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# Enhanced thermodynamic stability of β-lactoglobulin at low pH

## A possible mechanism

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The thermodynamic stability of β-lactoglobulin (β-Lg) was studied at acidic and near-neutral pH values using equilibrium thermal-unfolding measurements. Transition temperature increased with a decrease in pH from 7.5 to 6.5 and 3.0 to 1.5, suggesting an increase in the net protein stability. Determination of the change in free energy of unfolding and extrapolation into the nontransition region revealed that β-Lg increases its stability by increasing the magnitude of the change in free energy of unfolding at the temperature of maximum stability, as well as by increasing the temperature of maximum stability. The relative difference in the change in free energy of unfolding at 70 °C (with a reference pH of 7.5) was positive and its magnitude increased with a decrease in pH from 7.0 to 1.5. van't Hoff plots of thermal unfolding of β-Lg at all pH values studied were non-linear and the measured changes in the enthalpy and entropy of unfolding for β-Lg were high and positive. The relative magnitude of change of both enthalpy and entropy at 70 °C (compared with pH 7.5) increased with a decrease in pH up to 1.5. A possible mechanism for the increased stability of β-Lg at low pH is discussed.

## INTRODUCTION

β-Lactoglobulin (β-Lg), which is found in the milk of many mammals including cow, goat, dog, pig, deer, horse, dolphin etc. [1,2], is one of the most thoroughly studied proteins. It has been obtained in the crystal form [3] and the X-ray crystallographic structure of the protein has also been elucidated [4,5]. However, the biological role of this protein is not established. It is known that β-Lg avidly binds ($K_a$ $5 \times 10^7$ $M^{-1}$) one retinol molecule per monomer [6]. Recently a homology between β-Lg and human retinol-binding protein has been recognized [2,7]. In the three-dimensional structure of β-Lg a cavity that can accommodate a single retinol molecule has been assinged [7]. It has also been reported that specific receptors for β-Lg–retinol complex exist in the intestine of neonatal calf [7]. These experimental data suggest that the transport of retinol from mother to offspring could be the biological role of β-Lg. Although this is an attractive proposal some questions need to be answered before it can unequivocally be accepted. One of the most important questions concerns the nature of the structural stability of β-Lg with regard to overcoming the acidic conditions of the gastrointestinal tract of the young animal during the transport of retinol since most proteins are denatured and hydrolysed under these conditions. Although it is known qualitatively that β-Lg is stable at acid pH values [8,9], the nature of the acid stability of β-Lg at the molecular level is not understood.

In this study we investigated the acid stability of β-Lg in a quantitative manner by determining the thermodynamic stability of β-Lg using equilibrium thermal unfolding at low and near-neutral pH values and, based on these data, we propose a possible mechanism for its stability against acid denaturation.

## MATERIALS AND METHODS

Bovine β-Lg (three times crystallized) was purchased from Sigma (St. Louis, MO, U.S.A.), deionized by dialysis against distilled water and lyophilized before use. It was found to be 97% pure as judged by both h.p.l.c. and SDS/polyacrylamide-gel electrophoresis. All the experiments were done using double-distilled water to avoid the effect of buffer ion itself on the stability of β-Lg. Experimental protein solutions were prepared as follows. A stock β-Lg solution of approx. 5 mg/ml by wt. at pH 6.8 was prepared. Since the absorptivity of β-Lg in the u.v. range changes with a decrease in pH (see the Results and discussion section) determination of exact protein concentration of the experimental solutions using $A^{1\%}_{1\,cm,278}$ value requires the determination of these at different pH values. To obviate these difficulties the exact protein concentration of stock β-Lg (pH 6.8) was determined by measuring its absorbance at 278 nm and using an absorptivity value of 0.96 $ml \cdot mg^{-1} \cdot cm^{-1}$ [10]. The pH of this solution was adjusted to the desired value and the final concentration was calculated by taking dilution (by the addition of alkali or acid) into account. From these solutions 1 mg/ml β-Lg solutions were prepared by appropriate dilution with distilled water (whose pH was adjusted to the desired value). All solutions were passed through a 0.45 μm filter to remove dust and other particulate impurities.

U.v. difference spectra of β-Lg at low pH values were measured on a Cary-219 double-beam spectrophotometer using matched 1 cm-pathlength quartz thermostatted cuvettes [11]. Equilibrium thermal unfolding of the protein at each pH value was monitored by u.v. difference spectroscopy. The temperature of the cuvette in the sample beam was controlled by circulating water

Abbreviation used: β-Lg, β-lactoglobulin.

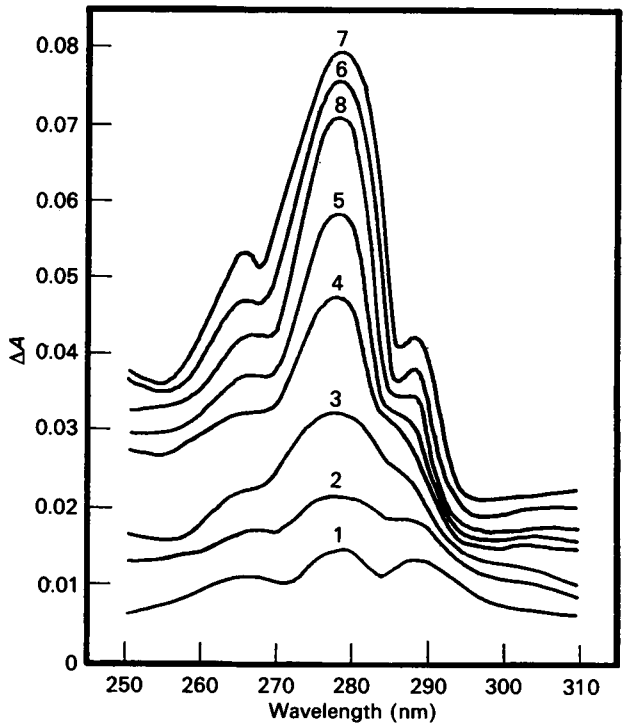


**Fig. 1. Acid-induced u.v. difference spectra of β-lactoglobulin**

Spectra of β-Lg at various pH values were recorded against the same protein at pH 7.5 as reference using 1 mg of protein/ml. Curves 1–8 were at pH values 7.0, 6.5, 6.0, 3.0, 2.5, 2.0, 1.5 and 1.0 respectively.

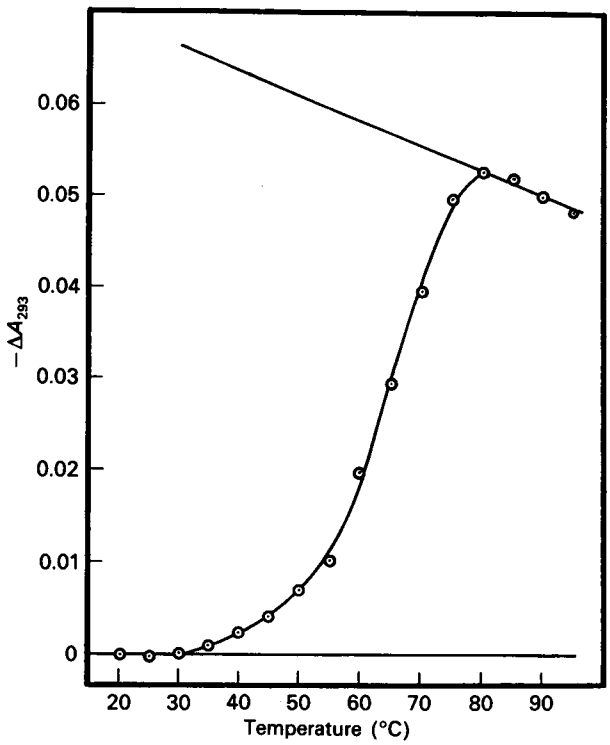


**Fig. 2. Equilibrium thermal unfolding curve of β-lactoglobulin at pH 7.5**

Thermal-induced difference in absorbance at 293 nm (with reference to the same protein at 20 °C) was recorded after equilibration at each temperature. Solid lines indicate the extrapolation of native and denatured states into the transition region. The protein concentration used for these experiments was 1 mg/ml.

of a given temperature through the cell housing from a Lauda circulating water bath (Brinkman Instruments Co., Westbury, NY, U.S.A.). The reference cuvette was controlled at 20 °C using a separate water bath (Endocal; Neslab Instruments Inc., Portsmouth, NH, U.S.A.). The two matched cuvettes were filled with the same protein solution (at a particular pH) and sealed with a Teflon stopper to avoid evaporation during thermal unfolding. After attainment of equilibrium at each temperature the difference in absorbance at 293 nm (which is a measure of the exposure of tryptophan residues from the protein interior upon unfolding) was recorded and plotted as a function of temperature. The reversibility of the thermal unfolding curves was assessed by heating β-Lg (1 ml) at pH 7.5 to different temperatures in the transition region and cooling back to 20 °C and comparing the absorbance of this sample at 293 nm with that of the unheated one at the same pH. This procedure was repeated at several pH values in the range 7.0 to 6.5 and 3.0 to 1.0 at 0.5 pH unit intervals.

## RESULTS AND DISCUSSION

Initially the conformational flexibility of β-Lg at different pH values was examined by u.v. difference spectroscopy. U.v. difference spectra of β-Lg as a function of pH (from pH 7.0 to 1.0) with β-Lg at pH 7.5 as the reference state are shown in Fig. 1. The difference spectra in the pH range below 6.0 and above 3.0 were not included because of the known anomalous behaviour of β-Lg involving octamerization in this pH range [1]. There was a progressive increase in the red shift in the spectrum as the pH of the protein solution was decreased from pH 7.5 to 1.5 below which a blue shift (with reference to pH 1.5) was observed. The red shift consisted of three peaks at 266, 278 and 288 nm. The red shift in the absorption spectrum could arise from the transfer of some chromophores from a polar exterior to the non-polar interior that probably results from a better folding of the protein [12] under these solution conditions. A blue shift in the protein spectrum at pH 1.0 (compared with that at pH 1.5) may arise from a slight unfolding of the protein. These results indicate that conformation flexibility of β-Lg decreases with a decrease in pH to 1.5 below which it increases again.

The stability of β-Lg was investigated more quantitatively by determining the conformational stability of β-Lg at different pH values. This was determined by monitoring the equilibrium thermal unfolding of β-Lg at pH values 7.5–6.5 and 3.0–1.0 employing u.v. difference spectroscopy. The equilibrium thermal-unfolding curve of β-Lg at pH 7.5 is shown (Fig. 2). The resulting unfolding curve was normalized using the relation [13]:

$$f_U = \Delta A(T) - \Delta A_N(T) / \Delta A_U(T) - \Delta A_N(T) \quad (1)$$

where $f_U$ is the fraction of the unfolded protein, $\Delta A$ is the observed difference in absorbance at 293 nm at temperature $T$, and $\Delta A_N$ and $\Delta A_U$ are the differences in absorbance (at the same wavelength) of the native and unfolded forms respectively at temperature $T$. The $\Delta A_N$ and $\Delta A_U$ values were obtained by extrapolation into the transition region [14,15]. Similarly the equilibrium

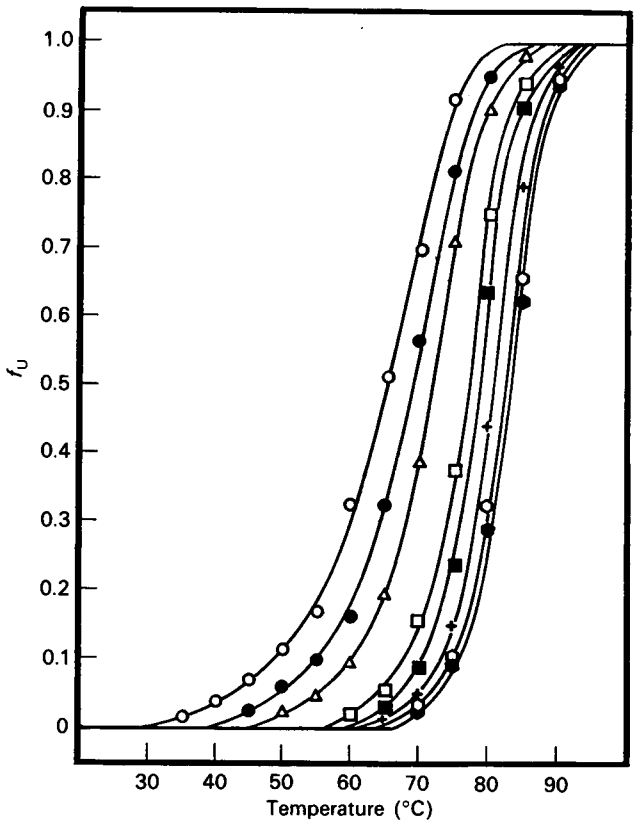


**Fig. 3. Normalized equilibrium thermal-unfolding curves for β-lactoglobulin at different pH values**

Equilibrium thermal unfolding data were collected and normalized as described in the text. The individual curves are at: pH 7.5 (○); pH 7.0 (●); pH 6.5 (△); pH 3.0 (□); pH 2.5 (■); pH 2.0 (○); pH 1.5 (●); pH 1.0 (+).

thermal-unfolding curves of β-Lg measured at pH values 7.0–6.5 and 3.0–1.0 (at 0.5 unit intervals) were also normalized (Fig. 3). $T_m$, the temperature at which the value of $f_U$ is 0.5, increased with a change in pH from 7.5 to 6.5 and 3.0 to 1.5 and decreased at pH 1.0 (Table 1). However, $T_m$ at pH 1.0 was higher than that at pH 7.5. An increase in the ionic strength of the protein solution caused by the addition of acid (which was used to change the pH of the system) may also contribute to the increased stability of the protein. Therefore, the effect of ionic strength on the stability of β-Lg was assessed as follows. Since maximum ionic strength of the protein sample is 0.1 (which is present in the pH 1.0 sample), the effect of this ionic strength on the transition temperature of β-Lg at pH 7.0 (where ionic strength is equal to zero) was studied. $T_m$ of β-Lg in the presence and absence of 0.1 M-KCl ($\Gamma/2 = 0.1$) was the same. This experiment suggests that an increase in the ionic strength of the protein sample by the added acid has negligible effect on the stability of the protein.

One of the advantages of equilibrium thermal-unfolding investigations is the evaluation of the thermodynamic parameters which yield important information regarding the forces that contribute to the stability of the folded state [13,16]. At all the pH values studied, the normalized equilibrium thermal-unfolding curves for β-Lg exhibited fairly abrupt increases in the fraction of the unfolded protein with temperature in the transition region (Fig. 3). This suggests that there was no accumulation of the intermediately unfolded protein species and thermal unfolding of the protein is dominated by two final states, native and the unfolded [16]. At all the pH values studied, the thermal-unfolding curve was reversible in the transition region as ascertained by the recovery of the initial absorbance at 293 nm of the heated and cooled samples. Since the unfolding of most of the proteins with single domains could be approximated by assuming a two-state model [13,17], the data of Fig. 3 were analysed by a two-state approximation to obtain the apparent equilibrium constant ($K_{app.}$) for protein unfolding reaction as follows:

**Table 1. Effect of pH on transition temperature ($T_m$) of β-lactoglobulin**

| pH | $T_m$ (°C) | $\Delta T_m$ (with ref. to pH 7.5) (°C) |
|---|---|---|
| 7.5 | 64.8 | |
| 7.0 | 69.2 | 4.4 |
| 6.5 | 71.9 | 7.1 |
| 3.0 | 77.2 | 12.4 |
| 2.5 | 78.8 | 14.0 |
| 2.0 | 82.5 | 17.7 |
| 1.5 | 83.3 | 18.5 |
| 1.0 | 81.4 | 16.6 |

$$K_{app.} = [U]/[N] = f_U/(1-f_U) \quad (2)$$

where U and N refer to the unfolded and native species. Thermodynamic parameters, free energy ($\Delta G^0_{app.}$), enthalpy ($\Delta H^0_{app.}$) and entropy ($\Delta S^0_{app.}$) of unfolding were determined from the standard equations (3)–(5):

$$\Delta G^0_{app.} = -RT\ln K_{app.} \quad (3)$$

$$\Delta H^0_{app.} = -R[\delta(\ln K_{app.})\delta(1/T)]_p \quad (4)$$

and

$$\Delta S^0_{app.} = (\Delta H^0_{app.} - \Delta G^0_{app.})/T \quad (5)$$

where $R$ is the gas constant. $\Delta G^0_{app.}$ values as a function of temperature at different pH values were non-linear (Fig. 4) and could be fitted to the equation [18]:

$$\Delta G^0_{app.} = A + BT + CT^2 + DT^3 \quad (6)$$

where $A$, $B$, $C$ and $D$ are empirical constants. The values of the coefficients of the free energy curves at different pH values are given in Table 2. To estimate the temperature of maximum stability ($T_{max.}$) of β-Lg (where $\delta\Delta G^0_{app.}/\delta T$ is zero) at each pH value the $\Delta G^0_{app.}$ values were extrapolated below the transition region up to 5 °C [18] using eqn. 6 (Fig. 4). As the pH was decreased from 7.5 to 1.5 the $T_{max.}$ shifted towards higher temperatures and the magnitude of $\Delta G^0_{app.}$ at the $T_{max.}$ increased and decreased at pH 1.0 (Fig. 4). This observation is in contrast to that made for ribonuclease for which the $T_{max.}$ shifted slightly towards lower temperatures with a decrease in pH in addition to an increase in $\Delta G$ at $T_{max.}$ [18].

For a better measure of the conformational stability of β-Lg at different pH values the relative change in the free energy of unfolding ($\Delta\Delta G^0_{app.}$) brought about by the change of pH of the system with reference to a pH of 7.5 at 70 °C was calculated. Thus:

$$(\Delta\Delta G^0_{app.})_{70} = (\Delta G^0_{app.}, \text{pH})_{70} - (\Delta G^0_{app.}, \text{pH } 7.5)_{70} \quad (7)$$

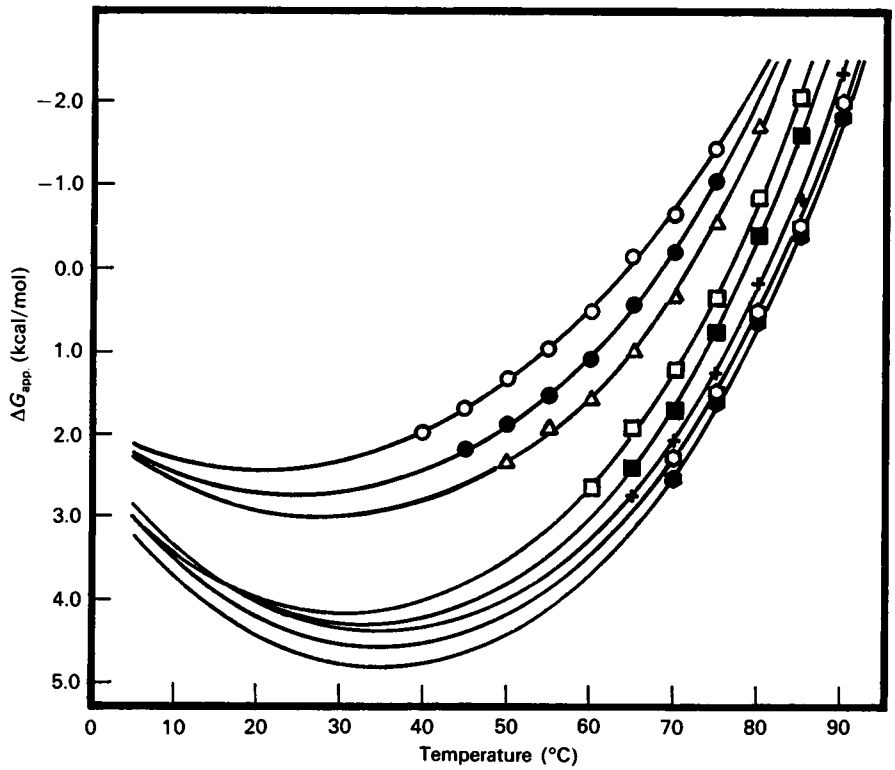


**Fig. 4. Temperature-dependence of the free energy change for the thermal unfolding of β-lactoglobulin at different pH values**

The points represent the experimental data and the solid curves correspond to the theoretical values calculated from eqn. (6) using least squares analysis of the observed data and curve fitting procedures at each pH. The individual curves are at: pH 7.5 (○); pH 7.0 (●); pH 6.5 (△); pH 3.0 (□); pH 2.5 (■); pH 2.0 (○); pH 1.5 (●); pH 1.0 (+).

**Table 2. Values of coefficients for the free energy change upon unfolding of β-lactoglobulin at different pH values**

Expressed as $\Delta G^0_{app.} = A + BT + CT^2 + DT^3$ where $\Delta G^0_{app.}$ is in kcal/mol; $T$ is temperature on Kelvin scale.

| pH | $10^{-2} \times A$ | $B$ | $10^3 \times C$ | $10^6 \times D$ |
|---|---|---|---|---|
| 7.5 | −0.87±0.09 | 0.54±0.10 | −5.41±0.11 | −0.84±0.48 |
| 7.0 | 0.34±0.07 | −0.70±0.06 | 3.62±0.54 | −5.46±1.21 |
| 6.5 | 1.27±0.23 | −1.65±0.11 | 6.83±0.71 | −9.03±1.73 |
| 3.0 | 1.10±0.20 | −1.54±0.10 | 6.89±0.48 | −9.11±1.08 |
| 2.5 | 1.17±0.14 | −1.63±0.09 | 7.04±0.29 | −9.5±0.45 |
| 2.0 | 1.60±0.09 | −2.02±0.14 | 8.16±0.79 | −10.55±1.35 |
| 1.5 | 1.53±0.09 | −1.96±0.13 | 8.00±0.59 | −10.43±0.88 |
| 1.0 | 0.74±0.10 | −1.24±0.07 | 5.84±0.36 | −8.27±0.69 |

$\Delta\Delta G^0_{app.}$ values as a function of decreasing pH were positive and increased up to pH 1.5 and decreased at pH 1.0 (Fig. 5). This observation indicates that lowering pH from 7.5 to 1.5 destabilizes the unfolded state or enhances the stability of the folded state. Since the overall net conformational stability of globular proteins ranges from about 5 to 15 kcal/mol [15,17], an increase in the $\Delta\Delta G^0_{app.}$ value by about 3.0 kcal/mol with a change in pH from 7.5 to 1.5 may enhance the stability of β-Lg as indicated by a $T_m$ increase of 18.5 °C (Table 1). The relative increase in $\Delta G^0_{app.}$ at low pH values compared with that at pH 7.5 could arise from (*a*) an increase in $\Delta G^0_{app.}$ at the $T_{max.}$, (*b*) a shift in the $T_{max.}$ upwards, or (*c*) a more pronounced flattening of the unfolding curve making $\Delta S^0_{app.}$ upon denaturation small [19]. Since there is no flattening of the unfolding curve with decrease in pH (Fig. 3) β-Lg seems to follow mechanisms (*a*) and (*b*) to increase the stability at low pH values (Fig. 4). Mechanism (*a*) results in a wider range of stability and apparently involves additional stabilizing interactions [19]. To understand the nature of the additional stabilizing forces, enthalpy ($\Delta H^0_{app.}$) and entropy ($\Delta S^0_{app.}$) of protein unfolding were determined from the van't Hoff plots.

van't Hoff plots of the equilibrium thermal unfolding of β-Lg at all the pH values studied were non-linear. At each pH value the slope of the curve increased with an increase in temperature. This may reflect the change in the heat capacity of the non-polar groups that are exposed to the polar water exterior from the non-polar interior upon unfolding [20]. From these curves $\Delta H^0_{app.}$ and the corresponding $\Delta S^0_{app.}$ values at 70 °C were

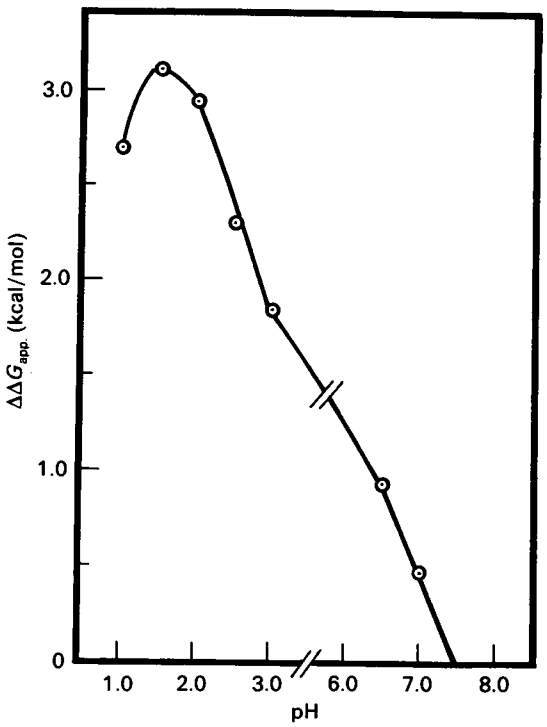


**Fig. 5. pH dependence of $\Delta\Delta G^0_{app.}$ of β-lactoglobulin at 70 °C (with reference to a pH of 7.5)**

**Table 3. Enthalpy ($\Delta H^0_{app.}$) and entropy ($\Delta S^0_{app.}$) of thermal unfolding of β-lactoglobulin at various pH values**

| pH | $\Delta H^0_{app.}$ (kcal/mol) | $\Delta\Delta H^0_{app.}$ (kcal/mol)* | $\Delta S^0_{app.}$ (e.u.) | $\Delta\Delta S^0_{app.}$ (e.u.)* |
|---|---|---|---|---|
| 7.5 | 47.80 | | 141.22 | |
| 7.0 | 51.74 | 3.94 | 151.34 | 10.12 |
| 6.5 | 53.53 | 5.73 | 155.23 | 14.01 |
| 3.0 | 57.70 | 9.90 | 164.73 | 23.51 |
| 2.5 | 59.65 | 11.85 | 169.12 | 27.90 |
| 2.0 | 63.68 | 15.88 | 178.95 | 37.73 |
| 1.5 | 65.98 | 18.14 | 185.07 | 43.85 |
| 1.0 | 61.32 | 13.52 | 172.80 | 31.58 |

* These values were calculated with reference to those at pH 7.5 (at 70 °C).

calculated. Both these values were high and positive (Table 3). $\Delta H^0_{app.}$ for unfolding of a protein in aqueous solution may be contributed primarily by four factors as follows [16,18,20,21,23]

$$\Delta H^0_{app.} = \sum_1^N \Delta H^0_\phi + \Delta H^0_s + \Delta H^0_h + \Delta H^0_{vw} \quad (8)$$

where $\Delta H^0_\phi$, $\Delta H^0_s$, $\Delta H^0_h$ and $\Delta H^0_{vw}$ are the enthalpy changes resulting from the exposure of hydrophobic side-chain residues to the polar solvent medium, rupture of salt bridges, breakage of peptide–peptide hydrogen bonds and decrease in van der Waals forces respectively which result from the unfolding of the protein. The same also holds good for the observed $\Delta S^0_{app.}$ [16,18,20,21, 23] except that it also contains the conformational entropy ($\Delta S^0_c$) term which tends to oppose the total attractive forces that are responsible for the folded state [17,22]. Thus:

$$\Delta S^0_{app.} = \sum_1^N \Delta S^0_\phi + \Delta S^0_s + \Delta S^0_h + \Delta S^0_{vw} + \Delta S^0_c \quad (9)$$

Since $\Delta S^0_c$ is independent of the solvent composition [24] such as varying $H^+$ concentration the observed increase in $\Delta H^0_{app.}$ and $\Delta S^0_{app.}$ values (Table 3) could be attributed only to the intramolecular bonding responsible for the folded state. $\Delta H^0_\phi$, $\Delta H^0_s$, $\Delta S^0_\phi$ and $\Delta S^0_s$ have a negative sign while $\Delta H^0_h$, $\Delta H^0_{vw}$, $\Delta S^0_h$ and $\Delta S^0_{vw}$ have the opposite sign [16,18,20,21,23]. Because both $\Delta\Delta H^0_{app.}$ and $\Delta\Delta S^0_{app.}$ values are positive (Table 3) the primary forces responsible for an increase in the net stability of β-Lg associated with a decrease in pH from 7.5 should be either hydrogen bonding or van der Waals forces or both.

At pH 7.5 β-Lg is negatively charged compared with the isoelectric point (pH 5.2). It has seven net negative charges at pH 7.5 and only about four at pH 6.5 per monomer [1]. This may facilitate a closer packing of the groups in the protein interior at pH 6.0 compared with 7.5 as shown by u.v. difference spectra (Fig. 1). This could increase hydrophobic interactions in the protein interior and ultimately result in the increased van der Waals attractions [25]. As the pH is decreased, below the pI, the net positive charge on the protein increases. At pH 3.0 the net positive charge is 15 units/monomer and at pH 1.5 it is about 18 units/monomer (compared with the pI). This may result in the destabilization of the close packing due to mutual repulsion and weakened van der Waals interactions. However, both the u.v. difference spectra and $\Delta\Delta G^0_{app.}$ measurements revealed that there is an increase in the protein stability with a decrease in pH down to 1.5. The increased stability at low pH values could only be contributed by additional hydrogen bonding in the protein due to titration of carboxy groups in the pH range 3.0–1.5 (since $pK_{int.}$ of α, β and γ carboxy groups in proteins ranges from 2.8 to 4.6 [26]. Either two titrated carboxy groups or one amide group and one titrated carboxy group could form a pair of hydrogen bonds [27]. The loss of localized unfavourable electrostatic interactions of the ionized carboxylates upon titration could also be a possible cause for the increased stability of β-Lg at low pH.

Thus, the present study suggests a possible mechanism by which β-Lg could increase its stability in the acid pH range and become more resistant to the acidic conditions that prevail in the stomach (abomasum) of the neonatal calf. This unique property of β-Lg, unlike other milk proteins (which unfold at low pH values [1]), may render it suitable for transporting retinol from mother to offspring via β-Lg-specific receptors in small intestine [7].

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