and lyophilized.

The TH and HIC fractions (F_A−F_D) were analyzed by reversed-phase HPLC (RP-HPLC) to determine the composition of fractions previously isolated at preparative scale. RP-HPLC was performed with a LKB system (Pharmacia, Canada) consisting of a controle module (model 2152), two pumps (model 2150), a variable wavelenght detector (model 2151) set at 220 nm, a temperature control system (model 2155), and a Hewlett-Packard integrator (model 3396A). RP-HPLC analysis was carried out on a C_{18} column (Beckman Ultrasphere, 250 × 4.6 mm i.d.) under the following conditions: column temperature, 39 °C; flow rate, 1 mL/min; solvent A, 0.11% (v/v) TFA in water; and solvent B, 60% (v/v) acetonitrile in water with 0.1% (v/v) TFA. Elution was obtained with a linear gradient of from 0−60% solvent B in 30 min, and 60−100% solvent B from 33 to 40 min.

Differential Scanning Calorimetry (DSC). The thermal behavior of bovine β-lg alone and in the presence of nonfractionated hydrolysate (TH) or peptide fractions (F_1−F_8 and F_A−F_D) was studied by DSC analysis, according to the method of Ma and Harwalkar (1988). The equipement was a DuPont 910 thermal analyzer (DuPont, Wilmington, DE) equipped with a 910 cell base and a high-pressure cell. The heating rate was 5 °C/min in the temperature range of 25−100 °C. All DSC experiments were performed in triplicate with 12.5 μL of β-lg solution (2.5 mg/15 μL) β-lg:TH or β-lg:peptide solutions (2.5 mg:0.8 mg/15 μL) prepared in 0.1 M acetate buffer for pH 4.6 and 5.1, and prepared in 0.1 M phosphate buffer for pH 6.8, 7.5, and 8.0. These pH values were selected for the following characteristics of bovine β-lg: octameric form (pH 4.6); isoelectric point (pH 5.1); pH of milk (pH 6.8); Tanford transition (pH 7.5); and monomeric form (pH 8.0; Townend et al., 1960, 1969; Timasheff and Townend, 1961; Tanford et al., 1959). Because of the small volumes of mixtures (12.5 μL), pH values after addition of β-lg and peptide fractions could not be measured. All the samples were prepared at room temperature and sealed into coated aluminum pans. A sealed empty pan containing 12.5 μL of the buffers was used as reference, and indium standards were used for temperature and energy calibration. After heating the samples to 100 °C, they were then cooled at room temperature and immediately reanalyzed; no endothermic response was observed indicating that the protein had irreversibly denatured. Also, no endothermic response was obtained for peptides solutions prepared with TH or peptide fractions alone (F_1−F_8 and F_A−F_D).

The denaturation temperature (T_D) and heat of enthalpy (ΔH) were computed by the thermal analyser, and the width at half peak height ($\Delta T_{1/2}$) was determined from the thermograms. The van't Hoff enthalpy (ΔH_{vH}) was estimated with the following formula: $\Delta H_{vH} = 4RT_D2/\Delta T_{1/2}$, where R is the gas constant and T_D is the midpoint of the thermal transition (Privalov et al., 1971). Also, ratios ΔH:ΔH_{vH} were calculated to assess the presence of intermediates in the unfolding reaction and its reversibility (Lumry et al., 1966; Privalov and Khechinashvili, 1974).

Mass Spectrometric Analysis of Peptide Fractions. Each of the peptide fractions (F_1−F_8 and F_A−F_D) were dissolved in 100 μL of 50% acetonitrile in water containing 0.1% (v/v) TFA and mixed gently and thoroughly. Except for F_1 that was analyzed without dilution, the peptide samples were diluted 1:10 with 0.1% TFA. All samples were then mixed with the matrix for peptide analysis (α-cyano-4-hydroxycinnamic acid in a 1:1 ratio) and scanned for their mass assignments. Following a quick scan of all the samples, 0.25 pmol of the internal standard (substance P, 1348.7 Da) was mixed with each peptide samples. Peptide spectra were obtained in duplicate with a Finnigan MAT (Bremen, Germany) LaserMat 2000 time-of-flight mass spectrometer. Each spectrum has been printed with two scales; first from 1 to 10 000 Da and second from 500 to 5000 Da.

Chemical Analyses. Protein contents (N × 6.38) of the WPI and TH were determined in triplicate by the Kjeldahl method (IDF 20B, 1993) performed on a Büchi block digestor 430 equipped with a distillation unit 323 (Brinkman Instruments, Montreal). Lactose content was evaluated in duplicate according to the HPLC method of Doyon et al. (1991). Calcium, potassium, and sodium were determined spectrophotometrically by atomic absorption (Ca) and emission (K, Na) with an Instrumentation Laboratory Spectrophotometer (model IL751, Wilmington, MA).

Molecular weight distribution profiles of the water-soluble proteins from WPI and their TH were determined by high-performance size-exclusion chromatography (HPSEC; LKB system) with a TSK-G 2000 SW column according to the method of Vijayalakshmi et al. (1986). Protein standards were BSA (66 200), ovalbumin (43 000), β-lg (18 400), adrenocorticotropin (ACTH; 4390), bacitracin (1411), and L-leucyl-L-leucyl-L-leucine (358). The total area of chromatograms was separated in three ranges of molecular weight (>5000, 2000−5000, <2000) and expressed in percentage of the total area.

Statistical Analysis. Results were expressed as mean ± standard error and treated by ANOVA followed by Dunnett's test with the Instat 2.0 computer program.

Table 1. Chemical Composition (Percent) and Molecular Weight Distribution of Protein Components of WPI and Their TH

	WPI	TH
component		
protein (%)	92.8	88.3
lactose (%)	0.9	0.6
minerals (mg/100 g)		
Ca	75	93
Na	246	1668
K	109	194
molecular weight distribution (%)[a]		
>5000	88.5	6.2
5000−2000	4.0	20.3
<2000	7.5	73.5

[a] Molecular weight distribution was calculated from the integration of the total area of the chromatogram obtained from HPSEC. The chromatogram was separated in three ranges of molecular weight and expressed in percentage of the total area.

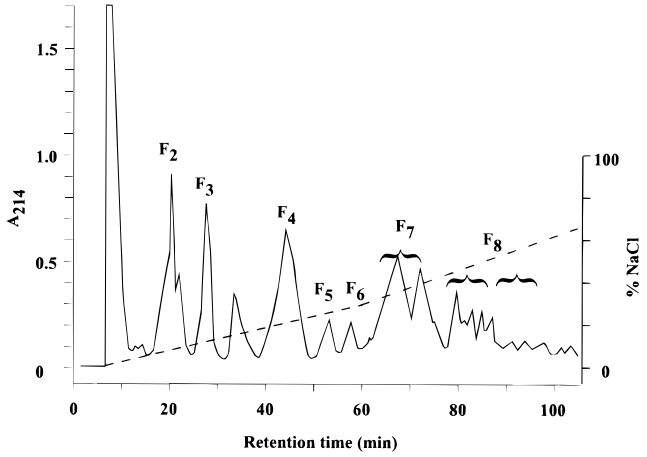

Figure 1. AEC profile of TH fractionated in eight peptide fractions (F_1−F_8). Conditions were as follows: column, TSK gel DEAE-5PW (150 × 21.5 i.d.); 20 mM tris buffer (pH 8.0); linear gradient, 0−65% of 0.5 M NaCl in same buffer over 120 min; flow rate, 6 mL/min; detection, 214 nm.

RESULTS AND DISCUSSION

The chemical composition and the molecular weight distribution of protein components from WPI and TH are given in Table 1. Ultrafiltration, performed at the end of the tryptic hydrolysis to remove enzyme and nonhydrolyzed protein, resulted in a slight decrease of protein and lactose content, whereas the mineral content was increased. The markedly higher sodium content of TH is explained by the pH adjustment with NaOH during enzymatic hydrolysis (Turgeon and Gauthier, 1990). The molecular weight distribution of protein components indicated that WPI is mainly composed of peptides >5000 Da (88.5%), whereas the TH contains a high concentration (93.8%) of small peptides (MW <5000). The high concentration of small peptides in the TH suggests a high degree of tryptic hydrolysis of WPI after 45 min.

To prepare different peptide fractions, the TH was fractionated by two methods: AEC and HIC. From AEC (Figure 1), a well-defined distribution was obtained, allowing the isolation of eight peptide fractions of increasing degree of ionic charge that were designated F_1−F_8. All the fractions obtained by AEC were desalted by dialysis to avoid salt effects in DSC analysis. On the other hand, the HIC performed at preparative scale provided only four peptide fractions, and their RP-HPLC profile (Figure 2) revealed overlapping in peptide composition, mainly for the fractions F_A, F_B, and F_C.

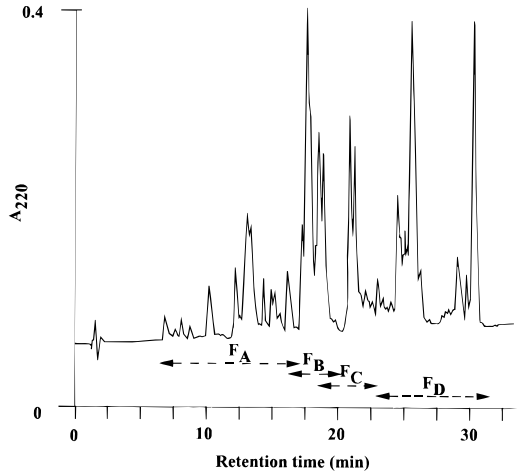

Figure 2. RP-HPLC profile of TH indicating the peptide composition of the four fractions (F_A−F_D) isolated by HIC at preparative scale. Conditions were as follows: column, C_{18} Ultrasphere (250 × 4.6 mm i.d.); column temperature, 39 °C; solvent A, 0.11% (v/v) TFA in water; solvent B, 60% (v/v) acetonitrile in water with 0.1% (v/v) TFA; linear gradient, 0−60% solvent B in 30 min and 60−100% solvent B from 33 to 40 min; flow rate, 1 mL/min; detection, 220 nm.

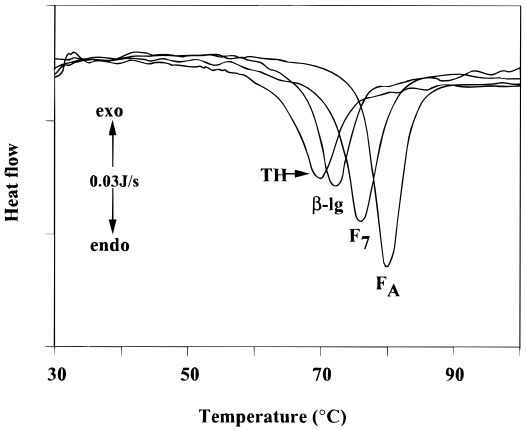

Figure 3. DSC thermograms of β-lg alone and in the presence of TH or peptide fractions obtained by AEC (F_7) or HIC (F_A). Samples were prepared in 0.1 M phosphate buffer at pH 6.8. The heating rate was 5 °C/min.

Typical DSC thermograms obtained at the pH of milk (6.8) for β-lg alone and in the presence of TH or peptide fractions obtained from AEC (F_7) or HIC (F_A) fractionation are shown in Figure 3. At this pH, β-lg exhibited a maximum T_D of 72 °C. A similar T_D value (75 °C) was determined by Puyol et al. (1994) for a β-lg solution (8%) prepared in acetate buffer at pH 6.5 and using the same heating rate (5 °C/min). In the presence of TH or peptide fractions (F_7 and F_A), the position and the shape of the denaturation peak of β-lg are affected. The nonfractionated hydrolysate (TH) decreased T_D, whereas an increase of T_D was observed in the presence of peptide fractions isolated by chromatographic separation of the TH (F_7 and F_A).

The effect of all peptide fractions (TH, F_1−F_8, F_A−F_D) on the T_D of β-lg as a function of pH is illustrated in Figure 4. The corresponding values of ΔH_D and $\Delta T_{1/2}$ of β-lg are given in Table 2. The T_D, identified by the peak temperature in a heat flow versus temperature graph, measures the thermal stability of the protein (Harwalkar and Ma, 1990). The ΔH_D value, calculated by the integration of the area under the endothermic peak, provides an estimate of the thermal energy

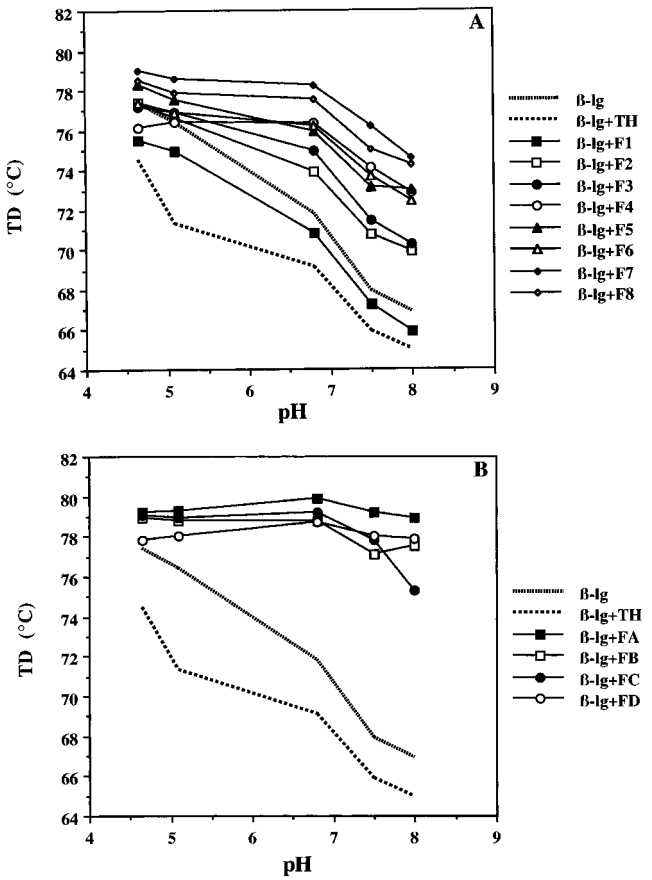

Figure 4. Effect of pH on T_D of β-lg alone, β-lg in the presence of TH or peptide fractions obtained from (A) AEC (F_1-F_8) or (B) HIC (F_A-F_D). Samples were prepared in 0.1 M acetate buffer for pH 4.6 and 5.1, and in 0.1 M phosphate buffer for pH 6.8, 7.5 and 8.0. The heating rate was 5 °C/min.

required to denature the protein. Enthalpy is usually correlated with the content of ordered secondary structure of a protein (Koshiyama et al., 1981). However, tertiary structure and protein−protein interactions are also involved, and for mixtures of β-lg and peptide fractions, more complex interaction and/or protein aggregation can also occur during the calorimetric transition. The $\Delta T_{1/2}$ value, which is the width of the calorimetric transition at half peak height, evaluates the cooperativity of protein unfolding. If denaturation occured within a narrow range of temperature (low $\Delta T_{1/2}$ value), the transition is considered highly cooperative (Privalov et al., 1971). All of these parameters are strongly dependent on the heating rate (°C/min) used for the DSC measurements.

As presented in Figure 4 and Table 2, denaturation of β-lg was greatly influenced by pH. When the pH is raised from 4.6 to 8.0, decreases of T_D and ΔH_D and an increase of $\Delta T_{1/2}$ are observed. The drastic decrease of T_D at neutral and alkaline pH may be related to the unfolding of β-lg, decreasing the content of ordered secondary structure, and lowering ΔH_D requirements for the denaturation of β-lg. Also, the high value of $\Delta T_{1/2}$ at alkaline pH indicates that the partial denaturation is less cooperative than at pH near the isoelectric point of β-lg (pH 5.1). These results are in agreement with other studies reporting that β-lg possesses a maximum T_D near it's isoelectric point that is decreased when the pH value is elevated from 6.0 to 8.0 (Rüegg et al., 1977; de Wit and Klarenbeek, 1981; Paulsson et al., 1985; Harwalkar and Ma, 1990). These observations are explained by the low net charge of β-lg at a pH value close to its isoelectric point, minimizing intermolecular electrostatic repulsion and promoting hydrophobic and van der Waals bonds (Xiong, 1992). When the pH value is increased above 6.0, β-lg becomes negatively charged (Lyster, 1970). In fact, β-lg possesses four net negative charges per monomer at pH 6.5, and about seven charges at pH 7.5 (McKenzie, 1971; Kella and Kinsella, 1988). The repulsion between the negative charges contained in the protein is accentuated by the phosphate anions contained in the buffer (0.1 M NaH_2PO_4), which may bind to positively charged amino groups in the β-lg subunits (Xiong, 1992). Also, the reactivity of the thiol group increases at pH values above 6.0, enhancing disulfide interchange and destabilizing the protein structure (de Wit and Klarenbeek, 1984). According to McSwiney et al. (1994), polymerized protein material resulting from heat treatment at pH 7.0 was induced via disulfide bond cross-linking.

The presence of nonfractionated TH in the β-lg solution decreases T_D and ΔH_D at all pH values under study (Figure 4 and Table 2). As indicated by higher $\Delta T_{1/2}$ values, this denaturation system is less cooperative than β-lg alone. These results suggest that TH accentuated the effect of pH denaturation of β-lg, and the thermal behavior of β-lg in the presence of TH seems similar to that observed with urea (Pearce and Kinsella, 1978). This solute disrupts the hydrogen-bonded structure of water and faciliates protein unfolding by weakening hydrophobic interactions (Pearce and Kinsella, 1978). Also, urea increases the "permeability" of water (Kinsella, 1982) for the hydrophobic core of the protein, causing loss of protein structure and heat stability.

The effects of peptide fractions on the thermal denaturation of β-lg at different pH values are summarized in Figure 4 and Table 2. The interpretation of these data needs to be made in light of some physicochemical properties of the peptide fractions, such as their peptide composition, molecular mass, the charge at pH 8.0, and the hydrophobicity of the peptides. These characteristics are summarized in Table 3. Although the data were calculated assuming an even distribution of peptides among the fractions, these values are useful for further qualitative interpretation of the results. As observed in Figure 4 and Table 2, the unfolding of β-lg, more prominent at pH values between 6.8 and 8.0, is significantly reduced by the presence of peptide fractions, as illustrated by higher T_D and ΔH_D values. The protecting effect of the peptide fractions obtained by AEC (Figure 4A) on β-lg unfolding is a function of the degree of their ionic charge (F_2-F_8). F_1-F_4 possess low anionic charges (Table 3) and are found to be inactive in this assay, whereas F_7 has the highest negative charge (−13), and also has better stabilization properties. At pH values above the isoelectric point of β-lg (pH 5.1), the total net charge of the protein is negative. When the protein is in the presence of negatively charged peptide fractions, such as F_7 or F_8 (Table 3), an electronegative repulsion between peptides and β-lg can be hypothesized. This mutual repulsion may compresses the β-lg molecule, resulting in a more compact structure in the alkaline solution and leading to a molecule that is less susceptible to unfolding by thermal treatment. Hence, we could speculate that negatively charged molecules could protect β-lg from thermal and pH-induced denaturation.

Peptide fractions obtained by HIC (F_A-F_D) also thermally stabilize β-lg with higher T_D and ΔH_D values over the pH range under study (Figure 4B and Table

Table 2. Effect of pH on ΔH and $\Delta T_{1/2}$ of β-lg Alone and in the Presence of TH or Peptide Fractions Obtained from AEC (F_1−F_8) or HIC (F_A−F_D) Chromatography[a]

	pH 4.65		pH 5.1		pH 6.8		pH 7.5		pH 8.0	
sample	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)
β-lg	9.95[a]	4.23[a]	9.49[a]	3.97[a]	7.22[a]	5.43[a]	6.30[a]	5.83[a]	5.87[a]	5.93[a]
β-lg+TH	9.00[a]	5.33[c]	6.51[b]	6.63[c]	6.59[a]	6.70[c]	5.21[a]	6.17[a]	4.85[a]	6.63[a]
β-lg+F_1	9.10[a]	4.83[c]	9.68[a]	5.73[c]	6.78[a]	6.80[c]	6.56[a]	7.50[c]	4.55[a]	8.87[c]
β-lg+F_2	9.73[a]	4.33[a]	11.24[a]	4.67[c]	8.27[a]	5.47[a]	5.85[a]	5.87[a]	5.49[a]	5.77[a]
β-lg+F_3	10.25[a]	4.43[b]	10.37[a]	4.20[a]	7.50[a]	5.90[a]	8.74[a]	7.17[c]	9.13[b]	6.53[a]
β-lg+F_4	8.31[a]	5.77[c]	9.65[a]	4.97[c]	9.50[a]	4.93[a]	8.34[a]	5.47[a]	7.05[a]	5.67[a]
β-lg+F_5	10.31[a]	4.50[c]	10.64[a]	4.77[c]	10.09[b]	5.87[a]	8.73[a]	6.40[c]	8.20[a]	7.93[c]
β-lg+F_6	11.06[a]	4.73[c]	11.49[a]	5.07[c]	10.30[b]	5.70[a]	8.90[a]	6.33[b]	8.27[a]	6.30[a]
β-lg+F_7	10.31[a]	5.07[c]	9.23[a]	4.70[c]	10.52[b]	4.87[a]	10.90[c]	5.43[a]	9.77[c]	5.73[a]
β-lg+F_8	8.69[a]	4.87[c]	8.33[a]	4.90[c]	9.26[a]	5.27[a]	8.32[a]	5.77[a]	7.18[a]	6.10[a]
β-lg+F_A	10.27[a]	5.20[c]	10.55[a]	4.77[c]	10.60[b]	4.30[b]	10.60[c]	4.50[c]	10.15[c]	4.50[b]
β-lg+F_B	9.25[a]	4.77[c]	10.58[a]	4.77[c]	10.38[b]	4.50[a]	10.37[c]	5.00[c]	8.94[b]	4.63[a]
β-lg+F_C	9.60[a]	4.80[c]	8.86[a]	4.57[b]	10.98[c]	4.27[b]	10.87[c]	4.90[c]	9.30[b]	4.17[a]
β-lg+F_D	8.69[a]	6.23[c]	10.09[a]	5.63[c]	10.62[b]	4.60[a]	9.64[b]	5.00[c]	10.61[c]	4.93[a]

[a] Values are the means ± SEM of three randomized determinations. For all samples, standard deviations were <1% of the mean. One-way analysis of variance was performed on the columns of data ($p < 0.05$). When this test indicated that groups were not homogeneous, Dunnett's test was then applied to compare numerical results with those with β-lg alone: means with superscripts b ($p < 0.05$) and c ($p < 0.01$) are significantly different from β-lg (superscript[a]).

Table 3. Qualitative Peptides Composition[a] and Physicochemical Characteristics of the Peptide Fractions Obtained from AEC (F_1−F_8) or HIC (F_A−F_D) of TH

		theor mass	exptl mass	charge at	fraction											
sequence	$H_{\phi av}$[b]	(Da)	(Da)	pH 8.0	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_A	F_B	F_C	F_D
1−8	1.34	934	934	1	X								X	X		
9−14	1.14	674	674	0									X			
15−40	1.18	2709	2708	−1			X									
21−40	1.05	2031	2031	−1	X	X						X				X
41−60	1.37	2315	2314	−3				X		X	X					X
61−69[c]+149−162	0.94	2722	2721	−2							X	X			X	
61−70[c]+149−162	0.96	2851	2850	−1					X		X				X	
61−69	0.79	1122	1123	−1							X	X				
61−70	0.87	1250	1249	0							X					
76−83	1.76	904	903	2										X		
78−83	2.03	675	675	1										X		
92−100	1.41	1066	1066	−1	X						X			X	X	
92−101	1.42	1194	1195	0	X									X		
125−135	0.85	1246	1246	−4							X					
125−138	0.97	1637	1636	−4										X	X	
142−148	1.54	838	837	2									X	X		
149−162	1.03	1660	1660	−1					X	X	X	X			X	
total charge					−1	−1	−1	−3	−2	−4	−13	−5	3	1	−9	−4

[a] Obtained by mass spectrometry. [b] Average hydrophobicity was calculated according to the method of Bigelow (1967). [c] Peptides linked by a disulfide bond.

2). Stabilization of β-lg against thermal denaturation by hydrophobic peptides seems to be related to the hydrophobicity of the peptide fractions (Table 3) rather than to the negative charges of the fractions. In fact, F_A, F_B, and F_D fractions, with increasing hydrophobicity, produced a corresponding stabilizing effect. Only the F_C fraction did not follow this rule. This exception can be related to the high negative charge (−9) of F_C, which makes this fraction share same similarities with F_7 in terms of T_D profiles. It was previously reported that nonpolar amino acid residues of peptides probably interact inside the hydrophobic pocket in β-lg formed by the β-barrels (Dufour and Tomasz, 1990). Also, β-lg is known to bind small hydrophobic molecules, such as free fatty acids and triglycerides, aromatic hydrocarbons, alkanone flavors, and retinol (Dufour and Tomasz, 1990). The introduction of a hydrophobic fatty acid side chain to β-lg induces a compact conformation through hydrophobic interactions, and promotes the folding of proteins and the formation of additional internal hydrogen bonds, thus restoring and causing a marked shapening of the endothermic peak and low $\Delta T_{1/2}$ value (Harwalkar and Ma, 1990; Puyol et al., 1994). The heat stability of β-lg is controlled by the balance of polar and nonpolar residues (Bigelow, 1967); the more important the proportion of nonpolar residues, the greater is the stability of β-lg to thermal denaturation (Harwalkar and Ma, 1990).

The ratios between apparent enthalpy and van't Hoff enthalpy (ΔH_D:ΔH_{vH}) were calculated for native β-lg and β-lg complexed with TH or peptide fractions (results not shown). This analysis is used to assess the reversibility of the denaturation transition. When ratios are <1, the denaturation transition is irreversible (McKenzie and Ralston, 1971; Paulsson and Dejmek, 1990). In our experiments, calculated ratios are all <1. These data suggest that the transition is irreversible.

In summary, the thermal denaturation of β-lg is greatly influenced by the pH of protein solution and the presence of specific peptide fractions. Further DSC studies with purified peptides, combined with circular dichroism and optical rotary dispersion measurements for structural determination, would be necessary to reinforce the aforementioned hypotheses. Also, lower protein concentrations should be used to prevent possible protein gelation during calorimetric transition in DSC experiments. The results suggest that nonfractionated hydrolysate accentuated the effect of pH and

thermal denaturation of β-lg through a mechanism similar to that in urea. Peptide fractions with high negative charges or high hydrophobicity can stabilize the structure of β-lg against thermal treatment at neutral and alkaline pH. For peptide fractions with high negative charges, their presence seems to favor a more compact structure of β-lg in alkaline solution, leading to molecule less susceptible to unfolding by thermal treatment. The protective effect of hydrophobic peptides probably resulted from their interaction with the hydrophobic core of β-lg, promoting a more compact structure of the molecule and the formation of additional internal hydrogen bonds. The denaturation behavior of β-lg was studied in buffered system, so prediction of the impact of these peptide fractions in a more complex system, such as milk, remains difficult. In this system, peptides could prevent interaction between denatured β-lg and the surface of casein micelles. Thus, further work is needed to evaluate the effect of the milk constituents and the real potential of these peptide fractions for the improvement of the stability of β-lg to the heat treatment applied in the dairy industry.

ABBREVIATIONS USED

AEC, anion-exchange chromatography; β-lg, β-lactoglobulin; DSC, differential scanning calorimetry; ΔH, heat of enthalpy; ΔH_{vH}, van't Hoff enthalpy; $\Delta T_{1/2}$, width at half peak height; HIC, hydrophobic interactions chromatography; MW, molecular weight; RP-HPLC, reversed-phase high-performance liquid chromatography; T_D, denaturation temperature; TH, tryptic hydrolysate; WPI, whey protein isolate.

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