Comparative Analysis of Chemical and Thermal Denatured β-Lactoglobulin AB in the Presence of Casein

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Abstract

Chemical denaturation and thermal denaturation of β-lactoglobulin AB in the absence and presence of αS-casein were measured at pH 2.0. It has been observed that ΔG₀°, (Gibbs free energy change in absence of denaturant at 25 °C) of β-lgAB: αS-casein ratios 1:1, 1:1.5 and 1:2 is increased. We report that the functional dependence of ΔG₁°, (Gibbs free energy change) of protein in the absence and the presence of αS-casein concentration is linear. The values of Tₐₜ (midpoint of denaturation), ΔHₐₜ (enthalpy change at Tₐₜ), and ΔCₚ (constant-pressure heat capacity change) under a given solvent condition were measured. It has been observed that each sugar stabilizes the protein in terms of Tₐ and ΔG₀°. The temperature that corresponds to maximum protein stability, Tₛ, is increased in the presence of these osmolytes. The same trend was also observed for Tₖ, the temperature corresponding to zero enthalpy change of denaturation.

Keywords: β-lactoglobulinAB; αS-casein; protein stability; Chemical denaturation; thermal denaturation

1. Introduction

Dairy products are an important area of food science, and in these products the milk proteins are the main surface active agents. Besides controlling coalescence and affecting the mechanical properties of the system, proteins also enable other physical processes like foaming, thickening and gelling [1].
Bovine β-lactoglobulin (BLG), the most abundant whey protein, is a lipocalin that has been studied extensively in the past six decades [2]. Although this globular protein has a well defined structure [3–6] and has been shown to bind a variety of ligands at more than one binding site with different modes [7–10], its true function is still an enigma.

Attempts have been made to improve the thermal stability of BLG by adding different compounds. One way to monitor this improvement is through increases in denaturation temperature [11]. The addition of 10 mM sodium dodecyl sulfate increases the denaturation temperature of BLG A and B by 3.4 and 4.4 °C, respectively [12]. Also, Zhang et al. [13] showed that in the presence of dextran sulfate, the denaturation temperature of BLG was about 4.6 °C higher.

Another approach is to determine how various compounds alter the aggregation process. Toward that end, mixtures of BLG and caseins have the potential to alter aggregation. Caseins are unusual as they are neither globular nor fibrous proteins in nature [14]. The four caseins differ markedly in many respects, including charge, hydrophobicity, and calcium sensitivity [15]. Molecular chaperone-like properties have been reported with β-casein (BCN) and αS-casein (ACN) [16, 17,18]. Molecular chaperones are able to stabilize proteins from unfolding, aggregating, and precipitating under stressed conditions, such as elevated temperature [18].

In the present work, the roles of αS-casein (ACN) as Molecular chaperone-like on the thermodynamic stability of β-lactoglobulins AB during heat stress and chemical denaturation have been extensively studied at various casein concentrations.

2. Materials and methods

2.1 Materials
Commercially lyophilized bovine β-lactoglobulin AB, αS-casein (ACN) and Guanidinium Chloride (extra pure) were purchased from Sigma Chemical Co., and used without further purification. All salts used for buffer preparation were analytical grade and dissolved in double distilled water. The 50 mM glycine buffer pH 2.0 was used as buffer. All of the solutions were used freshly after preparation.

2.2 Preparation of β-lgA Solution
Protein stock solutions were filtered using 0.45μm Millipore filter paper. The concentration of β-lgAB was determined experimentally using a value of 17,600 M⁻¹ cm⁻¹ for the molar absorption coefficient (ε) at 280 nm and pH 2.0. For fluorescence measurements all solutions were prepared in 0.05 M glycine-HCl buffer at pH 2.0 and equilibrated 30 min at 25 °C. Isothermal denaturation of β-lgAB by GdnHCl in the absence and presence of αS-casein at pH 2.0 and 25.0 °C was measured in a CARRY Eclipse Fluorescence Spectrophotometer. The β-lgAB: αS-casein ratios were 1:1, 1:1.5 and 1:2. Protein concentrations used for
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the absorption in the ranges 7–10 μM. The concentrations of GdnHCl in buffer solutions were determined refractometrically using tabulated values of the solution refractive index [19].

2.4 Thermal Denaturation of β-IgAB

Thermal denaturation studies were carried out in a Lambda 35 UV-Vis double beam spectrophotometer with a heating rate of 1 °C/min. The requirement for equilibrium conditions was achieved by this scan rate. Each sample was heated from 20 to 95 °C. The change in absorbance of β-Ig AB in the absence αs-casein ratios 1:1, 1:1.5 and 1:2 at pH 2.0 with increasing temperature were followed at 293 nm. The basic observation was a heat-induced transition curve, i.e. a plot of an optical property against temperature. To obtain values of Tm (the midpoint of the transition curve) and ΔHm (the enthalpy change upon denaturation at Tm), a nonlinear least-squares analysis was used to fit all the data points of the transition curve according to this relation [20]:

\[
y(T) = \frac{y_N(T) + y_N(T) \exp\left[ -\frac{\Delta H_m}{R} \left( \frac{1}{T} - \frac{1}{T_m} \right) \right]}{1 + \exp\left[ -\frac{\Delta H_m}{R} \left( \frac{1}{T} - \frac{1}{T_m} \right) \right]}
\]

(1)

Where \( y(T) \) is the optical property at temperature \( T(K) \), \( y_N(T) \) and \( y_D(T) \) are the optical properties of the native and denatured protein molecules at T, respectively, and R is the gas constant. In the analysis of the transition curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and denatured protein molecules (i.e., \( y_N(T) = a_N + b_N T + c_N T^2 \) and \( y_D(T) = a_D + b_D T + c_D T^2 \), where \( a_N, b_N, c_N, a_D, b_D, \) and \( c_D \) are temperature-independent coefficients) [21,22]. A plot of ΔHm versus Tm gave the value of ΔCp, the temperature-independent heat capacity change at constant pressure. ΔGD(T), the value of ΔG at any temperature T was estimated using Gibbs–Helmholtz equation with values of Tm, ΔHm and ΔCp,

\[
\Delta G = \Delta H_m(1 - \frac{T}{T_m}) - \Delta C_p[(T_m - T) + T \ln \frac{T}{T_m}]
\]

(2)

3. RESULTS

3.1 GdnHCl-induced Denaturation of β-IgAB

The denaturation of β-IgAB by GdnHCl is shown in Fig. 1 at pH 2.0 and 25 °C by observing changes in the Trp-fluorescence emission intensity at 330 nm (F330). It is seen in this figure that the denaturation induced by GdnHCl exists in a sigmoidal fashion therefore suggesting that a two-state model applies to the β-IgAB denaturation in agreement with previously reported data [23].
Fig. 1. Transition curves of GdnHCl-induced denaturation of β-lgA at pH 2.0 and 25 °C.

Assuming a two-state model of denaturation, optical transition data were converted into $\Delta G_D^o$, the Gibbs energy change using the relation,

$$
\Delta G_D^o = -RT \ln \left( \frac{y - y_N}{y_D - y} \right)
$$

Where $y$ is the observed optical property and $y_N$ and $y_D$ are, respectively, the properties of the native and denatured protein molecules under the same experimental conditions in which $y$ has been determined. $\Delta G_D^o(-5.4 \leq \Delta G_D^o(\text{kJ mol}^{-1}) \leq 5.4)$ [24] was plotted against [D], the molar concentration of the denaturant, and a linear least-squares analysis was used to fit the ($\Delta G_D^o, [D]$) data to the relation,

$$
\Delta G_D^o = \Delta G_D^o(\text{H}_2\text{O}) - m_d[D]
$$

Where $\Delta G_D^o(\text{H}_2\text{O})$ is the value of $\Delta G_D^o$ at 0 M denaturant and $m_d$ gives the linear dependence of $\Delta G_D^o$ on [D]. Fig 2. shows GdnHCl-induced denaturation curves of β-lgAB in the presence various ratios of αs-casein. Each curve, which was measured three times, was analyzed for $\Delta G_D^o(\text{H}_2\text{O})$, $m_d$ and $C_m$, the midpoint of chemical denaturation($=\Delta G_D^o(\text{H}_2\text{O})/m_d$) using Eq.(4). The results are shown in Table 1.

**Table 1.** Parameters characterizing the denaturation of β-lgA by guanidinium chloride in the presence of various caseins' ratios at pH 2.0 and 25 °C

<table>
<thead>
<tr>
<th>caseins' ratios</th>
<th>$\Delta G_D^o(\text{H}_2\text{O})$ (kJ mol$^{-1}$)</th>
<th>$C_m$ (M)</th>
<th>$m_d$ (kJ mol$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>17.74</td>
<td>3.095</td>
<td>5.732</td>
</tr>
<tr>
<td>1.00</td>
<td>18.41</td>
<td>3.898</td>
<td>4.723</td>
</tr>
<tr>
<td>1.50</td>
<td>22.08</td>
<td>3.984</td>
<td>5.542</td>
</tr>
<tr>
<td>2.00</td>
<td>20.19</td>
<td>3.906</td>
<td>5.169</td>
</tr>
</tbody>
</table>
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Fig. 2. Transition curves of GdnHCl-induced denaturation of \( \beta \)-lgAB at pH 2.0 and 25 °C in the presence of buffer(♦), 1:1(□), 1:1.5(▲) and 1:2(○) ratios of casein.

3.2 Thermal Denaturation of \( \beta \)-lg AB in the presence and absence Casein

Fig. 3 shows the representative denaturation curves of \( \beta \)-lg AB in the absence \( \alpha_S \)-casein and presence of \( \beta \)-lgAB: \( \alpha_S \)-casein ratios 1:1, 1:1.5 and 1:2. The values of \( T_m \), \( \Delta T_m \), \( \Delta H_m \) and \( \Delta \Delta H_m \) (the difference between \( \Delta H_m \) in the presence and absence of casein) for \( \beta \)-lgAB in the presence casein are collected in Table 2.

Fig. 3. Thermal denaturation curves of \( \beta \)-lactoglobulin AB in presence of buffer(♦), 1:1(■), 1:1.5(▲) and 1:2(○) ratios of casein.
Table 2. Stability parameters of β-lgAB in the presence of various ratios of casein at pH 2.0

<table>
<thead>
<tr>
<th>casein (αS-casein ratio)</th>
<th>Tm (K)</th>
<th>ΔTm (K)</th>
<th>ΔHm (kJ.mol⁻¹)</th>
<th>ΔΔHm (kJ.mol⁻¹)</th>
<th>ΔΔGD o (kJ.mol⁻¹)</th>
<th>%ΔΔGD o</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>353.7</td>
<td>0.00</td>
<td>382.2</td>
<td>0.00</td>
<td>34.23</td>
<td>0.00</td>
</tr>
<tr>
<td>1.00</td>
<td>354.2</td>
<td>0.50</td>
<td>384.6</td>
<td>2.40</td>
<td>34.62</td>
<td>1.13</td>
</tr>
<tr>
<td>1.50</td>
<td>354.8</td>
<td>1.10</td>
<td>388.5</td>
<td>6.30</td>
<td>35.27</td>
<td>3.03</td>
</tr>
<tr>
<td>2.00</td>
<td>355.1</td>
<td>1.40</td>
<td>389.8</td>
<td>7.60</td>
<td>35.48</td>
<td>3.65</td>
</tr>
</tbody>
</table>

4. DISCUSSION

Concerning the overall data can be describe that transitions N↔D of β-lgAB follow two-state mechanism and the dependence of ΔGD o on [GdnHCl] is linear under all experimental conditions. There are several documents that certify this assumption; including number tests for a two-state denaturation transition: (1) obtain protein denaturation results with different instrumental methods. These yield the same transition curve for a two state process, (2) measure the kinetics of denaturation. A two-state process, yields simple 1st order kinetics. By contrast, a three-state process exhibits bi-phasic 1st order kinetics, (3) check for an S-shaped denaturation curve. Three-state will normally produce a "double-S" profile unless the instrumental technique is sensitive to X or D states but not both when a simple S-shaped denaturation curve will be produced [25]. With respect to the above discussion we used two-state mechanism for analysis of denaturation curves. Values of ΔGD o(H2O) and Cm obtained from the analysis of the GdnHCl-induced transition curves of β-lgAB in the presence various ratios of αS –casein are collected in Table 1. It is observed that ΔGD o(H2O) of β-lgAB increases in the presence of casein. However, the following trend is observed for ΔGD o(H2O) and Cm: 1.5> 2.0 > 1.0

It can be calculated the percent stabilization of the protein, %ΔΔGD o by case as follow [26]:

%ΔΔGD o =100 × [(ΔGD o in the presence casein -ΔGD o in the absence of casein)]/ΔGD o in the absence of casein

(5)

The results are given in Table 3. β-lgAB: αS -casein ratio1:1.5 is found to induce remarkable stability of β-lgAB against chemical denaturation with respect to other ratios. However, differences in the capacity of the 1:2 with other ratios to stabilize the β-lgAB can be explained on the basis of aggregation of β-lgAB in this ratio.
Table 3. The percent stabilization of β-lgAB by casein at pH 2.0 and 25 °C

<table>
<thead>
<tr>
<th>caseins' ratios</th>
<th>%ΔΔG_D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1.00</td>
<td>3.80</td>
</tr>
<tr>
<td>1.50</td>
<td>24.48</td>
</tr>
<tr>
<td>2.00</td>
<td>13.82</td>
</tr>
</tbody>
</table>

Thermal denaturation’s results, ( Fig 3.), show that at the conditions of this study, the transition can be assumed as a change between two states and an intermediate state are not clear in this case. To convert the reversible heat-induced optical transition data into thermodynamic parameters, the following assumptions were made. First, the transition between N and D states follows a two-state mechanism. Second, the temperature dependencies of Y_N and Y_D are parabolic. Third, casein have no effect on the conformational ΔC_P of β-lg AB. Making use of the first two assumptions, the thermal transition curves were analysed according to eqn (1), and the analysis yielded values of T_m and ΔH_m with their uncertainties. Data fitting was done using Sigma Plot 10 software [27]. The values of T_m, ΔT_m, ΔH_m and ΔΔH_m (the difference between ΔH_m in the presence and absence of αS-casein for β-lg AB) are collected in Table 3.

Calculated denaturation temperatures show that T_m for β-lactoglobulins AB in buffer is 353.7 K. It is seen in Fig. 4 (also see Tables 3.) that T_m of β-lactoglobulins AB at pH 2.0 increases linearly with an increase in the concentration of αS-casein. Making use of the third assumption (independence of ΔC_P from casein concentration), we have determined ΔC_P from the linear plot of ΔH_m and T_m values at pH 2.0. The Value of ΔC_P in the presence of different concentrations of αS-caseins is 5.59 for β-lactoglobulins AB. A DSC study of thermal and cold denaturation of β-lactoglobulin was reported that in aqueous solutions at pH 2.0 (0.1 M KCl/HCl) ΔC_P=5.58 ± 0.7 kJ mol^{-1}K^{-1} [28].

Thermal stability curve, i.e., the variation of ΔG_D(T) versus T, was constructed for β-lg AB in the presence of various concentrations of casein and shown in Fig. 4. Table 3. presents the values of ΔG_D (Gibbs free energy change at 25 °C) at different ratios of αS-casein for β-lg B. correspond to the experimental measurements and the dashed lines have been calculated using eqn (2).
The value of $T_S$ was obtained exactly from Fig. 5 (temperature of the maximum point in plot of $\Delta G^0$ against $T$). The values of $T_S$ and $\Delta H_S$ were used in eqn (3) to estimate $T_H$, the temperature at which the enthalpy changes of denaturation equals zero [29].

$$T_H = T_S - (\Delta H_S / \Delta C_p) \quad (6)$$

Since $\Delta H$ at $T_S$ ($\Delta H_S$) is equal to $\Delta G$ at $T_S$ ($\Delta G_S$; the maximum of $\Delta G$) according to $\Delta G = \Delta H - T \Delta S$ with $\Delta S = 0$ at $T_S$, eqn (6) may be simplified to

$$T_H = T_S - (\Delta G_S / \Delta C_p) \quad (7)$$

Another important thermodynamic parameter that can be determined from thermal stability profiles is $T_G'$, the temperature at which the Gibbs energy change of denaturation is zero but the entropy change of denaturation is negative. $T_G'$ characterizes the cold denaturation of a protein and can be derived from continuing the left side of the thermal stability curve. This parameter was estimated from the following equation [30]:

$$T'_G = \frac{T_m^2}{3T_m - 2T_H} \quad (8)$$

The estimated values of $T_m$ and $T_H$ were used to determine $T'_G$ with the help of eqns (5). The values of $T'_G$, $\Delta T_G'$ (the difference between $T'_G$ in the presence and absence of osmolytes), $T_H$, $\Delta T_H$ (the difference between $T_H$ in the presence and absence of osmolytes), $T_S$ and $\Delta T_S$ thus obtained at various ratios of $\alpha_S$–casein are given in Tables 4. for $\beta$-lg AB.

Table 4. The values of $T'_G$, $\Delta T'_G$, $T_H$, $\Delta T_H$, $T_S$ and $\Delta T_S$ associated with thermal denaturation of $\beta$-lg A in the absence and presence of various ratios of casein at pH 2.0

<table>
<thead>
<tr>
<th>casein</th>
<th>$T'_G$ (K)</th>
<th>$\Delta T'_G$ (K)</th>
<th>$T_H$ (K)</th>
<th>$\Delta T_H$ (K)</th>
<th>$T_S$ (K)</th>
<th>$\Delta T_S$ (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>254.6</td>
<td>0.0</td>
<td>284.9</td>
<td>0.0</td>
<td>291.2</td>
<td>0.0</td>
</tr>
<tr>
<td>1.00</td>
<td>255.6</td>
<td>1.0</td>
<td>285.9</td>
<td>1.0</td>
<td>292.3</td>
<td>1.1</td>
</tr>
<tr>
<td>1.50</td>
<td>255.7</td>
<td>1.1</td>
<td>286.2</td>
<td>1.3</td>
<td>292.4</td>
<td>1.2</td>
</tr>
<tr>
<td>2.00</td>
<td>255.9</td>
<td>1.3</td>
<td>286.3</td>
<td>1.4</td>
<td>292.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>
T\textsubscript{G}′ increases with rising \(\alpha\_S\)–casein concentration. It means that the cold resistance of \(\beta\)-lg AB decrease with rising \(\alpha\_S\)–casein concentration. Changes in \(T\_H\) show an increase at all concentrations of \(\alpha\_S\)–casein. Following Baldwin’s suggestion that a protein has the least solubility at \(T\_H\) [31], it seems that \(\alpha\_S\)–casein increases the solubility of \(\beta\)-lactoglobulins AB at all concentrations. The temperature at which \(\beta\)-lg AB have the most stability, \(T\_S\), follows the same trend as \(T\_H\). It can be asserted that \(T\_S\) is related to the rate of \(\Delta G\_D\) changes with temperature. Therefore, the more \(T\_S\) increases, the more the rate of change of \(\Delta G\_D\) increases with temperature. On the other hand, the sensitivity of the thermodynamic stability of the protein increases with temperature.

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References


[26] I. Haque, R. Singh, A. A. Moosavi-Movahedi, F. Ahmad, Effect of polyol osmolytes on ΔGΔ, the Gibbs energy of stabilisation of proteins at different pH values Biophys. Chem. 117 (2005), 1-12.


