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Structural and Conformational Basis of the Resistance of β-Lactoglobulin to Peptic and Chymotryptic Digestion

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The structural basis accounting for the resistance of β-lactoglobulin (β-Lg) to peptic and chymotryptic digestion was studied. Native β-Lg was resistant to peptic and chymotryptic digestibility because of its stable conformation. Heating at 50, 60, and 70 °C for 15 min did not affect resistance of β-Lg, while heating at 80 and 90 °C significantly decreased its resistance to proteolysis. Surface polarity measurements and fluorescence spectra revealed that very little change in conformation of β-Lg occurred upon heating at 50, 60, or 70 °C. Measurable changes in the conformation of β-Lg occurred at 80 and 90 °C, increasing its susceptibility to hydrolysis. Cleavage of S–S bonds caused extensive changes in conformation and significantly decreased the resistance of β-Lg to peptic and chymotryptic hydrolysis. The results suggest that disruption of native conformation exposes susceptible peptide bonds and decreases the resistance of β-Lg to proteolytic digestion.

The nutritive value of a protein is related to its amino acid composition and the bioavailability of these amino acids. Milk proteins in general and whey proteins, β-lactoglobulin (β-Lg) in particular, have a high content of essential amino acids (Hambraeus, 1982; McKenzie, 1971). However, in experimental animals, native β-Lg is resistant to gastric digestion and apparently remains intact after it passes through the stomach/abomasum (Miranda and Pelissier, 1983; Yvon et al., 1984), and thus its component amino acids may be nutritionally unavailable. Recently, Jakobsson et al. (1985) reported the presence of immunoreactive bovine β-Lg in human milk and correlated its presence to the development of colic in breast-fed babies.

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Since β-Lg is thermolabile, heat processing may alter its digestibility characteristics and render it biologically available. Milk undergoes heat processing such as preheating, pasteurization, sterilization, concentration, dehydration, etc., which affects the structure and properties of milk/whey proteins, either reversibly or irreversibly. The effect of heat on the denaturation of β-Lg has been reviewed by McKenzie (1971). Dupont (1965a,b) and Dewit and Swinkels (1980) showed that reversible conformational change/denaturation of β-Lg occurred below 70 °C, but above this temperature, denaturation resulted in irreversible polymerization. There is limited information concerning the effects of heat-induced conformational changes of β-Lg on its resistance to proteolytic digestion especially at pH 1–2 where pepsin digestion occurs.

β-Lg contains four S–S groups per dimer of molecular weight 36000 (McKenzie, 1971). Intramolecular S–S bonds

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maintain the structural integrity and increase the stability of proteins (Cantor and Schimmel, 1980). Reduction of these bonds destabilizes the conformation of β-Lg. The S-carboxymethylation of β-Lg in the presence of β-mercaptoethanol increased the susceptibility of protein to peptic, tryptic, and chymotryptic digestibility (Otani, 1981).

The structural and conformational basis of the resistance of β-Lg to digestion and methods to reduce it are of theoretical and practical interest. Hence, the effects of heating at pH 6.8 and of disulfide bond cleavage on the in vitro digestibility of β-Lg by pepsin at pH 2.0 and α-chymotrypsin at pH 8.0 were studied. The changes in proteolytic digestion following heating and S–S bond cleavage were correlated with conformational changes in the protein.

MATERIALS AND METHODS

Materials. β-Lactoglobulin (a mixture of A and B variants) was isolated from fresh cow skim milk by the procedure of Aschaffenberg and Drewry (1957), lyophilized, and stored at 4 °C under desiccation. The α-chymotrypsin (type II, activity 40–60 units/mg of protein), pepsin (activity 2500–3500 units/mg of protein), and the hemimagnesium salt of 8-anilinonaphthalene-1-sulfonate (ANS) were purchased from Sigma Chemical Co., St. Louis, MO. ANS was recrystallized three times from hot water and used. All other chemicals used in this study were of reagent grade.

Methods. *Peptic Digestibility.* Pepsin hydrolysis was carried out at 37 °C in 0.1 N HCl (adjusted to pH 2.0 with NaOH) with a protein to enzyme ratio of 200:1. To 15 mL of 0.1% β-Lg solution, equilibrated in a constant-temperature water bath at 37 °C for 15 min, was added 75 μL of 0.1% pepsin solution (in 0.1 N HCl, pH 2.0) and the digestibility was assessed by the trichloroacetic acid (TCA) precipitation method adopted from Shyamasundar and Rao (1980). Aliquots (1 mL) of the digest were removed at regular intervals during digestion, and 1 mL of 20% TCA was added to terminate the enzymatic reaction and precipitate the undigested protein. The resultant mixture was allowed to stand for 10 min and centrifuged at 5000 rpm for 20 min at room temperature, and the absorbance of TCA-soluble peptides in the supernatant was measured at 280 nm on a Cary 219 double-beam spectrophotometer. All analyses were done in triplicate.

Chymotrypsin Digestibility. The enzymatic reaction was carried out at 37 °C, pH 8.0, for a given time with a protein to enzyme ratio of 200:1. To 15 mL of 0.1% β-Lg solutions (in 0.1 N HCl containing 0.098 M $NaHCO_3$ and 0.002 M Na_2CO_3, pH 8.0) equilibrated in a constant-temperature water bath at 37 °C for 15 min was added 75 μL of 0.1% chymotrypsin solution (in buffer, pH 8.0), and proteolysis was monitored by TCA precipitation as described above.

Heat Treatment. Aliquots (20 mL) of 0.1% β-Lg solution in 0.1 N HCl (adjusted to pH 6.8 with NaOH) or 0.1 N HCl, 0.098 M $NaHCO_3$, 0.002 M Na_2CO_3, pH 6.8, were heated in screw cap tubes at specific temperatures in a thermostatically controlled water bath. Each solution was preheated for 3 min to attain the desired temperatures and held at the specified temperatures for 15 min. After heating, the tubes were cooled to 23 °C in water. The pH was readjusted to 2.0 using 5 N HCl or to 8.0 with 5 N NaOH, and the pepsin and chymotrypsin digestibility was determined at 37 °C as described above.

Preparation of S–S Bond Cleaved β-Lg. β-Lg in which the disulfide bonds were completely cleaved was prepared by the method of Kella and Kinsella (1985) using cupric ion catalyzed sufitolysis. β-Lg (200 mg) was dissolved in 200 mL of 0.1 M phosphate buffer, pH 7.0, containing 0.1 M sodium sulfite and the resultant mixture incubated at 40 °C for 15 min. To this was added an ammoniacal solution of cupric sulfate (400 μM), and oxygen was bubbled through the gas dispenser with gentle stirring. The kinetics of the reaction were followed by stopping the reaction (at regular intervals) by the addition of 0.2 M EDTA (40 mM) and estimating the remaining S–S bond content (Kella and Kinsella, 1985). The reaction was continued until all the S–S bonds were cleaved. When the sulfitolysis reaction was complete, the β-Lg was dialyzed against water containing 40 mM EDTA and then lyophilized.

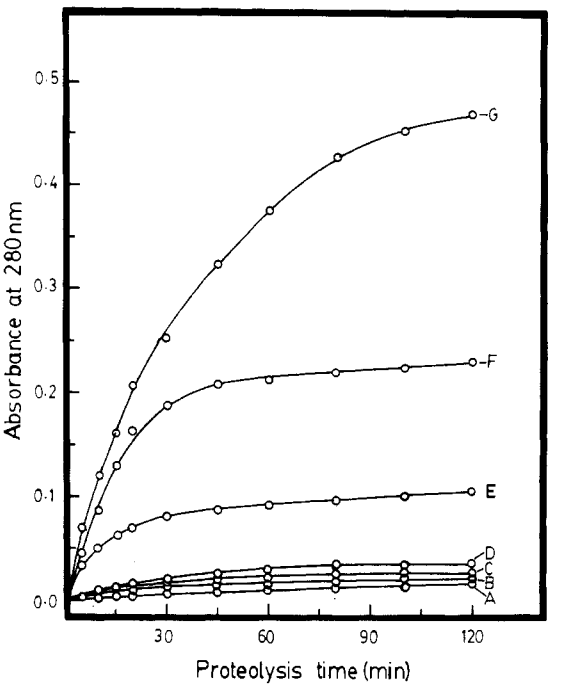

Figure 1. Kinetics of peptic hydrolysis of native, heat-treated, and S–S bond cleaved β-Lg at pH 2.0 and 37 °C as followed by the TCA precipitation method. Concentration of β-Lg was 1 mg/mL, and protein to enzyme ratio was 200:1: (A) native; (B) 50 °C heated; (C) 60 °C heated; (D) 70 °C heated; (E) 80 °C heated; (F) 90 °C heated; (G) S–S bond cleaved β-Lg.

Fluorescence Spectra. Ratiometric fluorescence spectra were recorded in a Perkin-Elmer fluorescence spectrophotometer, Model 650-40, using 1-cm-square cuvettes thermostated at 25 °C. Protein solutions (0.04 mg/mL) were excited at 280 nm, and the emission was automatically recorded at right angles to the excitation with a 5-nm band width in the range 300–400 nm.

Surface Polarity. The polarity of the hydrophobic binding sites on the protein surface was determined with ANS, according to Turner and Brand (1968). Protein solutions (0.1 mg/mL) containing 20 μM ANS were excited at 350 nm, and the emission was recorded at right angles to excitation, in the range of 400–500 nm with a 5-nm excitation band width at 25 °C. The fluorescence spectra in all cases were corrected for the emission of ANS blank; ν_F, the reciprocal of the emission maximum, was calculated in each case. From these values the surface polarity on Kosowar's Z scale was calculated from a standard curve (Turner and Brand, 1968).

RESULTS AND DISCUSSION

Peptic Digestibility. The time course of peptic hydrolysis of native β-Lg, heat-treated β-Lg, and S–S bond cleaved β-Lg is shown in Figure 1. The results show that native β-Lg is quite resistant to proteolysis by pepsin at pH 2.0. Heating at 50, 60, and 70 °C did not alter the

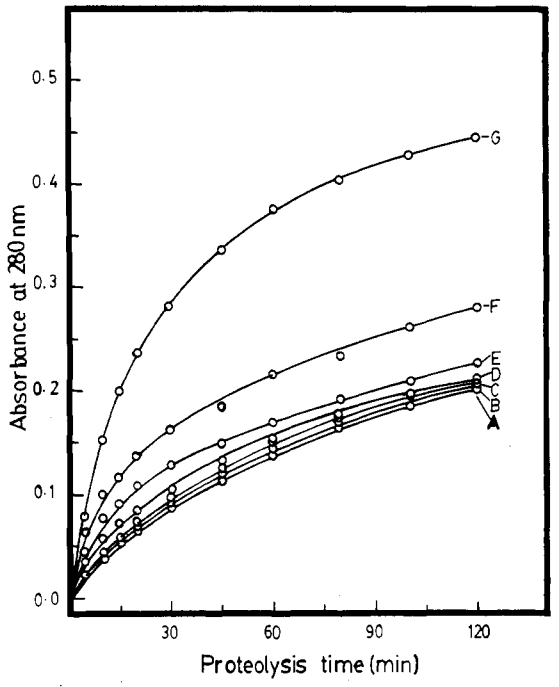

Figure 2. Kinetics of chymotryptic hydrolysis of native, heat-treated, and S–S bond cleaved β-Lg at pH 8.0 and 37 °C as followed by the TCA precipitation method. Concentration of β-Lg was 1 mg/mL, and protein to enzyme ratio was 200:1: (A) native; (B) 50 °C heated; (C) 60 °C heated; (D) 70 °C heated; (E) 80 °C heated; (F) 90 °C heated; (G) S–S bond cleaved β-Lg.

resistance of β-Lg to peptic digestibility. Heating at 80 °C increased the hydrolysis to some extent, while heating at 90 °C resulted in a rapid increase in hydrolysis of β-Lg up to 45 min where it reached a constant value.

Cleavage of the S–S bonds significantly increased the peptic digestibility of β-Lg. The proteolysis followed the order S–S bond cleaved β-Lg > 90 °C heated β-Lg > 80 °C heated β-Lg > 70 °C heated β-Lg ≥ 60 °C heated β-Lg ≥ 50 °C heated β-Lg ≥ native β-Lg.

Chymotryptic Digestibility. The kinetics of chymotryptic digestibility of native, heat-treated, and S–S bond cleaved β-Lg are shown in Figure 2. Proteolysis increased with time up to 2 h in all cases. Susceptibility to proteolysis gradually increased with heating temperature; however, the effect was more pronounced at 80 and 90 °C than at 50, 60, and 70 °C. A significantly greater increase in proteolysis was observed with S–S bond cleaved β-Lg compared to heat-treated β-Lg. The proteolysis followed the relative order S–S bond cleaved β-Lg > 90 °C heated β-Lg > 80 °C heated β-Lg > 70 °C heated β-Lg > 60 °C heated β-Lg ≥ 50 °C heated β-Lg ≥ native β-Lg.

Fluorescence Spectra. The conformational changes in β-Lg during heating at pH 6.8, subsequent cooling, and adjusting to pH 2.0 and 8.0 and in the S–S bond cleaved β-Lg at pH 2.0 and 8.0 (the optimum pH for pepsin and chymotrypsin, respectively) were followed by intrinsic fluorescence spectral measurements. The fluorescence emission spectra of native, heat-treated, and S–S bond cleaved β-Lg at pH 2.0 and 8.0, respectively, are shown in Figure 3a,b. Fluorescence emission at 335 nm as a function of temperature is presented in Figure 3c. Quenching of fluorescence emission intensity (F_{max}) with heating temperature and S–S bond cleavage was observed at pH 2.0 (Figure 3a). However, the changes in F_{max} are minimal at 50, 60, and 70 °C but are substantial at 80 and 90 °C (Figure 3c). An increase (red-shift) in the wavelength of maximum emission (λ_{max}) was observed with S–S bond cleaved β-Lg, whereas λ_{max} remained constant at 335 nm for native and heat-treated β-Lg at pH 2.0.

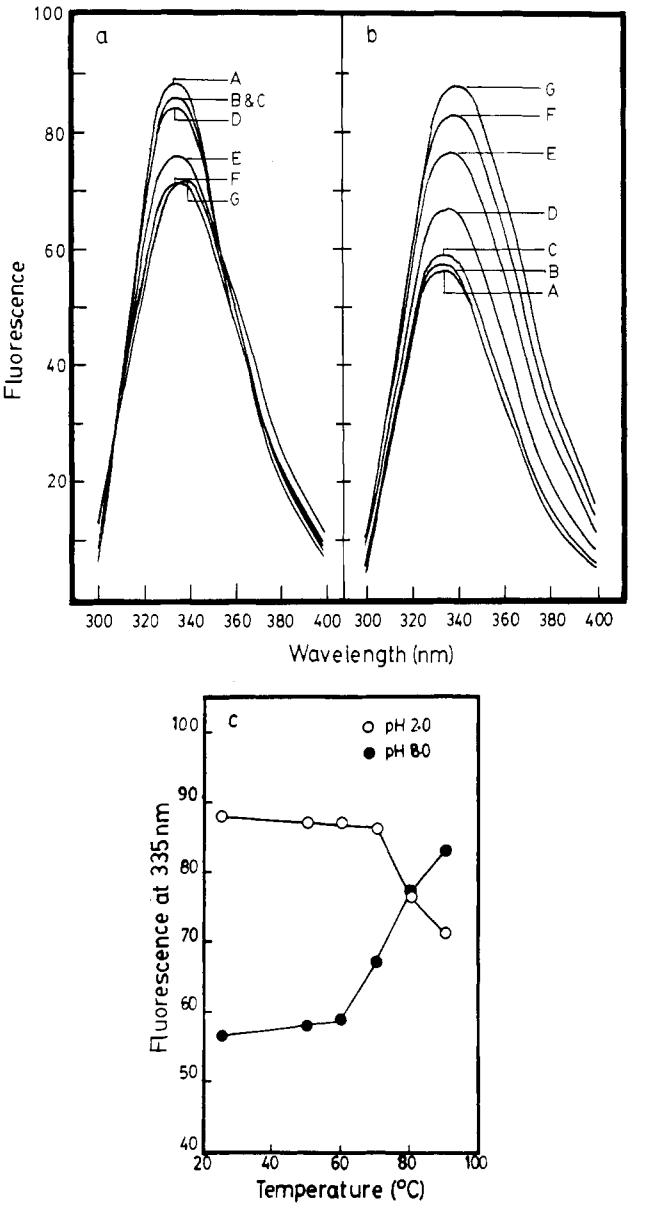

Figure 3. Fluorescence emission spectra of native, heat-treated, and S–S bond cleaved β-Lg: (a) pH 2.0; (b) pH 8.0; (c) fluorescence at 335 nm vs temperature. Key: (A) native; (B) 50 °C heated; (C) 60 °C heated; (D) 70 °C heated; (E) 80 °C heated; (F) 90 °C heated; (G) S–S bond cleaved β-Lg.

Mills (1976) reported quenching of F_{max} during heating of β-Lg at pH 6.4 from 20 to 90 °C and attributed this to the conformational changes leading to the exposure of tryptophan groups to the polar environment. The observed changes were reversible when the protein was cooled to 20 °C after heat treatments up to 70 °C, but above 70 °C, they became irreversible. Minimal changes in F_{max} up to 70 °C observed in the present study could be due to reversible conformational changes upon heating β-Lg at pH 6.8, subsequent cooling, and adjusting to pH 2.0.

An increase in F_{max} accompanied by a red-shift in λ_{max} was observed with an increase in heating temperature and S–S bond cleavage of β-Lg at pH 8.0 (Figure 3b). The F_{max} values at pH 8.0 for native β-Lg and β-Lg heated at 50 and 60 °C are almost the same, whereas the values increased at 70, 80, and 90 °C (Figure 3c). Interestingly, the F_{max} values for native β-Lg and heat-treated β-Lg up to 70 °C were low at pH 8.0 compared to the values at pH 2.0 (Figure 3c). This means that heat-treated β-Lg has undergone substantial pH-induced conformational changes

Table I. Surface Polarity of Heat-Treated (pH 6.8) and S-S Bond Cleaved β-Lg as Estimated from the Emission Maxima of the Bound ANS at pH 2.0 and 8.0

	pH 2.0		pH 8.0	
β-lactoglobulin	$\nu_F \times 10^4$, cm^{-1}	Z	$\nu_F \times 10^4$, cm^{-1}	Z
native	2.062	92.00	2.083	91.25
heated at 50 °C	2.083	91.25	2.083	91.25
heated at 60 °C	2.083	91.25	2.105	90.75
heated at 70 °C	2.083	91.25	2.127	89.00
heated at 80 °C	2.105	90.75	2.127	89.00
heated at 90 °C	2.105	90.75	2.150	88.10
S-S bond cleaved	2.127	89.00	2.127	89.00

at pH 8.0 than at pH 2.0. The above phenomenon can be explained on the basis of earlier reports that β-Lg (dimer) dissociates to monomer and undergoes reversible conformational change above pH 7.5, causing the exposure of tryptophan and tyrosyl residues to solvent (Townend et al., 1969; Zimmerman et al., 1970). β-Lg contains four tryptophan groups per dimer; two of them are "buried" and the other two are "exposed" or four of them are partly "buried" (McKenzie, 1971). β-Lg (dimer) is known to dissociate to the monomer in the region pH 6–9 as the temperature is increased from 20 to 45 °C and to aggregate above 65 °C with increasing time involving SH oxidation and S–S interchange (McKenzie, 1971). It is conceivable that heating above 65 °C has probably buried one of the tryptophan groups irreversibly, and subsequent conformational changes at pH 8.0 did not expose it to the solvent, hence the increase in F_{max} at 70, 80, and 90 °C. Kronman (1976) reported an increase in F_{max} with heating temperature for α-lactalbumin in 0.15 M KCl.

Walker et al. (1966, 1967) have shown that although a fluorophore may be buried in the hydrophobic interior of the molecule, it may still form an excited-state complex, or "exciplex" with a polar residue, which would have a λ_{max} of the order of 350 nm. The red-shift in the λ_{max} from 335 to 340 nm with an increase in temperature (70–90 °C) of β-Lg at pH 8.0 could be due to the formation of an exciplex.

Surface Polarity. The extrinsic fluorescence emission spectra of the ANS conjugates of native β-Lg at pH 2.0 and 8.0 (corrected for the fluorescence of the ANS blank) showed emission maxima at 485 and 480 nm, respectively. A decrease (blue-shift) in emission maxima and an increase in fluorescence emission intensity were observed with an increase in heating temperature and S–S bond cleavage of β-Lg at both pH values. From the emission maxima, ν_F values (which are the measure of emission transition energy) and Z (surface polarity) values were calculated (Table I). Native β-Lg gave Z values of 92.00 and 91.25 at pH 2.0 and 8.0, respectively. The values decreased with an increase in heating temperature and S–S bond cleavage of β-Lg at both pH values, indicating the exposure of hydrophobic groups to polar environment.

Protein digestibility by a proteolytic enzyme affects the nutritional quality of a protein. The rate of proteolysis depends upon the conformation of proteins (Green and Neurath, 1954). Changes in the conformation, which alter the number of accessible peptide bonds, alters the rate of proteolysis (Mihalyi, 1978). At neutral pH, β-Lg exists as a dimer and below pH 3.5 the dimer reversibly dissociates due to the strong electrostatic repulsive forces that develop as the ionogenic groups are titrated (McKenzie, 1971). This pH-induced dissociation is not accompanied by gross changes in molecular conformation (Timasheff et al., 1966; Mills and Creamer, 1975; Teller et al., 1979). Kella and Kinsella (1988) suggested that acid stability of β-Lg could result from increased internal hydrogen bonding that arises between either two titrated carboxyl groups or one amide and one carboxyl group. Thus, the resistance of native β-Lg to peptic digestibility may reflect its stable conformation at pH 2.0. Pepsin has a specificity for tryptophan, tyrosine, phenylalmine, leucine, and isoleucine (Fersht, 1977). The resistance of native β-Lg to peptic digestibility indicates that these groups are not accessible to enzyme.

β-Lg undergoes temperature-dependent thermodenaturation and conformational changes, resulting in the exposure of hydrophobic areas (Brunner, 1977). A decrease in surface polarity or an increase in surface hydrophobicity (Table I) and quenching in F_{max} (Figure 3a) of β-Lg upon heating at pH 6.8 and adjusting to pH 2.0 upon cooling reflect the changes in conformation of the protein. The data indicate that heating at 50, 60, and 70 °C induced very little change in conformation of β-Lg or its resistance to proteolysis by pepsin. However, heating at 80 and 90 °C resulted in measurable changes in the conformation of β-Lg accompanied by increased susceptibility to proteolysis (Figure 1).

Chymotrypsin has a specificity for bonds associated with the hydrophobic side chains of phenylalanine, tyrosine, and tryptophan (Fersht, 1977). Low F_{max} values and surface polarity (Z) values at pH 8.0 compared to those at pH 2.0 indicate that heat-treated β-Lg has undergone extensive conformational changes at pH 8.0, resulting in the exposure of hydrophobic groups to the solvent, thus allowing access of the enzyme to a few strategic peptide bonds. These cleavages may then induce further structural changes, eventually allowing cleavage at many points (Mihalyi, 1978). This explains the higher rate of chymotryptic proteolysis at all heating temperatures including native β-Lg, compared to peptic hydrolysis.

Intramolecular disulfide bonds stabilize the native structure of protein molecules by lowering the entropy of unfolded forms of the chain (Anfinsen and Scheraga, 1975). Cleavage of S–S bonds decreased the surface polarity (Table I) and altered the fluorescence spectra (Figure 3a,b) at pH 2.0 and 8.0, reflecting exposure of hydrophobic groups to the polar environment and alterations in the environment of aromatic side-chain chromophores. The cleavage of S–S bonds resulted in a significant increase in peptic and chymotryptic susceptibility of β-Lg. Underdown and Dorrington (1974) reported that mild reduction and alkylation caused significant changes in the CD spectra and decreased the resistance of IgA proteins to papain digestion.

These results suggest that the structural integrity and conformational stability of β-Lg are related to its resistance to peptic and chymotryptic digestibility. The data indicate that the disruption of native structure and conformation exposes susceptible peptide bonds and decreases significantly the resistance of β-Lg to peptide and chymotryptic proteolysis. Cleavage of S–S bonds was more effective than heating (at 90 °C) in decreasing the resistance of β-Lg to gastric proteolysis. Thus, processes such as heating (>90 °C) or chemical modification through cleavage of S–S bonds using permissible food additives such as sulfite could be used to improve the digestibility of β-Lg.

In recent years, several investigators have reported that various biologically active proteins occurring in milk such as immunoglobulins, transferrin, lactoferrin, and lysozyme are resistant to proteolytic digestion because of their structural and conformational basis (Brown et al., 1970; Underdown and Dorrington, 1974; Brock et al., 1976; Rham and Isliker, 1977; Stone et al., 1979; Kato et al., 1985). β-Lg binds retinol and may function as a physiological retinoid carrier protein (Hemley et al., 1979; Fugate and Song,

1980), specific receptors for β-Lg–retinol complex exist in the intestine of neonatal calves (Papiz et al., 1986), and a homology between β-Lg and serum retinol binding protein exist (Pervaiz and Brew, 1985; Papiz et al., 1986). The present study indicates that the resistance of β-Lg to peptic and chymotryptic digestibility reflects its structural and conformational stability and is consistent with the suggestion that a biological role of β-Lg is the transport of retinol to specific receptors in the duodenum in neonatal calves (Pervaiz and Brew, 1985). Hence resistance to proteolytic degradation at pH 2.0 in the stomach is an important requirement consistent with this function.

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Registry No. Pepsin, 9001-75-6; chymotrypsin, 9004-07-3.

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