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EFFECTS OF MEDIUM AND CHEMICAL MODIFICATION ON THERMAL CHARACTERISTICS OF β-LACTOGLOBULIN*

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Abstract

The thermal properties of β-lactoglobulin (β-LG) were studied by differential scanning calorimetry (DSC) under different medium conditions. *pH*, neutral salts, protein perturbants, and polyols all affected the DSC characteristics of β-LG. Acylation with fatty acids also changed the thermal properties, particularly peak width at half-height. The results suggest that the structural stability of β-LG is controlled by non-covalent forces, particularly electrostatic and hydrophobic interactions. Disulfide bonds did not contribute to the thermal response of β-LG. Fatty N-acyl-amino acids caused marked increases in thermal stability and decreases in denaturation enthalpy, and additional peaks were observed in the presence of some palmitoyl derivatives.

Keywords: β-lactoglobulin, DSC, protein modification, thermal properties

Introduction

β-Lactoglobulin (β-LG) is a major thermolabile milk protein which undergoes conformational and structural changes during processing, particularly treatments involving heat. Since the functionality of food proteins is determined by the molecular structure of these polymers under special conditions [1, 2], these conformational changes in β-LG would have profound influence on the functional properties of products such as whey protein isolates, of which β-LG is the major constituent. Thermoanalytical techniques such as differential scanning calorimetry (DSC) have been used extensively to monitor changes in physicochemical properties of proteins during food processing [3, 4].

In the present study, DSC was used to study the effects of medium composition on the conformation of β-LG. Many food processing steps, such as *pH* adjustment and salt addition, can be simulated by changing the medium composition. The addition of reagents that modify protein structure, such as

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urea, sodium dodecyl sulfate and reducing agents, can provide information on the chemical forces involved in the stabilization of protein structure.

Materials and methods

Materials

β-Lactoglobulin (3X crystallized) was purchased from Sigma Chemical Company, St. Louis, MO and was used without further purification. Fatty N-acylamino acids were prepared from appropriate amino acids and succinimidyl esters of fatty acids [5, 6] or from fatty acid chlorides [7]. All chemicals used were of analytical grade.

Fatty acid modification

Succinimidyl esters of fatty acids were used for acylating the free amino groups of β-LG, according to the method of Paquet [8, 9]. Solutions of β-LG, in saturated sodium bicarbonate, were added to either 7 or 15 equivalents of succinimidyl esters of the appropriate fatty acid in dioxane and stirred for 5 h. The reaction mixture was extensively dialysed and freeze-dried. The dried samples were washed with chloroform to remove un-reacted fatty acids.

Differential scanning calorimetry

The thermal characteristics of β-LG were examined by differential scanning calorimetry (DSC), using a DuPont 1090 Thermal Analyzer equipped with a 910 DSC cell base and a high pressure cell. Liquid samples (10 μL) were pipetted into the pan, or solid samples (about 1 mg) were weighed into the pan, and 10 μL of appropriate buffer were added. A sealed empty pan was used as reference, and Indium standards were used for temperature and energy calibration. The pans were heated from 25 to 120°C at 10°C min^{-1}. The peak or denaturation temperature (T_d), peak width at half-height ($\Delta T_{1/2}$), and heat of transition or enthalpy (ΔH) were computed from the thermograms as described previously [10]. The average standard deviations of T_d, $\Delta T_{1/2}$ and ΔH were ±0.8°C, ±0.4°C, and ±1.2 J g^{-1}, respectively.

Results and discussions

pH

The effects of *pH* on the DSC characteristics of β-LG are summarized in Figs 1 and 2. β-LG exhibited a maximum T_d at *pH* 3 to 4, and a minimum $\Delta T_{1/2}$ value between *pH* 4 and 6 (Fig. 1). There were marked decreases in both T_d and ΔH, and a marked increase in $\Delta T_{1/2}$, at alkaline *pH* (Fig. 1). At *pH* 8 and 9, a small second transition peak at around 130°C was observed (Fig. 2).

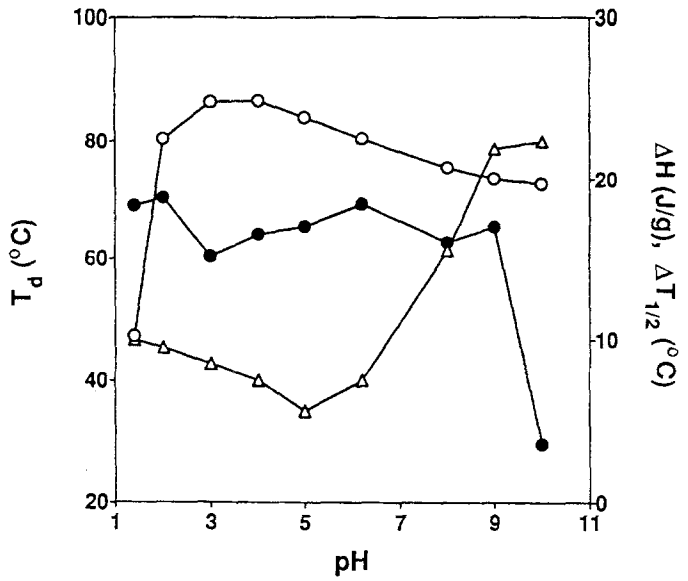

Fig. 1 Effect of *pH* on the DSC characteristics of β-LG. Protein samples (10%) were prepared in distilled water, and *pH* was adjusted by the addition of 0.2 *N* HCl or NaOH. o - T_d ; ● - ΔH; Δ - $\Delta T_{1/2} T_{1/2}$

These observations are in general agreement with other reports [11–15]. Similar to other globular proteins, β-LG has maximum thermal stability near its isoelectric point. At *pH* values far from the isoelectric point, proteins are unfolded due to intramolecular charge repulsion leading to rupture of hydrogen bonds and of hydrophobic interactions [11–15]. Enthalpy of denaturation is correlated with the content of ordered secondary structure of a protein [16], and *pH*-induced unfolding of β-LG will lead to a decrease in the content of ordered secondary structure and a reduction in enthalpy. Peak width at half-height has been used to evaluate the cooperativity of protein unfolding [17]. If denaturation occurred within a narrow range of temperature (low $\Delta T_{1/2}$ value), the transition was considered highly cooperative. The present data indicate that the partially denatured β-LG was a less cooperative system than was the native protein.

The appearance of a second transition peak between 130 and 140°C at *pH* 7–9 was also reported by De Wit and Klarenbeck [13] and Paulsson *et al.* [15]. It was attributed to partial stabilization of β-LG during denaturation near 80°C, followed by destabilization (induced by cleavage of disulfide bonds) of the residual protein structure at higher temperature.

Although DSC can be used to measure the denaturation temperature and the enthalpy change associated with thermal unfolding, most "denatured" proteins including β-LA [18] are not completely unfolded and retain some secondary structures when heated above their T_d, as determined by techniques such as

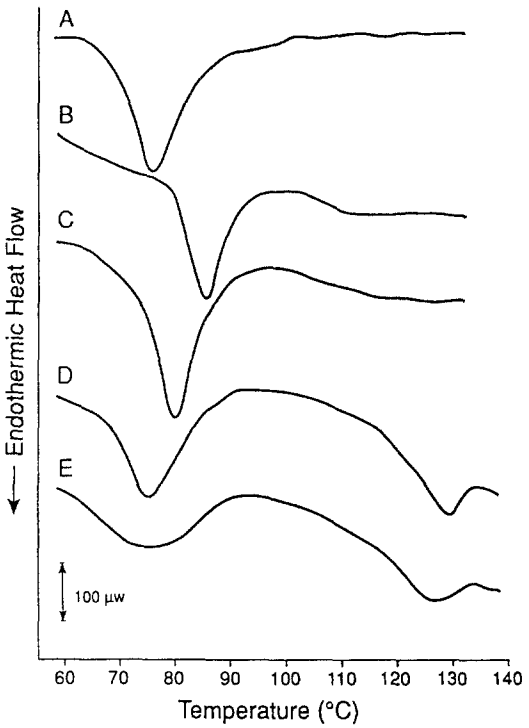

Fig. 2 Effect of *pH* on the DSC curves of β-LG. Protein samples (10%) were prepared in distilled water, and *pH* was adjusted by the addition of 0.2 *N* HCl or NaOH. A: *pH* 1.4; B: *pH* 3.0; C: *pH* 6.2; D: *pH* 8.0; E: *pH* 9.0

Fourier-transform infrared spectroscopy (FTIR) [19]. Small changes in protein conformation due to heating can be detected using monoclonal antibodies (mAbs). The binding affinity of two mAbs of β-LA increased indicating a conformational change in the random coil region when the protein was heated above 67°, well below the T_d determined by DSC [20].

Salts

The effects of NaCl and $CaCl_2$ on the thermal characteristics of β-LG are summarized in Fig. 3. The denaturation temperature was progressively increased with increases in the concentration of both salts, while the enthalpy was initially decreased, followed by either increases (NaCl) or a levelling off ($CaCl_2$). The type of anion in the medium also has a great influence on the thermal stability of β-LG (Table 1). At 1.0 *M* anion concentration, the T_d of β-LG was progressively lowered following the order Cl^-, Br^-, I^-, and SCN^-. The enthalpy values were not affected markedly.

The heat stability of proteins is controlled by the balance of polar and nonpolar residues [21], such that the larger the proportion of nonpolar residues, the

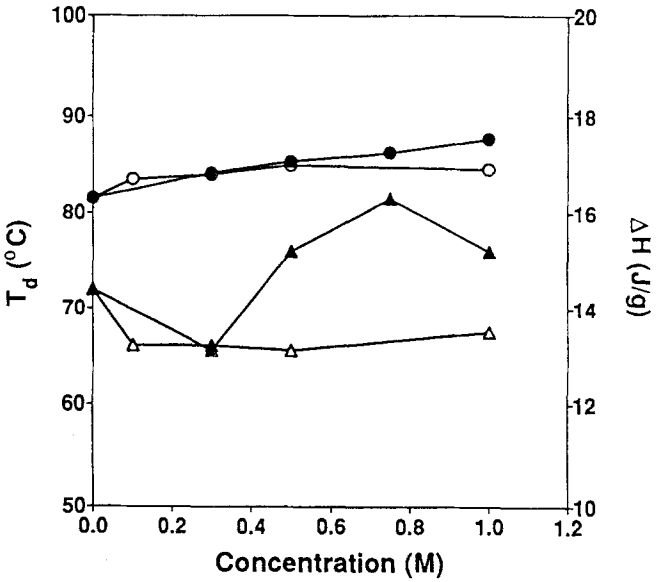

Fig. 3 Effect of sodium chloride and calcium chloride on the DSC characteristics of β-LG. Protein samples (10%) were prepared in salt solutions without *pH* adjustment. o - T_d (in NaCl); ● - T_d (in $CaCl_2$); Δ - ΔH (in NaCl); ▲ - ΔH (in $CaCl_2$)

higher the heat stability. Salts can perturb the conformation of proteins by influencing the electrostatic interaction with the charged groups and polar groups, and by affecting the hydrophobic interaction via a modification of the water structure [22, 23]. The degree to which water structure is affected depends on the nature of cations and anions, following the lyotrophic series [24]. Cations and anions at the higher order of the series (e.g., Ca^{++} and SCN^-) could reduce the free energy required to transfer the nonpolar groups into water, and thereby weaken intramolecular hydrophobic interactions and enhance the unfolding tendency of proteins [25], thus lowering both T_d and ΔH. The present data suggest that hydrophobic interaction plays an important role in the stabilization of protein structure in β-LG.

Table 1 Effect of anion on DSC characteristics of β-lactoglobulin[a]

Anion[b]	T_d/°C	ΔH/J g^{-1}
Cl^-	89.4	11.7
Br^-	87.2	13.6
I^-	83.9	12.5
SCN^-	80.3	13.8

[a] Average of two determinations.

[b] Sodium salts at 1.0 *M* concentration were used without *pH* adjustment.

T_d: denaturation temperature; ΔH: enthalpy of denaturation.

Sugars and polyols

Figure 4 shows the effect of some sugars and polyols on the thermal transition characteristics of β-LG. The thermal stability was enhanced by these additives, except for ethylene glycol, which lowered the T_d of β-LG. The enthalpy was either increased or decreased, but did not follow any specific pattern. The peak width at half-height remained unchanged (data not shown), indicating that the cooperativity of the thermal transition was not affected.

The protective effect of sugars against thermal denaturation of proteins has been well documented [26–29]. The extent of stabilization (increased T_d) by these sugars or polyols depends upon both the type of polyols and the nature of proteins [27]. The stabilization seems to arise from the effect of polyol on water structure, which in turn determines the strength of the hydrophobic interactions. Polyols reduce the driving force for transfer of hydrophobic groups from aqueous to nonpolar environments [27]. Although ethylene glycol is a polyol, it has the specific characteristic of lowering the dielectric constant of the medium and interacts with nonpolar side chains of proteins, thus weakening hydrophobic in-

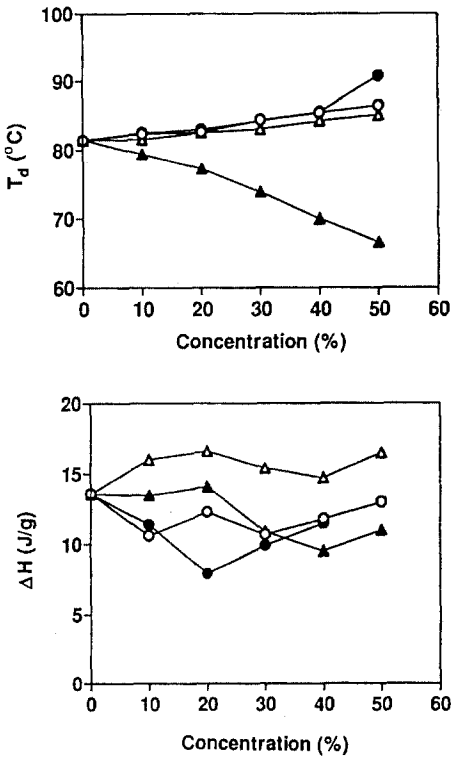

Fig. 4 Effect of sugars and polyols on the DSC charactersitics of β-LG. Protein samples (10%) were prepared in sugar and polyol solutions without *pH* adjustment.
o - sucrose; ● - glucose; Δ - glycerol; ▲- ethylene glycol

teractions and lowering thermal stability [26]. It has also been suggested that protein stability depends on the excess chemical potential of water [30]. Sugars lowered the excess chemical potential of water and increased the cohesion of water molecules in the solution, thus stabilizing globular protein conformation.

Protein structure perturbants

Several chemical reagents, known to modify protein conformation, were used to assess their effects on thermal characteristics of β-LG. Both T_d and ΔH of β-LG were lowered by urea and sodium dodecyl sulphate (SDS), whereas the $\Delta T_{1/2}$ was markedly increased (Table 2). At low concentrations of SDS (≤5 *mM*), however, T_d was increased while $\Delta T_{1/2}$ was decreased.

Table 2 Effect of protein perturbants on DSC characteristics of β-lactoglobulin[a]

Additive	T_d/°C	ΔH/J g^{-1}	$\Delta T_{1/2}$/°C
No additive	81.5	13.6	5.0
3 M urea	75.8	12.9	6.3
6 M urea	66.7	9.6	7.5
1 mM SDS	82.3	12.5	5.0
5 mM SDS	84.2	10.1	4.3
10 mM SDS	80.5	11.8	9.0
20 mM SDS	76.4	7.4	13.0
10 mM DTT	81.3	13.3	n.d.
10 mM DTT+10 mM SDS	80.9	11.5	n.d.

[a] Averages of two determinations. Proteins was dispersed in distilled water with or without additives.

T_d: denaturation temperature; ΔH: enthalpy of denaturation; $\Delta T_{1/2}$: peak width at half-height; n.d.: not determined.

Urea effectively disrupts the hydrogen-bonded structure of water and facilitates protein unfolding by weakening hydrophobic interactions [31]. Urea is also regarded as a "cosolvent", or a compound in binary aqueous solvents, which preferentially binds to protein, and is associated with destabilization of native structure [32]. SDS is an anionic detergent generally regarded as a protein denaturant [33]. Low concentrations of SDS may stabilize proteins against denaturation by highly specific interactions, presumably between cationic groups of proteins and anionic groups of SDS [34]. The resulting hydrophobic side chains of SDS may be entropically transferred to the interior of the protein molecules, leading to an increase in thermal stability. At higher detergent concentrations, the binding seems to be non-specific and causes charge repulsion between protein chains, leading to either unfolding or lowering in thermal stability. Similar to the effect of extreme *pH*'s, urea and SDS (at higher concentrations) caused loss of cooperativity in β-LG, as indicated by the increases in peak width at half-height.

β-LG exists as a dimer in milk and as an octamer near its isoelectric *pH*, and the monomers are linked by non-covalent bonds. At extreme *pH*, β-LG exists as a monomer which contains two disulfide bonds and a sulfydryl group. To assess the contribution of disulfide bonds to the thermal characteristics of β-LG, dithiothreitol (DTT), a reducing agent, was added to the medium with or without SDS. DTT did not cause marked changes in T_d or ΔH of β-LG in the absence or presence of SDS (Table 2). These results do not support the view that disulfide bonds contribute to thermal stability of β-LG, whereas other proteins containing disulfide bonds generally have higher T_d and ΔH values than similar proteins without disulfide linkages [31].

Fatty N-acylamino acids

Table 3 shows the effect of some fatty N-acylamino acids on the thermal transition characteristics of β-LG. There were marked increases in T_d and decreases in ΔH values, with the exception of lauroyl methionine methyl ester. The peak width at half-height was generally increased in the presence of the derivatives (Table 3). Additional peaks were observed in some curves (Figs 5 and 6). In some instances, e.g., lauroyl methionine methyl ester (Fig. 5C), the additional peak was attributed to the additive (demonstrated by a separate scan of the derivative; data not shown), and the transition was reversible (confirmed by a re-scan; data not shown), as opposed to the non-reversible protein transition.

Table 3 Effect of fatty N-acylamino acids on DSC characteristics of β-lactoglobulin[a]

Derivative	T_d/°C	ΔH/J g^{-1}	$\Delta T_{1/2}$/°C
Control	75.7	12.4	8.0
Lauroyl tryptophan	85.1	6.5	13.0
Lauroyl phenylalanine	84.6	5.8	10.0
Lauroyl methionine	83.5	5.6	10.5
Lauroyl methionine methyl ester	76.3	9.2	8.0
Myristoyl tryptophan	85.4	6.2	8.5
Myristoyl phenylalanine	84.2	5.5	10.5
Myristoyl methionine	84.7	5.5	10.5
Palmitoyl tryptophan	85.3	7.8	8.0
Palmitoyl phenylalanine	84.3	8.5	8.0
Palmitoyl methionine	84.3	6.6	9.5

[a] Average of two determinations. Protein samples (10%) were prepared in 0.1 *M* sodium phosphate buffer, *pH* 6.8, and 0.05 mg of fatty N-acilamino acid were added to 10 μL of protein solution
T_d: denaturation termperature; ΔH: enthalpy of denaturation; $\Delta T_{1/2}$: peak width at half-height.

The addition of palmitoyl methionine (Fig. 5E) and palmitoyl tryptophan (Fig. 6) led to the appearance of a sharp endotherm near 100°C. These additives showed a large endothermic peak near 80°C (Fig. 6F), and both the additive transitions and the 100°C peak were reversible upon re-scan (data not shown). The addition of increasing amount of palmitoyl tryptophan led to a gradual shift of the β-LG peak to higher temperature, and progressive increases in the 100°C transition (Fig. 6). At high derivative concentration, there was the appearance of a small transition near 75°C (Fig. 6E).

Fatty N-acylamino acids have been found to possess significant antibacterial properties [36, 37], high nutritive value [38], and good emulsifying properties [39, 40], suggesting their potential as multi-functional food additives. In a recent study [41], it was observed that fatty N-acylamino acids improved the functional properties of egg white and whey protein, and some of the changes could be attributed to modification of the thermal characteristics of the proteins. Additional endothermic peaks were also observed in some scans [41].

Fatty N-acylamino acids are surface-active agents composed of hydrophobic fatty acids and hydrophilic amino acids, and a proper lipophile-hydrophile balance is required for both optimal antimicrobial and functional properties

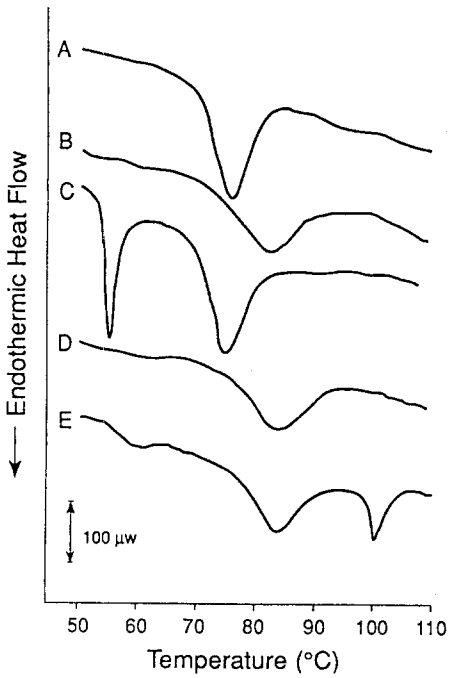

Fig. 5 Effect of fatty N-acyl methionine on the DSC curves of β-LG. Protein samples (10%) were prepared in 0.1 *M* sodium phosphate buffer, *pH* 6.8, and 0.05 mg fatty N-acyl methionine was added to 10 µL of protein solution. A:control (no additive); B: lauroyl-methionine; C: lauroyl methionine methyl ester; D: myristoyl methionine; E: palmitoyl methionine

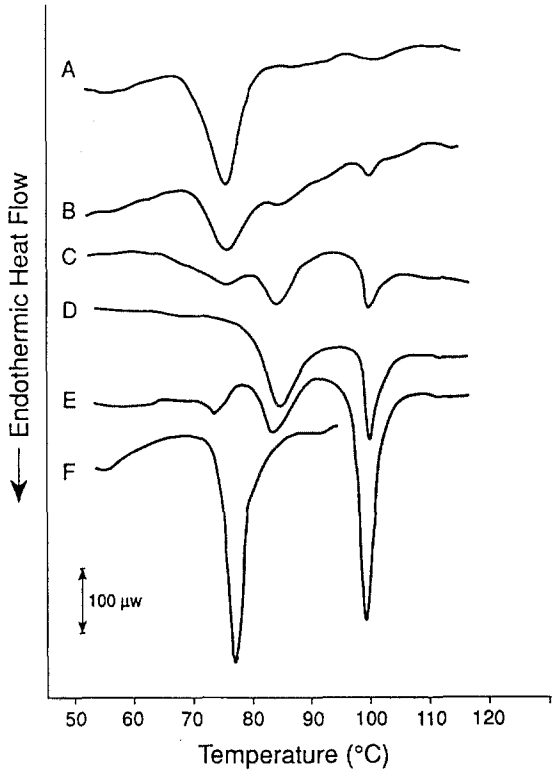

Fig. 6 Effect of palmitoyl tryptophan on the DSC curves of β-LG. Protein samples (10%) were prepared in 0.1 *M* sodium phosphate buffer, *pH* 6.8, and weighed palmitoyl tryptophan was added to 10μL of protein solution. A: control (no additive); B: 0.01 mg; C: 0.03 mg; D: 0.10 mg; E: 0.20 mg; F: 0.10 mg palmitoyl tryptophan without β-LG

[36, 41]. The ineffectiveness of the esters of active derivatives was due to the blocking of the hydrophilic carboxyl group of the amino acids. The fact that the DSC characteristics of β-LG were not affected by the ester of lauroyl methionine further indicates a close relationship between physicochemical properties of proteins and their conformation.

The extra endothermic peak observed near 100°C in the presence of some palmitoyl derivatives, could represent a product of the interaction between the additives and protein, similar to that observed in starch-lipid interactions [42]. The starch-lipid complexes, with transition temperatures (95–130°C) well above the melting endotherm of starch crystallites [43], are also thermoreversible [44, 45]. The re-appearance of a small transition around 75°C in the presence of high palmitoyl tryptophan concentration could be attributed to residual un-reacted additive.

Fatty acid modifications

The effect of lipophilization on DSC characteristics of β-LG is shown in Table 4. The use of organic solvent caused significant protein denaturation, as indicated by the marked decrease in enthalpy. Acylation with fatty acids restored some protein structure, with increases in ΔH value. The extent of changes in ΔH and T_d varied with the chain length of the fatty acids. Fatty acid modifications also caused a marked sharpening of the endothermic peak and decrease in $\Delta T_{1/2}$ value, and the C_{16} modified protein exhibited an additional endotherm near 100°C (Table 4).

Table 4 Effect of fatty acid modification on DSC characteristics of β-lactoglobulin[a]

Treatment	T_d/°C	ΔH/J g^{-1}	$\Delta T_{1/2}$/°C
Control	81.5	14.4	5.0
Dioxane treated	84.8	1.3	11.5
C_{12}-modified	83.2	2.2	9.0
C_{14}-modified	80.2	3.6	2.8
C_{16}-modified	83.7, 99.5	1.9, 2.2	2.8, 2.5
C_{18}-modified	88.4	5.5	2.5

[a] Average of two determinations. The control was non-modified protein dispersed in distilled water without *pH* adjustment.
T_d: denaturation termperature; ΔH: enthalpy of denaturation; $\Delta T_{1/2}$: peak width at half-height.

Covalent attachment of fatty acids to free amino groups of proteins is a chemical modification used to improve functionality of proteins, by deliberately introducing lipophilicity, making a protein more amphiphilic [46, 47]. The introduction of a hydrophobic fatty acid side chain to β-LG could enhance a compact conformation through hydrophobic interactions, and may promote the formation of additional hydrogen bonds, thus partly restoring the endothermic peak. The compact protein would also denature in a more cooperative manner, resulting in lower $\Delta T_{1/2}$ values. The appearance of the additional peak in the palmitic acid-modified protein can be attributed to complex formation similar to that observed in the presence of some palmitoyl amino acids. However, the additional endotherm was not thermoreversible, suggesting that the interaction product has properties different from those of the fatty N-acylamino acid β-LG complexes.

Conclusion

The present data showed that changes in medium, such as *pH*, neutral salts, protein perturbants, polyols, etc., and fatty acid modification have a profound influence on the thermal properties of β-lactoglobulin. The data suggest that

non-covalent forces, particularly electrostatic and hydrophobic interactions, contribute to the stability of β-lactoglobulin. The effect of some fatty N-acyl-amino acids on β-LG is more complex, suggesting the formation of an interaction product with high thermal stability.

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