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The Kinetics of Heat-Induced Structural Changes of β-Lactoglobulin

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ABSTRACT

Heat-induced structural changes of β-lactoglobulin were studied at temperatures ranging from 67.5 to 82.5°C, and at pH 7.5. These changes were monitored by measurement of surface hydrophobicity, thiol availability, and protein solubility. Kinetic studies were conducted to quantitatively describe the contribution of hydrophobic and SH/SS interchange reactions to the thermal structural changes of β-lactoglobulin. Results indicate that β-lactoglobulin is sensitive to heat-induced interchange reactions with consequences for protein solubility. The extent of changes measured by the increase in surface hydrophobicity and the decrease in slow-reacting SH groups content could be described by a first-order fractional conversion model and were characterized by activation energy values of 233.9 ± 8.6 and 148.2 ± 6.7 kJ/mol, respectively. The break in the Arrhenius plot suggested in literature for β-lactoglobulin denaturation was confirmed in this study only for the kinetics of exposed SH groups.
(**Key words:** β-lactoglobulin, structural change, surface hydrophobicity, sulfhydryl/disulfide interchange reaction)

Abbreviation key: ANS = 1-aniline 8-naphthalene sulfonate, **DTNB** = 5,5′-dithio-bis(2-nitrobenzoic acid), **FI** = fluorescence intensity.

INTRODUCTION

Whey proteins are used as food ingredients because of their high nutritional value and interesting physicochemical properties (Kinsella and Whitehead, 1989; Hoffman et al., 1997). β-Lactoglobulin is the main protein in whey, comprising about 50% of the total whey proteins in bovine milk. At room temperature and physiological pH of milk, β-LG exists mainly as a noncovalently linked dimer stabilized by hydrogen bonds (de Wit, 1998). It is a water-soluble, globular protein and consists of 162 amino acid residues, including 5 cysteine residues and 2 tryptophan residues (Trp^{19} and Trp^{61}) (Kinsella and Whitehead, 1989). Four of the cysteine residues form disulfide bonds (Cys^{106}–Cys^{119} and Cys^{66}–Cys^{160}) and one is a free thiol group, located in position 121. The amino acid sequence of β-LG reveals that many hydrophobic amino acids are located near the Cys^{121} group and Cys^{106}–Cys^{119}, whereas fewer hydrophobic residues are located near Cys^{66}–Cys^{160} (Shimada and Cheftel, 1989). The latter disulfide bond is at the C-terminal side of the polypeptide chain, whereas the former is buried in the inner core of the protein, and is, therefore, less available for intermolecular interchange reactions. In native β-LG, the free thiol group is masked in the hydrophobic interior of the protein and does not normally participate in a disulfide linkage (Bryant and McClements, 1998). The reactivity of the free thiol group can be markedly increased by protein unfolding induced by, for example, thermal treatment. Then, the free SH^{121} group promotes SH/S-S interchange reactions principally with the Cys^{66}–Cys^{160} bond of the same or of another β-LG molecule (Anema and McKenna, 1996). These newly formed SS bonds play an important role in the heat-induced aggregation and gelation of β-LG (Iametti et al., 1995; Anema and McKenna, 1996; Hoffman and van Mill, 1997). Noncovalent interchange reactions (hydrophobic, electrostatic, and steric) may also be involved in this complex process. The thermal denaturation of β-LG was found to be complex due to association of the nonnative monomeric β-LG units combined with the irreversible aggregation of its unfolded state. There are numerous reports on the thermal denaturation of β-LG in buffer solutions or in milk (Shimada and Cheftel, 1989; Laligant et al., 1991; Monahan et al., 1995; Iametti et al., 1996; Galani and Apenten, 1997; Hoffman and van Mill, 1997; Hoffman et al., 1997; Manderson et al., 1999). Although thermal denaturation of β-LG clearly involves some successive steps, the extent of contribution of both covalent and noncovalent interactions to the aggregation and gelation process is not fully elucidated. Extrinsic factors like pH, temperature, and ionic environment may affect molecular flexibility or stability, and there-

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fore protein-protein interactions (Harwalker and Ma, 1989; de Wit, 1998).

A kinetic study on the heat-induced structural changes in β-LG should lead to a better understanding of the relationship between heat treatment and its effect on the functional properties of β-LG, with the perspective of new applications of whey proteins in foods (de la Fuente et al., 2002). Additionally, a kinetic model for the thermal denaturation of β-LG is important for optimizing heat treatment of milk products so that the desired functional properties are achieved.

The objective of this study was to follow the heat-induced changes in surface hydrophobicity, thiol availability, solubility, and turbidity of β-LG solutions heated at pH 7.5. Kinetic studies were performed to quantitatively describe the contribution of hydrophobic and SH/SS interchange reactions to the thermal structural changes of β-LG.

MATERIALS AND METHODS

Bovine β-LG (90% pure) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Isothermal Treatment of β-LG Solutions

β-Lactoglobulin solutions (110 μL of 2.5 mg/mL in 0.02 *M* Tris-HCl buffer, pH 7.5) were heated in 1.5-mL flexible centrifuge tubes (Eppendorf, Eppendorf AG, Hamburg, Germany) in a thermostatically controlled water bath at constant temperatures between 67.5 and 82.5°C for 1 to 45 min. After heat treatment, samples were immediately transferred to ice-cold water to prevent further denaturation. Analysis of the heat-induced changes was performed exactly 2 min after thermal treatment.

Solubility

Diluted samples of treated and untreated β-LG solutions were centrifuged for 15 min (Eppendorf 201 centrifuge, Eppendorf AG) at 19,900 × *g* and 4°C. Protein concentration in the supernatant was determined using Sigma procedure no. TPRO-562. This method of protein quantification is based on the reduction of Cu^{2+} by protein in an alkaline environment. Bicinchoninic acid forms a colored complex with the Cu^{+} formed. The absorbance of the complex was measured at 562 nm. Bovine serum albumin was used as a standard, and all samples were assayed in duplicate. Solubility was expressed as the percentage of protein content in the supernatant compared with the total protein content of the untreated sample.

Turbidity

Turbidity was determined spectrophotometrically after diluting the samples to 1 mL in 0.02 *M* Tris-HCl buffer, at a wavelength of 600 nm and 20°C. One hundred percent turbidity was defined as 0% transmission of light.

Surface Hydrophobicity

The surface hydrophobicity (S_0) was determined spectrofluorometrically using 1-aniline 8-naphthalene sulfonate (**ANS**). A stock solution of ANS (8 m*M*) was prepared in 0.1 *M* phosphate buffer (pH 7.6). The treated and untreated protein solutions were diluted with the phosphate buffer (pH 7.6) to a final protein concentration in the range of 0.002 to 0.0125%. Excitation and emission wavelengths were fixed at 390 and 470 nm, respectively, with 5-nm slit widths. The relative fluorescence intensity (**FI**) of the dilutions with and without ANS was measured with a Cary-Eclipse spectrofluorimeter (Varian, Mulgrave, Victoria, Australia). The net relative FI for each sample was then calculated by subtracting the relative FI attributed to protein in buffer. The initial slope of the net relative FI vs. protein concentration plot was calculated by linear regression analysis and used as an index for protein surface hydrophobicity (Alizadeh-Pasdar and Li-Chan, 2000).

Analysis of Sulfhydryl Groups

The procedure using Ellman's reagent [5,5′-dithio-bis (2-nitrobenzoic acid); **DTNB**] was applied to determine the sulfhydryl group content. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used to calculate the amount of SH groups, expressed in μmoles per gram of protein (Beveridge et al., 1974). Three procedures were followed to determine the amount of total and exposed (surface) SH groups and free SH^{121} groups (slow-reacting SH groups).

Determination of surface and total SH groups. β-Lactoglobuin solutions from all heat-treatment groups were diluted with 990 μL of standard buffer (0.086 *M* Tris, 0.09 *M* glycine, 4 m*M* Na_2EDTA; pH = 8) for surface SH groups, or urea (8 *M* in standard buffer) for total SH groups. To these samples, 10 μL of DTNB (4 mg of DTNB/mL of standard buffer) was added. The absorbance at 412 nm was measured against a reagent blank after 2 min (total SH groups) or 15 min (surface SH groups) at 20°C (Ultrospec 2100 pro, Biochrom, Cambridge, UK).

Determination of slow reacting SH groups. Content of slow reacting SH groups was determined spectrophotometrically, based on a method described by