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Thermal stability of β-lactoglobulin B in the presence of sucrose, sorbitol and trehalose as osmolytes

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ABSTRACT

Thermal denaturation of β-lactoglobulin type B in the absence and presence of various concentrations of trehalose, sucrose and sorbitol as sugar osmolytes and polyols were measured by monitoring changes in the absorption coefficients at pH 2.0. These measurements gave values of T_m (midpoint of denaturation), ΔH_m (enthalpy change at T_m), and ΔCp (constant-pressure heat capacity change) under a given solvent condition. Using these values of ΔH_m, T_m and ΔC_p, ΔG_D° (Gibbs energy change), was determined at a given concentration of each sugar. It has been observed that each sugar stabilizes the β-lactoglobulin B in terms of T_m and ΔG_D°. The temperature that corresponds to maximum protein stability, T_S, is increased in the presence of these osmolytes. The same trend was also observed for T_H, the temperature corresponding to zero enthalpy change of denaturation.

Keywords: Protein stability; Sugar osmolytes; Thermal denaturation; β-lactoglobulin B

INTRODUCTION

β-Lactoglobulin (β-lg), the major protein of bovine whey, is a 162-amino acid – containing globular protein with a molar mass (MW) of 18362 $g.mol^{-1}$, and well-established primary, secondary, tertiary, and quaternary structures. Although the physiological function of β-lg is not clear, β-lg belongs to the lipocalin superfamily sharing the common β-barrel calyx structural feature arranged as an ideal site for hydrophobic ligands [1-3]. β-Lg is composed of anti-parallel β-sheets formed by nine strands labeled A to I, and one α-helix as determined by X-ray crystallography [4]. The tertiary structure of β-lg is strongly stabilized by two disulfide bonds (Cys66–Cys160 and Cys106–Cys119), which seem to play an important role in the reversibility of β-lg denaturation [5]. In 1955, it was found that bovine β-lg existed in two genetic forms that differed slightly in their electrophoretic behaviour on paper at pH 8.6. These forms are called β-lactoglobulin A (β-lgA) and β-lactoglobulin B (β-lgB) [6]. Although, several other β-lg genetic variants exist, A and B are predominant. Variant A differs in amino acid sequence from variant B at position 64 (AspA→GlyB) and 118 (ValA→AlaB). These differences result in distinct biophysical and biochemical properties of the variants, such as heat stability, self association properties and solubility [7].

Considerable time in nearly all fields of biochemical sciences is devoted to improving protein stability, which is the result of a balance between the intramolecular interactions of protein functional groups and their interaction with solvent environment [8-10]. Naturally occurring osmolytes are co-solvents that are used to protect organisms from denaturation by harsh

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environmental stresses. These molecules stabilise proteins, not by interacting with them directly but by altering the solvent properties of the surrounding water and hence the protein–solvent interactions[10]. Their effect seems to be general for all proteins. They have no inhibitory or enhancing effects on biological activity under physiological conditions hence are called compatible osmolyte [9,11]. Stabilizing osmolytes are chemically diverse and include such chemical classes as polyols, certain amino acids and their derivatives, and methylamine compounds [11]. There are various mechanisms that have been used to explain the observation on the effect of osmolytes on the protein denaturation equilibrium, native (N) state ↔ denatured (D) state [12-15]. The most widely used mechanism is due to Timasheff [14]. According to this mechanism osmolytes stabiliseNstate because they are preferentially excluded from the protein surface, for the preferential exclusion increases the chemical potential of the protein proportionately to solvent exposed surface area. Thus, by Le Chatelier's principle, osmolytes favour the more compact state, i.e., the N state over the structurally expanded state, i.e., D state. Hence according to this mechanism ΔG_D, the Gibbs free energy change associated with the denaturation process, N state ↔ D state, should increase in the presence of osmolytes, for $\Delta G_D = -RT\ln([D]/[N])$, where square bracket represents concentration. The most recent mechanism of stabilisation of proteins by osmolytes is due to Bolen and co-workers [15]. According to this mechanism osmolytes stabilise N state because of their overwhelming unfavourable interaction with the peptide backbone. Thus, this "osmophobic effect" favours the N state over the D state of proteins. Hence, according to this mechanism ΔG_D should increase in the presence of osmolytes.

Previous studies showed that osmolytes such as sugar and polyols effect on denaturation and have found that sugar and polyols have a stabilizing effect, increasing thermal denaturation temperature of β-lg and other globular proteins [16-25]. The main conclusion of these studies is that all osmolytes act independently on the protein, i.e., none of the osmolytes alters the efficacy of the other in forcing the protein to fold or unfold.

In the present work, the roles of trehalose, sucrose and sorbitol as sugar osmolytes on the thermodynamic stability of β-lactoglobulins B during heat stress have been extensively studied at various sugar concentrations.

EXPERIMENTAL SECTION

Chemicals

Commercially lyophilized bovine β-lg B was purchased from Sigma Chemical Co. Glycine was from Merck. D-sorbitol, D-mannitol, D-trehalose and D-sucrose were also obtained from Sigma. All of the used chemicals were analytical-grade reagents and used without further purification. Protein stock solutions were filtered using 0.45 μm milipore filter paper. The concentration of β-lgB was determined experimentally using a value of 17600 $M^{-1}.cm^{-1}$ for the molar absorption coefficient (ε) at 280 nm and pH 2.0. For optical measurements all solutions were prepared in 0.05 M glycine buffer at pH 2.0 and 25 °C .

Thermal Denaturation of β-lgB

Thermal denaturation studies were carried out in a Cary 300 UV-vis spectrophotometer with a heating rate of 0.5 °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-lg A at a fixed concentration of each osmolyte with increasing temperature was 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 T_m (the midpoint of the transition curve) and ΔH_m (the enthalpy change upon denaturation at T_m), a nonlinear least-squares analysis was used to fit all the data points of the transition curve according to this relation [26]:

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

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., $y_N(T)= a_N + b_NT + c_NT^2$ and $y_D(T)= a_D + b_DT + c_DT^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients) [27, 28]. A plot of ΔH_m versus T_m gave the value of ΔC_p, the temperature-independent heat capacity change at canstant pressure. $\Delta G_D(T)$, the value of ΔG_D at any temperature T was estimated using Gibbs-Helmholtz equation with values of T_m, ΔH_m and ΔC_p,

$$\Delta G=\Delta H_m(1-\frac{T}{T_m})-\Delta C_p[(T_m-T)+T\ln\frac{T}{T_m}] \quad (2)$$

RESULTS

All denaturation curves were measured at least three times. Fig. 1 shows the representative denaturation curves of β-lg B in the presence and absence af trehalose, sucrose and sorbitol. 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 dependeocies of Y_N and Y_D are parabolic. Third, osmolytes have no effect on the conformational ΔC_P of β-lgB. 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 [29].

The denaturation results in 0.75 and 1.0 M trehalose could not be analysed to estimate fitting parameters due to high dispersion of the experimental points. It seems the assumptions that have been made for analysing the transition curve failed under these conditions. The values of T_m, ΔT_m, ΔH_m and $\Delta\Delta H_m$ (the difference between ΔH_m in he precence and absence of osmolytes) for β-lg B in the presence of different concentrations of trehalose, sucrose and sorbitol are collected in Table 1.

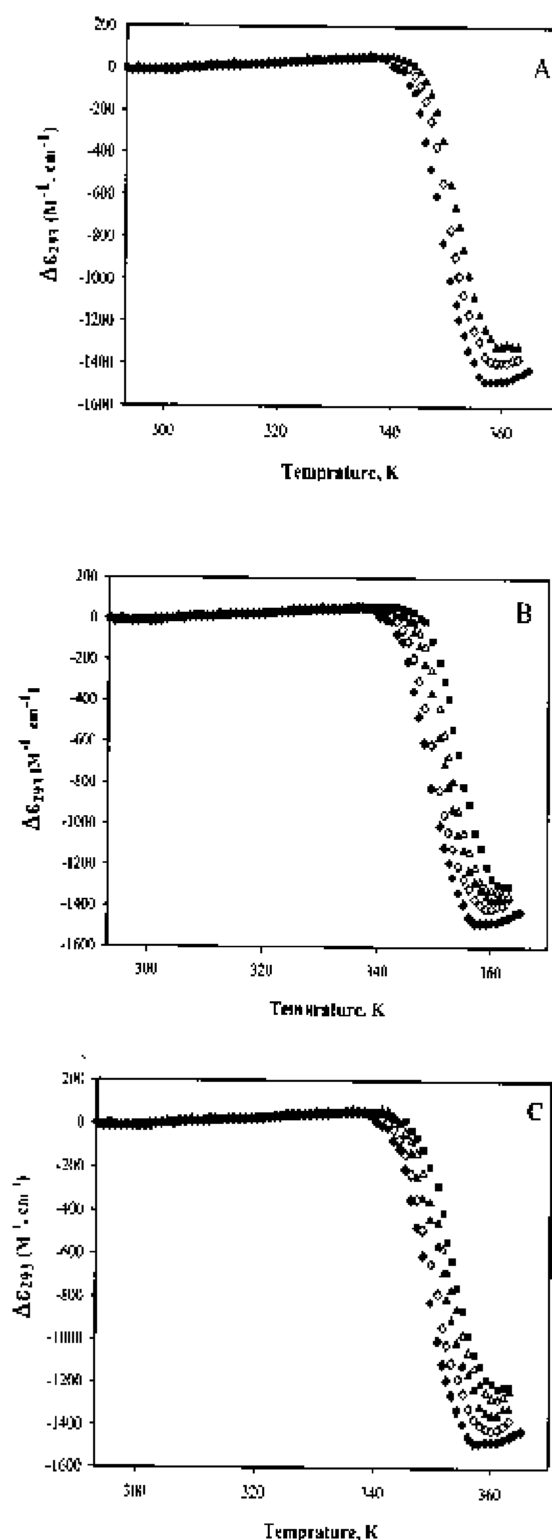

Fig. 1. Thermal denaturation curves of β-lactoglobulin B in the absence and presence of Trehalose (A), Sucrose (B) and Sorbitol (C). buffer (♦), 0.25 M (◊), 0.5 M (▲), 0.75 M (Δ),and 1M (■).

Making use of the third assumption (independence of ΔC_P from osmolyte concentration), we plotted ΔH_m as a function of T_m at each fixed concentration of an osmolyte. The value of 5.30 $kJ.mol^{-1}.K^{-1}$ obtained for ΔC_P of β-lg B. Thermal stability curve, i.e., the variation of $\Delta G_D(T)$ versus T, was constructed for β-lg B in the presence of various concentrations of osmolytes and shown in Fig. 2. Tables 1. present the values of ΔG_D° (Gibbs free energy change at 25 °C) at different concentrations of trehalose, sucrose and sorbitol for β-lg B. This table also shows $\%\Delta\Delta G_D^{\circ}$, the percent change in ΔG_D° of the protein due the presence of sugars(s); $\%\Delta\Delta G_D^{\circ}$ =100 [ΔG_D° (in the presence of sugar(s)) - ΔG_D° (in the absence of sugar)] /ΔG_D° (in the absence of sugar).

The value of T_S was obtained exactly from Fig. 2 (temperature of the maximum point in plot of ΔG° against T). The values of T_S and ΔH_S were used in eqn (3) to estimate T_H, the temperature at which the enthalpy changes of denaturation equals zero [30].

$$T_H = T_S - (\Delta H_S / \Delta C_p) \quad (3)$$

Since ΔH at T_S (ΔH_S) is equal to ΔG at (ΔG_S: the maximum of ΔG) according to ΔG T_S = $\Delta H - T\Delta S$ with $\Delta S = 0$ at Ts, eqn (3) may be simplified to

$$T_H = T_S - (\Delta G_S / \Delta C_p) \quad (4)$$

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[31]:

$$T_G' = \frac{T_m^2}{3T_m - 2T_H} \quad (5)$$

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 he precence and absence of osmolytes), T_H, ΔT_H(the difference between T_H in he precence and absence of osmolytes). T_S and ΔT_S thus obtained at various concentrations of osmolytes are given in Tables 2. for β-lg B.

Table 1. Stability parameters of β-lgB in the presence of various concentrations of sugar osmolytes and polyols at pH 2.0

Osmolytes	M	T_m/K	ΔT_m/K	ΔH_m($kJ.mol^{-1}$)	$\Delta\Delta H_m$($kJ.mol^{-1}$)	ΔG_D°($kJ.mol^{-1}$)	$\%\Delta\Delta G_D^{\circ}$
Control	0.00	348.2±1.2	0.0	411.2±2.3	0.00	39.9±0.6	0.00
Trehalose	0.25	350.2±1.7	2.0	416.9±2.4	5.70	40.4±0.6	1.30
	0.50	352.0±1.3	3.8	424.7±2.3	14.0	41.9±0.7	5.00
Sucrose	0.25	349.7±1.3	1.5	414.8±2.3	3.60	40.3±0.6	1.00
	0.50	351.5±1.5	3.3	422.4±2.4	11.0	41.5±0.6	4.00
	0.75	352.6±1.2	4.4	427.7±2.1	17.0	42.5±0.5	6.50
	1.00	354.6±1.3	6.4	443.1±2.6	32.0	45.3±0.4	14.0
Sorbitol	0.25	349.6±1.2	1.4	414.2±2.5	3.00	40.1±0.6	0.50
	0.50	351.4±1.6	3.2	421.9±2.3	11.0	41.4±0.7	3.80
	0.75	352.7±1.4	4.5	428.0±2.4	17.0	42.4±0.5	6.30
	1.00	354.0±1.3	5.8	435.5±2.6	24.0	43.9±0.4	10.0

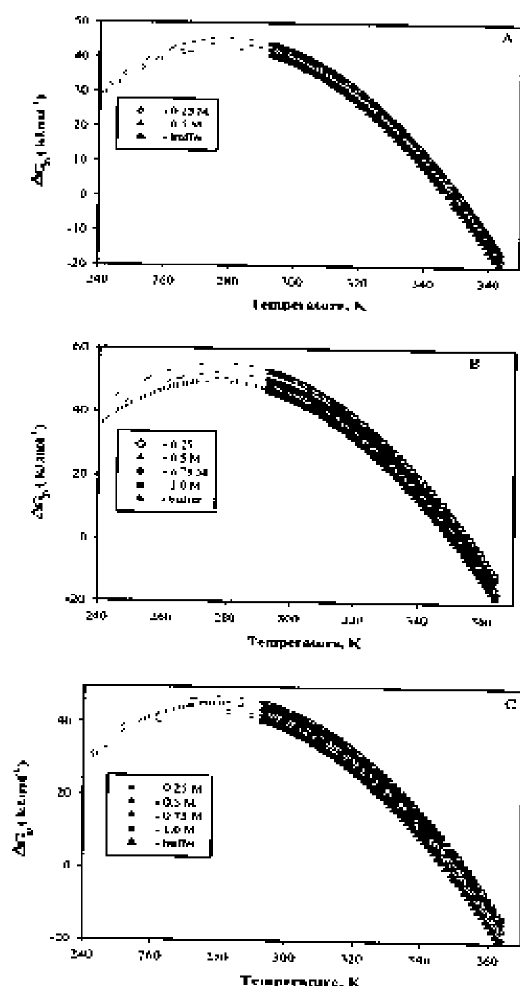

Fig. 2. Thermal stability curves for β-lactoglobulin B in the Trehalose (A), in the presence of Sucrose (B), and Sorbitol (C). The points joined by continuous lines correspond to the experimental measurements and the dashed lines have been calculated using eqn (2).

DISCUSSION

All thermodynamic quantities, given in Table 1 were obtained from the analysis of heat denaturation curves of β-lg B in the presence and absence of different sugars (e.g., see Figs. 1). This analysis according to eq. (1) assumes that the transition between the native and denatured states is a two-state process. Most authors state that the β-lg unfolding can be represented by a two state reversible transition between native and unfolded states N→U in the presence of osmolytes, polyols and etc. [21, 32-37]. It can be seen in Fig. 1 that y_D has a stronger dependency on temperature and osmolyte concentration than y_N, suggesting that osmolytes are more effective on the denatured state of β-lg B. In other words, osmolytes affect the denatured state of the protein more than its native state, leading to a change in protein stability. This case is more obvious for sorbitol and sucrose than for trehalose. It seems that the effect of trehalose follows another mechanism.

Moreover, our curves in Fig. 1 shows that at the conditions of this study, the transition can be assumed as a change between two states and an intermediate state is not clear in this case.

Calculated denaturation temperatures show that T_m for β-lg B in buffer are 348.2 K. Recently, Chanasattru[38] showed the T_m value of the β -lg solutions without co solvent was 347.15 K and shifted to 349.15 and 359.15 K with the presence of 50 wt.% glycerol and 50 wt.% sorbitol, respectively. This result is also in good agreement with the data reported by Apenten and Galani [39,40] who gave value 81.2 °C for β-lg in 0.05M glycine –HCl buffer pH 2.6. Lapanje [41] found T_m to be 83.2°C, ΔH_m (414 $kJmol^{-1}$), and ΔG_D^o(41 $kJmol^{-1}$) for β-lg. The values of ΔG_D^o have been determined by substitution of corresponding values of ΔH_m, T_m and ΔC_P into eq. (3).

It is seen in Fig. 1 (also see Tables 1) that T_m of β-lg B at pH 2.0 increase linearly with an increase in the concentration of individual sugar. The ΔH_m values of many proteins remain unchanged in the presence of various osmolytes [22, 23, 42-47]. We have also observed that the ΔH_m of β-lg B in the presence of different sugars shows insignificant dependence on type and concentration of the sugar. This and earlier observations suggest that sugar osmolytes have no significant affinity on the protein.

We have determined $\Delta C_P = (\partial \Delta H_m / \partial T_m)p$ from the linear plot of ΔH_m and T_m values at pH 2.0. The value of ΔCp in the presence of different concentrations of sugars is 5.3 kJ $mol^{-1}K^{-1}$ for β-lg B. A DSC (differential scanning calorimetry) study of thermal and

cold denaturation of β-lg was reported that in aqueous solutions at pH 2.0 (0.1 *M* KCl/HCl) $\Delta C_P = 5.58 \pm 0.7$ kJ $mol^{-1}K^{-1}$ [48].

Our previous calculated parameters of β-lg A showed that ΔG_D° values of native β-lgA are greater than those of native β-lgB [49]. Thus, it can be concluded that native β-lgA has a higher thermal stability relative to native β-lgB. These data are in a good agreement with previous reports which suggest that the difference in the thermal behavior of β-lgA and β-lgB can be explained by the destabilization of the core of the β-lgB relative to β-lg -A, leaving a cavity formed by the loss of the two methyl groups as a result of the substitution ValA→AlaB [34, 50, 51].

The effect of sugars on protein stability have been explained in terms of preferential more favourable than the corresponding interaction with non-polar groups [55, 56]. Thus stabilizing/destabilizing osmolytes will be preferentially excluded/accumulated around protein backbone. This prediction is consistent with the thermodynamics of preferential interaction of stabilizing and destabilizing osmolytes [54, 57 and 58]. This new molecular mechanism for osmolyte-induced protein stability also predicts that osmolytes having the same fraction of the polar contact surface area will have the same effect on the protein denaturation equilibrium [55].

T_G' increases with rising sugars concentration. It means that the cold resistance of β-lg B decrease with rising sugars concentration. Changes in T_H show an increase at all concentrations of sugar osmolytes. Following Baldwin's suggestion binding and preferential exclusion of these cosolutes [10, 14, 52], which is supported by recent observations on the transfer-free energy of protein groups from the solvent water to the co-solvent aqueous solutions [53]. Both Timasheff's and Bolen's group have argued that the source of stabilization of protein by sugars is the shifting of denaturation equilibrium towards the N state [53, 54]. Thus, what effects co-solvents will have on the denaturation equilibrium, N state ↔ D state under the native condition will be known only by measuring ΔG_D° . It is seen from Tables 1 that the effect of sugars on ΔG_D° of protein increases with increasing sugar concentrations at pH 2.0. It is seen that the $\%\Delta\Delta G_D^{\circ}$ increases with the molar concentration of the additive.

Table 2. The values of T_G', $\Delta T_G'$, T_H, ΔT_H, T_S and ΔT_S associated with thermal denaturation of β-lgB in the absence and presence of various concentrations of sugar osmolytes and polyols

Osmolytes	M	T_G'/K	$\Delta T_G'$/K	T_H/K	ΔT_H/K	T_S/K	ΔT_S/K
Control	0.00	240.4±1.1	0.0	270.1±1.1	0.0	274.9±1.8	0.0
Trehalose	0.25	240.9±1.5	0.5	270.8±1.3	0.7	275.8±1.3	0.9
	0.50	241.3±1.6	0.9	271.3±1.2	1.2	276.2±1.4	1.3
Sucrose	0.25	241.0±1.2	0.6	270.8±1.4	0.7	275.6+1.7	0.7
	0.50	241.5±1.4	1.1	271.4±1.2	1.3	276.3±1.3	1.4
	0.75	241.6±1.4	1.2	271.5±1.3	1.4	276.5±1.3	1.6
	1.00	241.1±1.6	0.7	271.1±1.3	1.0	276.5±1.5	1.6
Sorbitol	0.25	240.9±1.3	0.5	270.7±1.5	0.6	275.6±1.4	0.7
	0.50	241.4±1.2	1.0	271.3±1.3	1.2	276.2±1.7	1.3
	0.75	241.5±1.3	1.1	271.4±1.4	1.3	276.4±1.8	1.5
	1.00	241.1±1.4	0.7	271.1±1.3	1.0	276.3±1.3	1.4

Although, there is no universal molecular theory that ean explain the mechanism by whieh these stabilizing osmolytes interact with proteins to affect their stability. However, a new mechanism based on the observation of transfer-free energy of the protein backbone from water to aqueous osmolyte solution predicts that the interaction between the protein backbone and osmolytes polar group is that a protein has the least solubility at T_H [59], it seems that sugar osmolytes increases the solubility of β-lactoglobulins B at all concentrations. The temperature at which β-lg B 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 ΔG_D changes with temperature. Therefore, the more T_S inereases, the more the rate of change of ΔG_D increases with temperature. On the other hand, the sensitivity of the thermodynamic stability of the protein increases with temperature. Of course, one should be careful in this interpretation, because this comment holds true as long as T_m is constant. Ravanmehr and Bordbar have reported similar results about stabilization of yeast alcohol dehydrogenase in the presence of sugar osmolytes[60].

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