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HEAT-INDUCED CHANGES IN SOLUBILITY AND SURFACE HYDROPHOBICITY 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 followed by measurement of surface hydrophobicity and protein solubility. Results indicate that β-lactoglobulin is sensitive to heat-induced interchange reactions with consequences for the protein solubility.

***Keywords**:* β-lactoglobulin, thermal treatment, surface hydrophobicity, and solubility.

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 (β-LG) is the main protein in whey, constituting about 50% of the total whey proteins in bovine milk. At room temperature and physiological pH of milk, β-LG exists mainly as a non-covalently linked dimer, stabilised by hydrogen bonds (deWitt, 1998). It is a water soluble, globular protein and consists of 162 amino acid residues, of which five are cysteine residues and two tryptophan residues (Trp^{19} and Trp^{61}) *(*Kinsella and Whitehead, 1989). Four of the cysteine residues are disulfide bound (Cys^{106}–Cys^{119} and Cys^{66}-Cys^{160}) and one is a free thiol group, located in position 121.

The thermal denaturation of β-LG was found to be complicated due to association of the non-native monomeric β-LG units combined with the irreversible aggregation of its unfolded state.

Although thermal denaturation of β-LG clearly involves some successive steps, the extent of contribution of both covalent and non-covalent interactions to the aggregation and gelation process is still not fully elucidated. Extrinsic factors like pH, temperature, and ionic environment may affect molecular flexibility or stability, and therefore protein – protein interactions (Harwalker and Ma, 1989, deWitt, 1998). A study on the structural heat-induced 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*)*. The aim of this study was to follow the heat-induced changes in surface hydrophobicity and solubility of β-LG solutions heated at pH 7.5.

Experimental

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

Isothermal treatment of β-lactoglobulin solutions: β-LG 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) in a thermostated water bath at constant temperatures between 67.5-82.5°C for 1-45 min. After thermal treatment, samples were immediately transferred to ice water to prevent further denaturation. Analysis of the heat-induced changes was always performed exactly 2 min after thermal treatment.

Solubility: Diluted samples of (un)treated β-LG solutions were centrifuged for 15 min (Eppendorf 201 centrifuge) at 19,900 g and 4°C. Protein concentration in the supernatant was determined using Sigma Procedure nr. TPRO-562. Bovine serum albumin was used as a standard. All samples were assayed in duplicate. Solubility was expressed as the percentage of protein content in the supernatant compared to the total protein content of the untreated sample.

Surface Hydrophobicity: The surface hydrophobicity (S_o) was determined spectrofluorimetrically using 1-(aniline)-naphtalene-8-sulfonate (ANS). A stock solution of ANS (8 m*M*) was prepared in 0.1

M phosphate buffer (pH 7.6). The (un)treated protein solutions were diluted with the phosphate buffer (pH 7.6) to a final protein concentration in the range of 0.002-0.0125%. Excitation and emission wavelengths were fixed at 390 and 470 nm respectively, with 5 nm slit widths. The relative fluorescence intensity (rel FI) of the dilutions with and without ANS was measured with a Cary-Eclipse spectrofluorimeter (Varian, USA). The netto relative FI for each sample was then calculated by subtracting the rel FI attributed to protein in buffer. The initial slope of the net rel FI versus protein concentration plot was calculated by linear regression analysis and used as an index for protein surface hydrophobicity (Alizadeh-Pasdar and Li-Chan, 2000). In all cases, R^2 values of 0.99 were noted for the linear regression analyses used to calculate S_o values.

Results and Discussions

Heat-induced changes in the solubility of β-LG solutions

Functional properties of whey proteins are often impaired by inevitable heat treatments during processing of food products. Many attempts have been made to predict desired functional characteristic in food products on the basis of functional properties of whey proteins (deWitt, 1998).

Functionality of β-LG depends on its solubility in aqueous solutions. The major forces that govern the solubility of β-LG are hydrophobic (de la Fuente *et al*., 1998). The activation of SH groups and hydrophobic patches as a consequence of unfolding during heat treatment results in a decrease of protein stability affecting its solubility.

Heating at a temperature between 70 and 75°C results in a minimal loss β-LG in solution, a decrease in solubility of only 10-20% compared with the native protein was observed after 45 min of heating, probably because at lower temperature, the intramolecular interchange reactions are favoured. These results are in good agreement with the data concerning heat-induced changes in surface hydrophobicity β-LG solutions as a function of temperature, as it can be seen in figure 1.

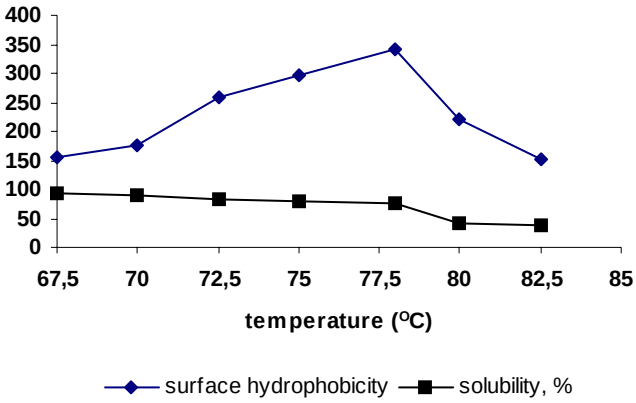

Fig. 1. Heat induced changes in solubility and surface hydrophobicity of β-LG after 45 minutes of heating at different temperatures

The maximum extent of unfolding coincides for both properties measured above 78°C. It should be noted that above pH 7.0, the dimer starts to dissociate to form non-native monomer. Thus, partial protein denaturation and its solubility coincide to some extent and good solubility of β-LG after thermal treatment under neutral conditions and at low ionic strength is theoretically expected (De Witt and Klarenbeek, 1983) and actually observed. When too many hydrophobic sites are exposed, due to thermal treatment, the hydrophobic interactions are enhanced, usually leading to a decrease in solubility. Irreversible changes of protein structure occurred above the denaturation temperature (figure 1) and results in reduced protein solubility. However, thermal treatment above 80°C results in protein aggregation, with a decrease in solubility of 60% (Figure 2).

This observation indicates that thermal denaturation of β-LG as measured by the changes in solubility involves two steps: an unfolding step (70-75°C) and an aggregation step (78-82.5°C), that mostly follows unfolding, leading to a major decrease in solubility.

The extent of unfolding required prior to aggregation is unknown but it is clearly affected by temperature and holding time.

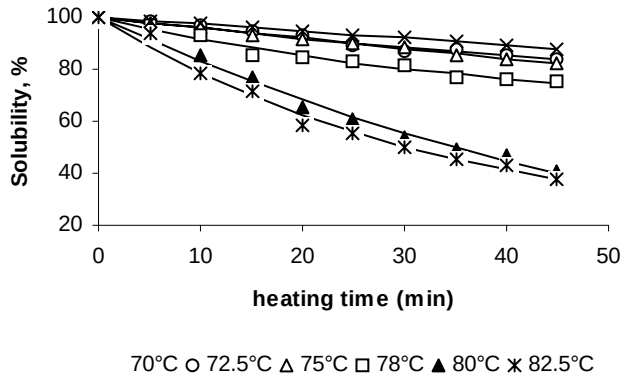

Fig. 2. Thermal denaturation curves of β-LG (2.5 mg/ml in Tris-HCl buffer 0.02 M, pH = 7.5) after heat treatment at different temperature as determined by the changes in solubility

Heat-induced changes in the surface hydrophobicity of β-lactoglobulin solutions

β-LG contains a high proportion of hydrophobic amino acid side chains, preferentially turned toward the inside of the molecule (Laligant *et al.,* 1991). When the molecule is unfolded, an increase in surface hydrophobicity is expected. The application of the ANS probe can provide information about the structural changes due to heat treatment (Manderson *et al.,* 1999). Thus, the measurement of surface hydrophobicity can be used as an approach for studying the protein-protein interactions (Alizadeh-Pasdar and Li-Chan, 2000), with further application for the manufacturing of gel with different characteristics (Monahan *et al.*, 1995). According to Shimada and Cheftel (1991), unfolding of β-LG molecules is followed by protein aggregation, through hydrophobic interactions and/or SH/SS interchange reactions, leading to a decrease in surface hydrophobicity.

Figure 3 shows that the ANS fluorescence intensity increases as a function of protein concentration at various temperature treatments. These linear relationships indicate that the increase in FI was not due to aggregation. This could arise from unfolded molecules with exposed hydrophobic sites that are more accessible to ANS, compare with native β-lactoglobulin.

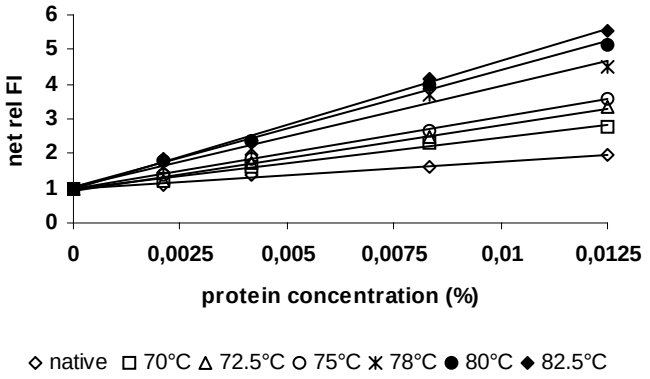

Fig. 3. The increase of relative fluorescence intensity of β-LG solutions after thermal treatment for 20 minutes at different temperature

A marked increase in surface hydrophobicity (S_o) upon heating of β-LG can be observed in Figure 4.

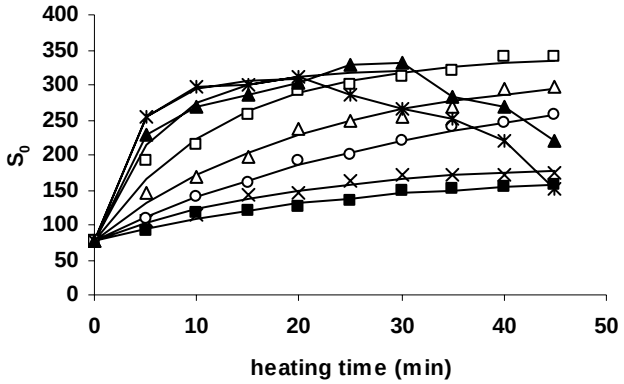

Fig. 4.Thermal denaturation curves of β-LG after heat treatment at different temperature as determined by the changes in surface hydrophobicity

This implies that during thermal treatment the molecules are unfolded leading to an exposure of the hydrophobic clusters, which can promote intra- and intermolecular interactions.

Surface hydrophobicity seems to increase to a plateau value. The maximum value for S_o was reached after 30 min of heating at 80°C,

being 4.2 times higher than the initial values (78.4±3.2). The increase in surface hydrophobicity due to heat treatment is considered a positive attribute for emulsifying and foaming capacities, as long as solubility is not lost (Moro *et. al*, 2001). At 80-82.5°C, however, S_o appears to decrease after reaching this maximum value, as can be seen clearly in Figure 4. After prolonged heating, too many hydrophobic sites are exposed, increasing the probability of intermolecular hydrophobic interactions and as a consequence, the protein aggregates. Monahan *et al.* (1995) observed an increase in surface hydrophobicity for the protein that was heated at temperature between 60-85°C.

Conclusions

Upon heating β-LG at neutral pH, the native dimers start to dissociate into monomer, leading to the exposure of the previously buried hydrophobic amino acids and the single free thiol group. Above 78°C, this is accompanied by the aggregation of the β-LG molecules as a result of hydrophobic interactions, with consequences for protein solubility.

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