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Comparative analysis of chemical and thermal denatured β-lactoglobulin A in the presence of sugar osmolytes

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ABSTRACT

Chemical denaturation and thermal denaturation of β-lactoglobulin A (β – lgA) in the absence and presence of various concentrations sugar osmolytes and polyols were measured by monitoring changes in the absorption coefficients at pH 2.0. It has been observed that ΔG_D° (H_2O), (Gibbs free energy change in absence of denaturant at 25 °C) of β-lgA in the presence of 10% (w/v) Trehalose, Sucrose, Sorbitol and Mannitol is increased. We report that the functional dependence of ΔG_D°, (Gibbs free energy change at 25 °C) of protein in the absence and the presence of sugar osmolytes on denaturant concentration is linear. Trehalose is found to induce remarkable stability of β-lgA against chemical denaturation. The 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 were measured. It has been observed that each sugar stabilizes the protein in terms of T_m and ΔG_D°.

Keywords: β-LactoglobulinA; Sugar osmolytes; Protein stability

INTRODUCTION

Bovine β-lactoglobulin (BLG) is a major whey protein of bovine milk with known primary, secondary and tertiary structures, however its biological function is still unknown [1]. It normally exists as a dimer with a subunit molecular mass approximately 18,400 kDa. Each monomer is comprised of 162 amino acid residues, with one free cysteine residue and two disulphide bridges [2]. The X-ray crystal structure of BLG (lattice X) at a resolution of 1.8 °A shows that the BLG monomer is calyx, a three-turn α-helix, and four short 310 helical fragments located in the N-terminal, AB, GH, and C-terminal loops [3, 4].

In 1955, it was found that bovine BLG existed in two genetic forms that differed slightly in their electrophoretic behavior on paper at pH 8.6. These forms are called β-lactoglobulinA(BLGA) and β-lactoglobulin B (BLG-B) [5]. Although several other BLG

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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 [6].

When it needs to maintain the osmotic pressure of living cells, nature has (through evolutionary selection) opted to do this by incorporating a number of compounds known as osmolytes into the cells. It is remarkable that the small numbers of these compounds span cellular organisms, plants, and animal vertebrates and invertebrates [7–9]. These compounds comprise polyols, sugars, methylamines, amino acids and their derivatives, and in some cases urea in combination with methylamines [8]. Among these chemical categories, carbohydrates are usually dominant solutes accumulated in organisms to protect the proteins in terms of loss of activity [10,11] and chemical [12,13] and thermal denaturations [14–18]. They have also been found to be effective stabilizers of proteins and biological assemblies when added at high concentrations [19–24].

There are various mechanisms that have been used to explain the observation on the effect of sugars on the protein denaturation equilibrium, native (N) state ↔ denatured (D) state [25–29]. According to one mechanism sugars stabilize N state because they are preferentially excluded from the protein surface, for the preferential exclusion increases the chemical potential of the protein proportionately to the solvent exposed surface area. Thus, by Le Chatelier's principle, sugar osmolytes favor more compact state, i.e., the N state over the structurally expanded state, i.e., D state. Hence according to this mechanism ΔGD, the Gibbs free energy change associated with the denaturation process, N state ↔ D state, should increase in the presence of osmolytes, for $\Delta G^0_D = -RT\ln([D]/[N])$, where square bracket represents concentration. According to the most recent mechanism of sugar osmolytes stabilization of proteins, Bolen and colleagues [30] used apparent water-to-osmolyte solution transfer free energies for side-chain and backbone models to interpret the increase in stability. They concluded that unfavorable interactions between the fully unfolded protein backbone and the osmolyte solution drive folding. That is, the decreased exposure of the backbone on folding is the major driving force for osmolyte-induced stabilization.

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 [19-23, 31-35]. 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 A during heat stress and chemical denaturation have been extensively studied at various sugar concentrations.

EXPERIMENTAL SECTION

Chemicals

Commercially lyophilized bovine β-lactoglobulin A (β-lgA), was purchased from Sigma Chemical Co. Guanidinium Chloride (Gdn HCl) (extra pure), Glycine and KCl were from Merck. D-Sorbitol, D-mannitol, D-Trehalose and D-Sucrose were also obtained from Sigma. These and other chemicals were analytical-grade reagents and used without further purification.

Preparation of β-lgA Solution

Protein stock solutions were filtered using 0.45μm milipore filter paper. The concentration of β-lgA 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 optical measurements all solutions were prepared in 0.05 M glycine-HCl buffer containing 0.1 M KCl at pH 2.0 and equilibrated 30 min at 25 °C.

Chemical Denaturation of β-lgA

Isothermal denaturation of β-lgA by GdnHCl in the absence and presence of 10% (w/v) Trehalose, Sucrose, Sorbitol and Mannitol at pH 2.0 and 25.0 °C was measured in a CARY model 300 UV/vis spectrophotometer with a Peltier-type temperature controller. Protein concentrations used for 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 [36].

Thermal Denaturation of β-lgA

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 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 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 [37]:

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

where $y(T)$ is the optical property at emperature $T(K)$, $y_N(T)$ 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) [38,39]. A plot of ΔH_m versus T_m gave the value of ΔC_p, the temperature-independent heat capacity change at constant 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

GdnHCl-induced Denaturation of β-lgA

The denaturation of β-lgA by GdnHCl is shown in Fig. 1 at pH 2.0 and 25 °C by observing changes in the difference absorption at 292 nm ($\Delta\varepsilon_{292}$). This is very similar to previous report [40]. 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-lgA denaturation in agreement with previously reported data [41]. The first transition is centered in the [GdnHCl], the molar concentration of GdnHCl, range 0–2.0 M and is represented here by the reaction N↔X, where X is the thermodynamically stable intermediate

state of the protein between its N (native) and D (denatured) states. The second transition occurs in the [GdnHCl] range 2.0–6.0 M and is represented by the reaction X↔D.

Assuming the process N↔X and X↔D designated as transition I, II follows a two-state mechanism, the thermodynamics parameters such as ΔG_I (Gibbs energy change associated with the transition I), ΔG_{II} (Gibbs energy change associated with the transition II), ΔG_I^o (ΔG_I value at zero [GdnHCl]), ΔG_{II}^o (ΔG_{II} value at zero [GdnHCl]), f_I (fraction of molecules in the intermediate state) and f_{II} (fraction of molecules in the D state), were calculated with using following relations :

$$f_I = \frac{(Y - Y_N)}{(Y_X - Y_N)} \quad (3)$$

$$\Delta G_I^{\,o} = -RT \ln\left[\frac{(Y - Y_N)}{(Y_X - Y_N)}\right] \quad (4)$$

$$\Delta G_I^{\,o} = \Delta G_I^{\,o}(H_2O) - m_I[GdnHCl] \quad (5)$$

$$f_{II} = \frac{(Y - Y_X)}{(Y_D - Y_X)} \quad (6)$$

$$\Delta G_{II}^{\,o} = -RT \ln\left[\frac{(Y - Y_X)}{(Y_D - Y_X)}\right] \quad (7)$$

The results are given in Table 1.

Table 1. Thermodynamic parameters characterizing the denaturation of β-lgA by guanidinium chloride at pH 2.0 and 25 °C

Transition	[GdnHCl]	$\Delta G_I^o(H_2O)$ / $\Delta G_{II}^{\,o}{}_X$ (kJ. mol^{-1})	C_{mI} / C_{mII} (M)	m_I / m_{II} (kJ. mol^{-1}.M^{-1})
N↔X	0-2	29.50 ± 0.95	1.45 ± 0.13	20.34 ± 0.92
X↔D	2-6	21.45 ± 1.15	3.23 ± 0.10	15.54 ± 0.80

Values of ΔG_I^o and ΔG_{II}^o in the range $-5.5 \leq \Delta G^o$kJ.mol^{-1} ≤ 5.5 were estimated as a function of [GdnHCl] and they were analyzed for $\Delta G_I^o(H_2O)$, $\Delta G_{II}^o(H_2O)$, m_I, C_{mI}, the midpoint of the transition I, C_{mI} ($=\Delta G_I^o(H_2O)/ m_I$), m_{II} and C_{mII} ($=\Delta G_{II}^o(H_2O)/ m_{II}$). The $\Delta G_{II}^{\,o\,X}$ is value of $\Delta G_{II}^{\,o}$ at 2 M [GdnHCl]. All results are in agreement with previous report[40].

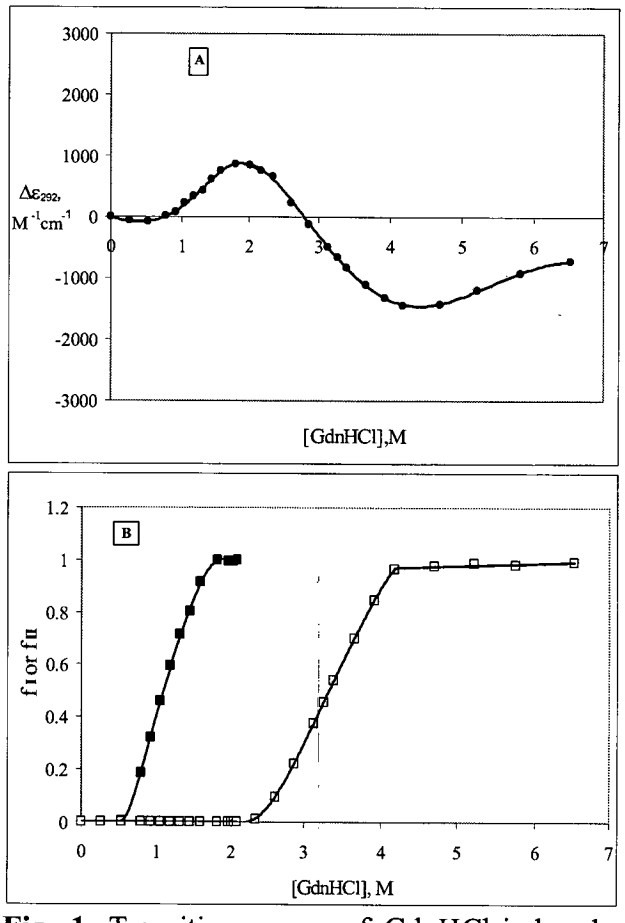

Fig. 1. Transition curves of GdnHCl-induced denaturation of β-lgA at pH 2.0 and 25 °C (A); The normalized transition curves (B), f_I(■) and f_{II}(□).

GdnHCl-induced Denaturation of β-lgA in the presence of Sugar osmolytes

GdnHCl-induced denaturation of β-lgA in the presence of 10% (w/v) Trehalose, Sucrose, Sorbitol and Mannitol was followed by measuring changes in $\Delta\varepsilon_{292}$ as a function of GdnHCl concentration at pH 2.0 and 25 °C.

Assuming a two-state model of denaturation, optical transition data were converted into ΔG_D, the Gibbs energy change using the relation,

$$\Delta G_D^{\ o} = -RT \ln\left[\frac{(Y - Y_N)}{(Y_D - Y)}\right] \qquad (8)$$

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. ΔG_D°(-5.4≤ ΔG_D°(kJ mol^{-1}) ≤5.4) [42] was plotted against [D], the molar concentration of the denaturant, and a linear least-squares analysis was used to fit the (ΔG_D°,[D]) data to the relation,

$$\Delta G_D^{\circ} = \Delta G_D^{\circ}(H_2O) - m_d[D] \qquad (9)$$

where ΔG_D° is the value of ΔG_D at 0 M denaturant and m_d gives the linear dependence of ΔG_D° on [D]. Fig 2. shows GdnHCl-induced denatration curves of β-lgA in the presence sugar osmolytes. Each curves, which was measured three times, was analyzed for ΔG_D° (H_2O), m_d and C_m (=ΔG_D° (H_2O) / m_d) using Eq. (9). The results are shown in Table 2. Fig 3. shows the linear dependence of ΔG_D° on [D].

Thermal Denaturation of β-lgA in the presence and absence Sugar osmolytes

Fig. 4 shows the representative denaturation curves of β-lg A in the presence and absence of trehalose, sucrose and sorbitol. The results were reported in previous paper [43].

Table 2. Parameters characterizing the denaturation of β-lgA by guanidinium chloride in the presence of various sugar osmolytes at pH 2.0 and 25 °C

Osmolytes (10% W/V)	$\Delta G^{\circ}_D(H_2O)$ (kJ mol^{-1})	C_m (M)	m_d (kJ $mol^{-1}.M^{-1}$)
Trehalose	56.43 ± 2.20	3.93 ± 0.15	14.36 ± 0.95
Sucrose	54.59 ± 3.35	3.56 ± 0.25	15.33 ± 0.84
Sorbitol	52.72 ± 4.40	3.40 ± 0.30	15.51 ± 0.87
Mannitol	51.90 ± 4.35	3.35 ± 0.45	15.49 ± 0.91

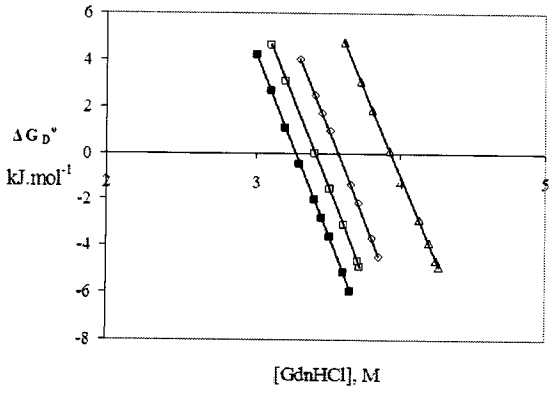

Fig. 2. Transition curves of GdnHCl-induced denaturation of β-lgA at pH 2.0 and 25 °C in the presence of 10% (w/v)Trehalose(Δ), Sucrose(◊), Sorbitol(□) and Mannitol(■). The lines correspond to fitting curves.

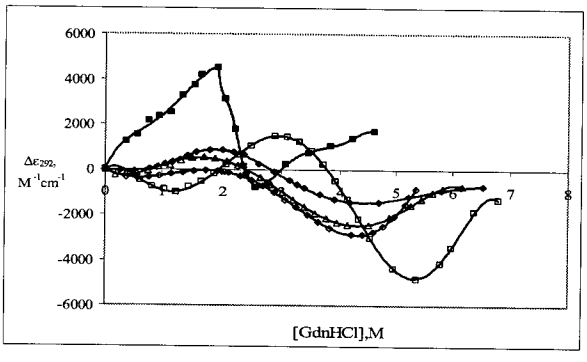

Fig. 3. ΔG_D° Versus [GdnHCl] plots for β-lgA at pH 2.0 and 25 °C in the presence of 10% (w/v) Trehalose(Δ), Sucrose(◊), Sorbitol(□) and Mannitol(■). The lines correspond to fitting curves.

Table 3. The percent stabilization of β-lgA by sugar osmolytes at pH 2.0 and 25 °C.

Osmolytes (10% W/V)	$\%\Delta\Delta G_D^{\circ}$
Trehalose	10.76
Sucrose	7.14
Sorbitol	3.47
Mannitol	1.86

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 the presence and absence of osmolytes) for β-lgA in the presence of different concentrations of trehalose, sucrose and sorbitol are collected in Table 4.

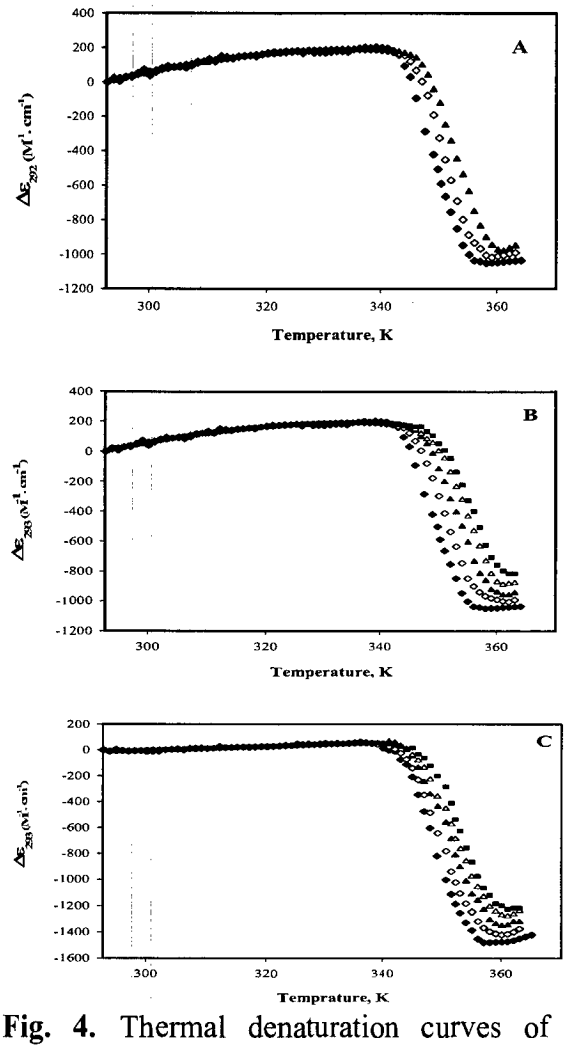

Fig. 4. Thermal denaturation curves of β-lactoglobulin A in the absence and presence of Trehalose (A), Sucrose (B) and Sorbitol (C), buffer (♦), 0.25 M (◊), 0.5 M (▲), 0.75 M (Δ),and 1M (■).

The values of 5.39 $kJ.mol^{-1}.K^{-1}$ obtained for ΔC_P of β-lg A. Tables 4. present the values of ΔG_D^o (Gibbs free energy change at 25°C) at different concentrations of trehalose, sucrose and sorbitol for β-lgA. This tables also show $\%\Delta\Delta G_D^o$.

Table 4. Stability parameters of β-lgA in the presence of various concentrations of sugar osmolytes and polyols at pH 2.0

Osmolyte	M	T_m (K)	ΔT_m (K)	ΔH_m ($kJ.mol^{-1}$)	$\Delta\Delta H_m$ ($kJ.mol^{-1}$)	ΔG_D^o ($kJ.mol^{-1}$)	$\%\Delta\Delta G_D^o$
Trehalose	·.··	351.0 ± 1.2	·.·	·448.2 ± 2.3	·.·0	44.9 ± 0.6	·.··
	0.25	351.9 ± 1.3	0.9	450.1 ± 2.4	1.9·	45.4 ± 0.5	1.1·
	·.*·	353.0 ± 1.4	2.0	453.1 ± 2.5	4.9·	46.1 ± 0.6	2.7·
Sucrose	0.25	351.8 ± 1.3	0.8	450.0 ± 2.4	1.8·	45.4 ± 0.6	1.1·
	·.*·	353.3 ± 1.5	2.3	455.0 ± 2.4	6.8·	46.5 ± 0.5	3.6·
	0.75	355.1 ± 1.4	4.1	464.5 ± 2.6	16.3	48.4 ± 0.4	7.8·
	١.··	356.5 ± 1.3	5.5	479.2 ± 2.5	31.0	51.2 ± 0.5	14.8
Sorbitol	8.25	351.7 ± 1.5	0.7	450.0 ± 2.3	1.8·	45.3 ± 0.5	0.9·
	·.*·	353.1 ± 1.6	2.1	453.7 ± 2.6	5.5·	46.3 ± 0.6	3.1·
	0.75	354.8 ± 1.5	3.8	462.2 ± 2.1	14.0	48.0 ± 0.4	6.9·
	١.··	356.0 ± 1.7	5.0	469.4 ± 2.4	21.2	49.2 ± 0.5	9.6·

DISCUSSION

According to the previous results [40], the GdnHCl-induced denaturation involves two steps. The first step (N↔X) involves the formation of the additional secondary structure and the second step (X↔D) represent the melting of all secondary structures in the protein. They showed that the N↔X transition involves the burial of Trp residue. The reason for saying this, is that the transfer of Trp from a less non-polar medium to a more non-polar medium is accompanied by an increase in the absorption in the region 280–292 nm and in the fluorescence emission spectrum in the region 330–350 nm.

They also represented the tertiary structure melts on the addition of GdnHCl above 2 M, for both Trp absorption at 292 nm and fluorescence emission at 335 nm decrease on transferring Trp from a non-polar environment to a polar environment.

The ΔG_D° ($=\Delta G_I^\circ$ (H_2O) + $\Delta G_{II}{}^{\circ\ X}$) of denaturation for the N↔D transition of β-lgA is 50.95 kJ mol^{-1}. That is in agreement with previous report [40].

Concerning the overall data can be describe that transitions N↔X and X↔D of β-lgA follow two independent two-state mechanism and the dependence of ΔG_I° and $\Delta G_{II}{}^\circ$ on [GdnHCl] is linear under all experimental conditions. There are several documents that certify this assumption; including a 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 [44]. According to the previous results [40], they observed that various measurements gave, within experimental errors, identical values of thermodynamics parameters. Thus, they assumed that GdnHCl-induced denaturation is composed of two distinct two-state processes. With respect to the above discussion we used two independent "two states models" for analysis of denaturation curve in the absence of osmolytes.

However, the abrupt change in the transition curve in the presence of manitol can be explained on the basis of "specific hydration model". Sugar molecules induce structure in the water molecules, surrounding them if the orientation of OH groups is such that some of the 0-0 spacings correspond with the 0-0 distance of 4.86 °A of the water lattice [45, 46]. So that mannitol behaves differently from sorbitol (its isomer) and other sugar osmolytes. On the other hand, differences in the capacity of the manitol with other sugar osmolytes to stabilize the globular protein structure can be attributed to differences in their preferential interactions with the protein surface [47, 21]. Two different physicochemical phenomena contribute to the preferential accumulation or exclusion of osmolytes and solvent molecules around proteins: steric exclusion and differential interactions [48].

An explanation of the differences in stabilizing effect between these sugars and polyols will obviously require a more rigorous approach than the one we have used. Our results imply that one cannot simply discuss the effect of these osmolytes on the conformational stability of β-lactoglobulin.

Values of ΔG_D° and C_m obtained from the analysis of the GdnHCl-induced transition curves of β-lgA in the presence of 10% (w/v) Trehalose, Sucrose, Sorbitol and Mannitol are given in Table 2. These sugars are as same as polyols. It is observed that ΔG_D° of β-lgA increases in the presence of all sugars. However, The following trend is observed for ΔG_D° and C_m:

Trehalose> Sucrose > Sorbitol > Mannitol.

This represents the stabilizing power of these osmolytes in comparison with each other. This stabilizing effect can be related to the following mechanism that has been explained in the following.

Thermal denaturation's results, (Fig 4.), 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. Calculated denaturation temperatures show that T_m for β-lactoglobulins

A in buffer are 351.0 K. It is seen in Fig. 4 (also see Tables 4) that T_m of β-lactoglobulins A at pH 2.0 increases linearly with an increase in the concentration of individual sugar.

We have determined $\Delta C_p = \left(\frac{\partial \Delta H_m}{\partial T_m}\right)_p$ from the linear plot of ΔH_m and T_m values at pH 2.0. Values of ΔC_p in the presence of different concentrations of sugars are 5.39 for β-lactoglobulins A. 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 $mol^{-1}K^{-1}$ [39].

The effect of sugars on protein stability have been explained in terms of preferential binding and preferential exclusion of these cosolutes [29]which is supported by recent observations on the transfer-free energy of protein groups from the solvent water to the co-solvent aqueous solutions [30]. 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 Tables4. 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 linearly with the molar concentration of the additive.

Overall, protein stability should depend upon a fine balance between favorable and unfavorable interactions of the native and the denatured protein states with the cosolvent molecules [49].

In summary, Trehalose, Sucrose, Sorbitol and Mannitol as sugar osmolytes and polyols stabilize proteins by shifting the denaturation equilibrium toward the native state at pH 2.0.

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REFERENCES

[1] T. V. Burova, Y. Choiset, V. Tran, T. Haertle, Protein Eng. 11 (1998) 1065.

[2] G. Kontopidis, C. Holt, L. Sawyer, J. Mol. Biol. 318 (2002) 1043.

[3] S. Brownlow, J.H. Morais Cabral, R. Cooper, D.R. Flower, S.J. Yewdall, I. Polikarpov, A.C.T. North, L. Sawyer, Structure 5 (1997) 481.

[4] F. Morgan, A. Venien, S. Bouhallab, D. Molle, J. Leonil, G. Peltre, D.Levieux, J. Agric. Food. Chem. 47 (1999) 4543.

[5] R. Townend, Arch. Biochem. Biophys. 109 (1965) 1.

[6] K.M.G. Oliveira, V.L. Valente-Mesquita, M.M. Botelho, L. Sawyer, S.T. Ferreira, I. Polikarpov, Eur. J. Biochem. 268 (2001) 477.

[7] P. H. Yancey, G. N. Somero, Biochem. J. 183 (1979) 317.

[8] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Science 217 (1982) 1214.

[9] G.N. Somero, Protons, Am. J. Physiol. 251 (1986) 197.

[10] C. Colaco, S. Sen, M. Thangavelu, S. Pinder, B. Roser, Biotechnology (N.Y.) 10(1992) 1007.

[11] P. Carninci, Y. Nishiyama, A.Westover, M. Itoh, S. Nagaoka, N. Sasaki, Y. Okazaki, M.Muramatsu, Y. Hayashizaki, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 520.

[12] L.S. Taylor, P. York, A.C. Williams, H.G. Edwards, V. Mehta, G.S. Jackson, I.G. Badcoe, A.R. Clarke, Biochim. Biophys. Acta 1253 (1995) 39.

[13] M. Sola-Penna, A. Ferreira-Pereira, A.P. Lemos, J.R. Meyer-Fernandes, Eur. J. Biochem. 248 (1997) 24.
[14] J.F. Back, D. Oakenfull, M.B. Smith, Biochemistry 18 (1979) 5191.
[15] J.C. Lee, S.N. Timasheff, J. Biol. Chem. 256 (1981) 7193.
[16] T. Arakawa, S.N. Timasheff, Biochemistry 21 (1982) 6536.
[17] T.Y. Lin, S.N. Timasheff, Protein Sci. 5 (1996) 372–381.
[18] G. Xie, S.N. Timasheff, Biophys. Chem. 64 (1997) 25–43.
[19] S.Y. Gerlsma, E.R. Stuur, Int. J. Pept. Protein Res. 4 (1972) 377.
[20] G. Xie, S.N. Timasheff, Protein Sci. 6 (1997) 222.
[21] G. Xie, S.N. Timasheff, Protein Sci. 6 (1997) 211.
[22] J.K. Kaushik, R. Bhat, J. Phys. Chem. B 102 (1998) 7058.
[23] Y.S. Kim, L.S. Jones, A. Dong, B.S. Kendrick, B.S. Chang, M.C. Manning, T.W.Randolph, J.F. Carpenter, Protein Sci. 12 (2003) 1252.
[24] S.N. Timasheff, Annu. Rev. Biophys. Biomol. Struct. 22 (1993) 67.
[25] A.J. Saunders, P.R. Davis-Searles, D.L. Allen, G.J. Pielak, D.A. Erie, Biopolymers 53 (2000) 293.
[26] G.T. Weatherly, G.J. Pielak, Protein Sci. 10 (2001) 12.
[27] D.W. Bolen, I.V. Baskakov, J. Mol. Biol. 310 (2001) 955.
[28] S.N. Timasheff, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9721.
[29] S.N. Timasheff, Biochemistry 41 (2002) 13473.
[30] Y. Liu, D.W. Bolen, Biochemistry 34 (1995) 12884.
[50]
[31] Z. Saadati, A.Kh. Bordbar, Protein J. 27 (2008) 455.
[32] L. D'Alfonso, M. Collini, G. Baldini, Eur J Biochem. 270 (2003) 2497.
[33] A. Kulmyrzaev, C. Bryant, DJ. McClements, J Agric Food Chem. 48 (2000) 1593.
[34] CM. Romero, JM. Lozano, J. Sancho, GI. Giraldo, 40 (2007) 423.
[35] L.M. Holthauzen, D.W. Bolen, Protein Sci. 16 (2007) 293–298.
[36] C.N. Pace, Methods Enzymol. 131, (1986) 266.
[37] V. Rishi, F.Anjum, F.Ahmad, W. Pfeil, Biochem. J. 329 (1998) 137.
[38] A. Sinha, S. Yadav, R. Ahmad, F. Ahmad, Biochem. J. 345 (2000) 711.
[39] S.Yadav, F. Ahmad, Anal. Biochem. 283 (2000) 207.
[40] T. Ali Dar, L. R. Singh, and F. Ahmad, Biophys. Chem. 127, (2007)140.
[41] L. D'Alfonso, M. Collini, and G. Baldini, Biochem. 41, (2002) 326.
[42] C. Tanford, Adv. Protein Chem. 23, (1968) 121.
[43] Z. Saadati, M. Aghaei, , H. Aghaei, (not publish)
[44] R. K. O. Apenten, S. Khokhar, and D. Galani, 16, (2002) 95.
[45] J. H. Stern, and M. E. O'Connor, J. Phys. Chem. 76, (1972) 3077.
[46] M. J. Tait, A. Suggett, F. Franks, S. Ablett, , and P. A. Quickenden, J. Solution Chem. 1, (1972). 131.
[47] T. Arkawa, R. Bhat, and S. N. Timasheff, Biochem. 29(7), (1990) 1924.
[48] D. J. McClements, Critical Reviews in Food Science and Nutrition. 45, (2002) 417.
[49] J.K. Kaushik, and R. Bhat, J. Biol. Chem. 278, (2003) 26458.