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Thermodynamic study of the influence of polyols and glucose on the thermal stability of holo-bovine α-lactalbumin

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Abstract Thermal stability of bovine α-lactalbumin in buffer and dilute aqueous solutions of erythritol, xylitol, sorbitol, inositol and glucose was evaluated by fluorescence spectroscopy and circular dichroism. Results show that at the selected conditions, the transition is reversible and is well described by a two-state model. At low concentration the cosolutes do not show a structure stabilizing effect, and some of them even destabilize the protein. At higher concentration, all of them stabilize the native protein conformation; however, the extent of stabilization is lower than the effect shown with other proteins, presumably due to the lactalbumin incomplete unfolding.

Keywords Bovine lactalbumin · Glucose · Polyols

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Introduction

Bovine α-lactalbumin (LA) is a small monomeric globular protein found in whey with a molecular weight of 14.2 kDa. This 123 aminoacid protein has four disulfides bonds, one of them linking two subdomains, and can bind several metals including Ca^{2+} [1, 2]. It can be found in two forms: when it is not bound to a Ca^{2+} ion is called apo-lactalbumin and the holo form refers to the protein bound to Ca^{2+} ions [3]. The calcium ion increases the thermal stability of the protein, producing only minor changes in its structure. α-Lactalbumin has potential applications in the pharmaceutical and dietary industries as bactericide [4], tumor cell apoptosis inducer [5] and gelation, emulsification and foaming agent [6]. For this reason it has been widely studied and has been considered a model metalloprotein [2, 7]. The thermal denaturation process of this protein has been studied under different buffer conditions however, results about the temperature effect on the α-lactoalbumin are not conclusive and show that the number of states involved in this process is very sensitive to the protein environment [8–12].

For a long time, it has been known that polyols and sugars stabilize proteins, but the mechanism has not been elucidated. Preferential interaction, surface tension increment, excluded volume, scaled particle theory, Wyman linkage functions and solvent exchange equilibria models have been used to try to explain the experimental observations, with different degrees of success [13–17]. Thermodynamic studies about protein behaviour in different solvents give valuable information about solute–solvent interactions and solvent effect on protein stability [18]. They show that the interactions between protein and solvent molecules are dominant at low concentrations. However, polyols–protein–water systems are generally studied

at high cosolute concentrations, where the stabilizing effect becomes evident [14].

In this work, we studied the influence of erythritol, xilytol, glucytol, inosytol and glucose at low concentrations on the thermal stability of the bovine holo-α-lactalbumin using fluorescence and circular dichroism (CD) in the far UV and near UV (CD). According to the obtained results the denaturation process can be represented by a two-state transition and the observed effect of the cosolutes on the protein stability is moderate. The CD spectra a high temperature suggest that the protein conserves some secondary structural elements in its denaturate state which could explain the moderate stability increment observed.

Materials and methods

Erythritol 99.9%, xilytol 99%, glucytol 99.9%, inosytol 99%, glucose 99.5% and holo-bovine lactalbumin type I (99% by SDS-PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), $CaCl_2$ and others reagents were analytical grade. A PIPES 5 mM, $CaCl_2$ 1 mM stock solution was prepared and degassed and given amounts of cosolutes were dissolved to achieve the desired cosolute concentration, afterwards, the lyophilized protein was dissolved. Water was distilled and deionized obtaining water with conductivity below than 1.5 μS/cm. The pH after cosolute and protein dissolution was 6.5. Protein concentration was measured by UV spectroscopy using a $E_{1\%} = 20.9\ g^{-1}\ L\ cm^{-1}$ at 280 nm [19].

Circular Dichroism spectra were obtained in the far and near UV region using a CHIRASCAN spectrophotometer from Applied Photophysics Ltd. (Surrey, UK). The spectra were recorded at 298.15 ± 0.1 K, except the spectra of the protein dissolved in inositol 0.9 m, which was obtained at 303.15 ± 0.1 K because of inositol low solubility. For near UV-CD spectra quartz cells of 10.00 mm pathlength were employed. In the case of the far UV-CD spectra, demountable cells of pathlength of 0.10 mm were used because of the strong buffer absorption below 200 nm. Thermal denaturation profiles were obtained following the CD signal at 222 and 270 nm, in the far and near CD UV spectra, respectively. A Peltier system was used to heat/cool the sample and the temperature inside the cell was recorded using a small temperature probe. The scan heating rate was 1 K/min.

Fluorescence intensity measurements were carried out using an Aminco-Bowman Series 2 spectrometer monitoring fluorescence intensity emission at 360 nm with excitation at 280 nm. Excitation and emission slit widths were 2 and 4 nm, respectively. The sample was heated using an external circulator bath attached to the cell holder. In all cases sample temperature was measured inside the cell using a temperature probe. The scan heating rate was 1 K/min.

Differential Scanning Calorimetry DSC was carried out with a VP-DSC MicroCal System (MicroCal, Northampton, MA, USA) and profiles were analyzed with Origin software. The protein sample in buffer was degassed and over pressed to 25 psig. The scan heating rate was 1 K/min. Buffer baseline was obtained by scanning 5 mM PIPES 1 mM $CaCl_2$ buffer at pH 6.5, at the correspondent heating rate to subtract it from experimental runs.

Reversibility of denaturation process was followed by fluorescence and DSC, heating the protein solution to 360 K during 5 min before cooling it down to room temperature. This process is repeated with the same sample, checking reproducibility of fluorescence and DSC readings.

The thermodynamic denaturation parameters are obtained from the thermal denaturation curves [20]. For a reversible denaturation process through a two-state transition between native and unfolded states, the denaturation equilibrium constant can be determined using the following equation:

$$K_D = \frac{Y_N - Y}{Y - Y_D} \tag{1}$$

where K_D is the apparent equilibrium constant, Y is the value of the property at temperature T, and Y_N and Y_D are the experimental data of the property for the native and denaturated protein, respectively. In practice, the Y_N and Y_D values are function of temperature and it is assumed that they have a linear dependence on it and can be expressed as:

$$\begin{aligned} Y_N &= Y_{N0} + m_N T \\ Y_D &= Y_{D0} + m_D T \end{aligned} \tag{2}$$

For a reversible process, the standard free energy ΔG° can be related to the equilibrium constant K_D through the expression:

$$\Delta G^{\circ} = -RT \ln(K_D) = \Delta H^{\circ} - T\Delta S^{\circ} \tag{3}$$

At Tm, the denaturation temperature, $\Delta G^{\circ} = 0$ and

$$\Delta S^{\circ}\,(Tm) = \frac{\Delta H^{\circ}(Tm)}{Tm} \tag{4}$$

Combining the above equations, the following equation is obtained.

$$Y = \frac{Y_{N0} + m_N T - (Y_{D0} + m_D T) e^{-\frac{\Delta G^{\circ}}{RT}}}{1 + e^{-\frac{\Delta G^{\circ}}{RT}}} \tag{5}$$

where:

$$-\frac{\Delta G^{\circ}}{RT} = -\frac{\Delta H^{\circ}(Tm)\left(1-\frac{T}{Tm}\right)+\Delta Cp\left(T-Tm-T\ln\left(\frac{T}{Tm}\right)\right)}{RT} \quad (6)$$

Using Eq. 6 experimental data are fitted to obtain the thermodynamic parameters that describe the thermal denaturation process.

The thermal denaturation of bovine lactalbumin was studied in buffer and in aqueous solutions of erythritol, xylitol, sorbitol and glucose at concentrations of 0.300, 0.600, 0.900, 1.200, and 1.500 m and in aqueous solutions of inositol at concentrations of 0.150, 0.300, 0.600, 0.750 and 0.900 m. Protein concentration was about 30 μmol/L.

Results and discussion

CD spectra in the far and near UV were obtained for the protein in buffer and the mixed solvent. The obtained CD-UV spectra are in good agreement with others previously reported at pHs 5.5, 8.0 and 7.8 [12, 21, 22]. Figure 1 shows the influence of the different cosolutes at its highest concentration on the protein far and near CD spectra. No significant changes can be observed so is evident that the cosolutes used in this work do not produce changes on the tertiary and secondary structural properties of bovine lactalbumin. The differences around 190 nm are due to the absorption shown by glucose at these wavelengths. The protein structural properties and the influence of the selected cosolutes were quantified with the CDPro [23] and CDNN [24] softwares. Results are shown in Table 1. Although, the protein helical fraction calculated with these programs is significant lower than the one obtained from the crystallography structure, results show that all fractions remain practically unchanged in the presence of the selected polyols and glucose. This is important because most of the models proposed to explain the influence of cosolutes on the stability of proteins assume that they do not affect the structure of the proteins [14].

Table 1 Secondary structural parameters of α-lactalbumin with different concentrations of various cosolutes

Cosolute	Molality (mol kg^{-1})	Elements of secondary structure (%)			
		α-helix	β-structure	Turns	Unordered
Buffer		34	16	21	30
Erythritol	1.50	34	16	21	30
Xylitol	1.50	33	17	20	30
Sorbitol	1.50	32	17	20	30
Inositol	0.90	35	15	20	30
Glucose	1.50	33	16	22	29

Figure 2 shows the obtained scans by DSC for the protein in buffer up to 360 K. The results obtained by DSC and fluorescence show that at the conditions of this study, reversibility of the thermal denaturation of holo-bovine lactalbumin, is higher than 90%. No dependency of the thermodynamics denaturation parameters was found neither with protein concentration in the 2–30 μmol/L range nor with scan velocity in the 0.5–1.5 K/min range.

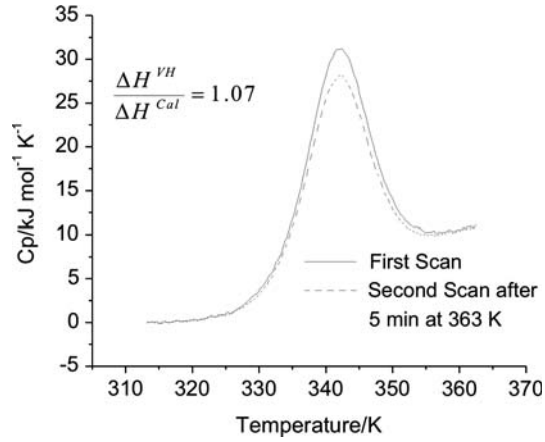

Fig. 2 Differential Scanning Calorimetry for the holo-bovine-α-lactalbumin in buffer at pH 6.5

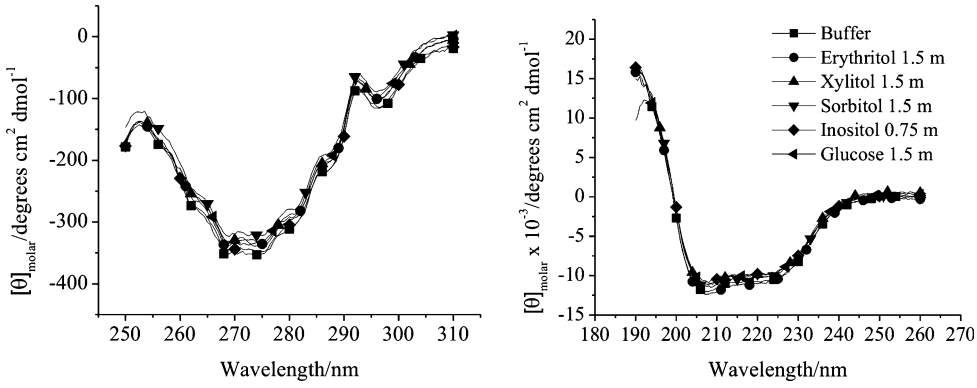

Fig. 1 Near (**a**) and far (**b**) UV-CD spectra of the holo-bovine-α-lactalbumin in buffer at pH 6.5 in the presence of erythritol, xylitol, sorbitol, inositol and glucose

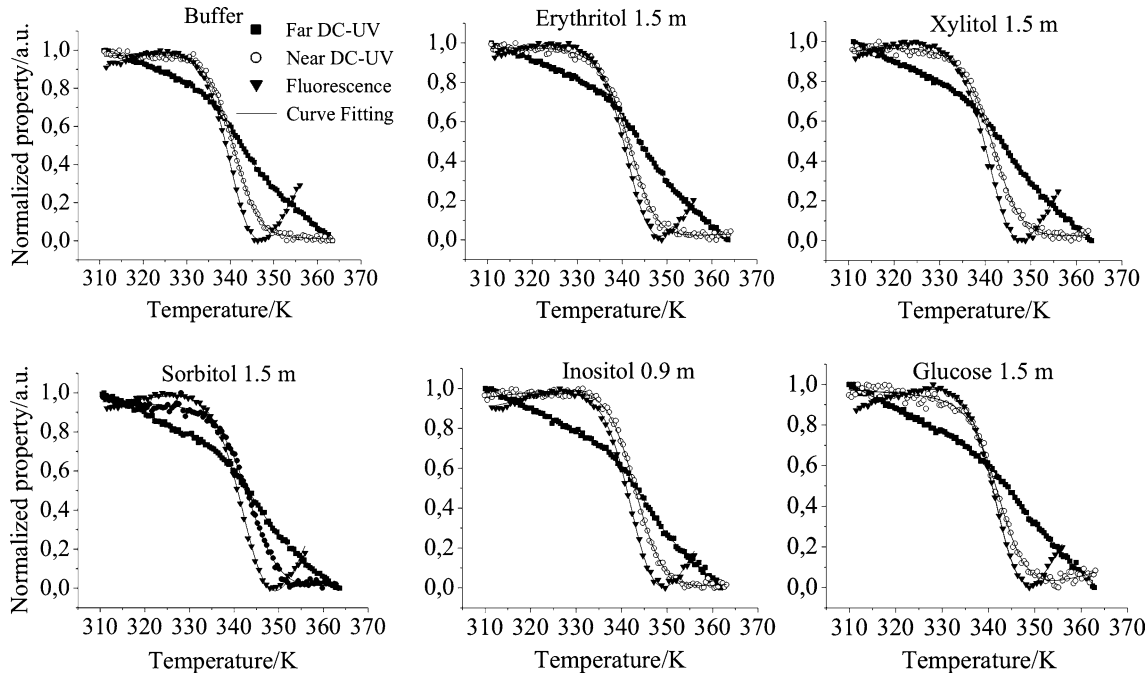

Fig. 3 Thermal denaturation curves by far and near UV-CD and Fluorescence Intensity for holo-bovine-α-lactalbumin in buffer at pH 6.5 at the highest concentration of erythritol, xylitol, sorbitol, inositol and glucose considered

In order to determinate if the lactalbumin unfolding can be represented by a reversible two-state transition between native and unfolded states or a multi state denaturation process, thermal denaturation profiles were obtained using fluorescence intensity and CD in the far UV and near UV. All data were normalized to represent signal change between 1 and 0. Figure 3 shows the CD thermal denaturation profiles of α-lactalbumin in buffer with the maximum concentration of the cosolutes employed. The fluorescence denaturation curves at the selected concentrations of polyols and glucose are shown in Fig. 4. The curves show that at the conditions of the study, the transition can be described by a two-state process and that the cosolutes employed do not affect this mechanism. Another evidence of the two-state model for this protein is that the relation between the calorimetric enthalpy determined by DSC which is a measurement of the heat energy uptake, and the van' Hoff enthalpy which depends on the shape of the endotherm and assumes a true two-state reversible equilibrium, is very close to 1 [25]. The value obtained in this study for this relation is 1.07.

According to these results, the denaturation parameters were determined fitting simultaneously the thermal denaturation curves obtained with far CD, near CD and fluorescence measurements, assuming a two-state process. Denaturation temperatures determined in this work are summarized in Table 2 while Tables 3 and 4 present the unfolding enthalpy and entropy of the process at denaturation temperature. The calculated denaturation parameters obtained in this work for the protein in buffer are in good agreement with others previously reported at similar conditions [11, 12, 26, 27]. Calculated denaturation temperature for holo-α-lactalbumin in buffer is 340.5 ± 0.4 K at pH 6.5.

From the results obtained it can be seen that at the higher concentrations employed, the cosolutes present a small stabilizing effect increasing the denaturation temperature but a lower concentrations the effect is very small or even they induce a denaturating behavior lowering the denaturation temperature. The destabilizing effect of erythritol at low concentrations has been previously reported [28]. However, there is not a clear effect of the number of OH groups of polyols and glucose on denaturation temperature. As the number of OH increases the stabilizing effect has a small increment. The stabilizing effect of these cosolutes increase in the following order: erythritol < xylitol < sorbitol ≈ glucose < inositol.

The results obtained show that polyols and glucose stabilize bovine lactalbumin, however, their effect on the thermal denaturation parameters is lower than the effect observed with other proteins in which the increments induced in *Tm* are two and three folds higher as can be seen in Fig. 5 [15, 29, 30]. In another study [31] that considers the interaction of bovine lactalbumin with sorbitol, glycerol and sucrose the results show that the stabilization effect is very similar to the one observed in this work and only at concentrations higher than 2.0 m, a significant stabilization of the native state of the protein is observed.

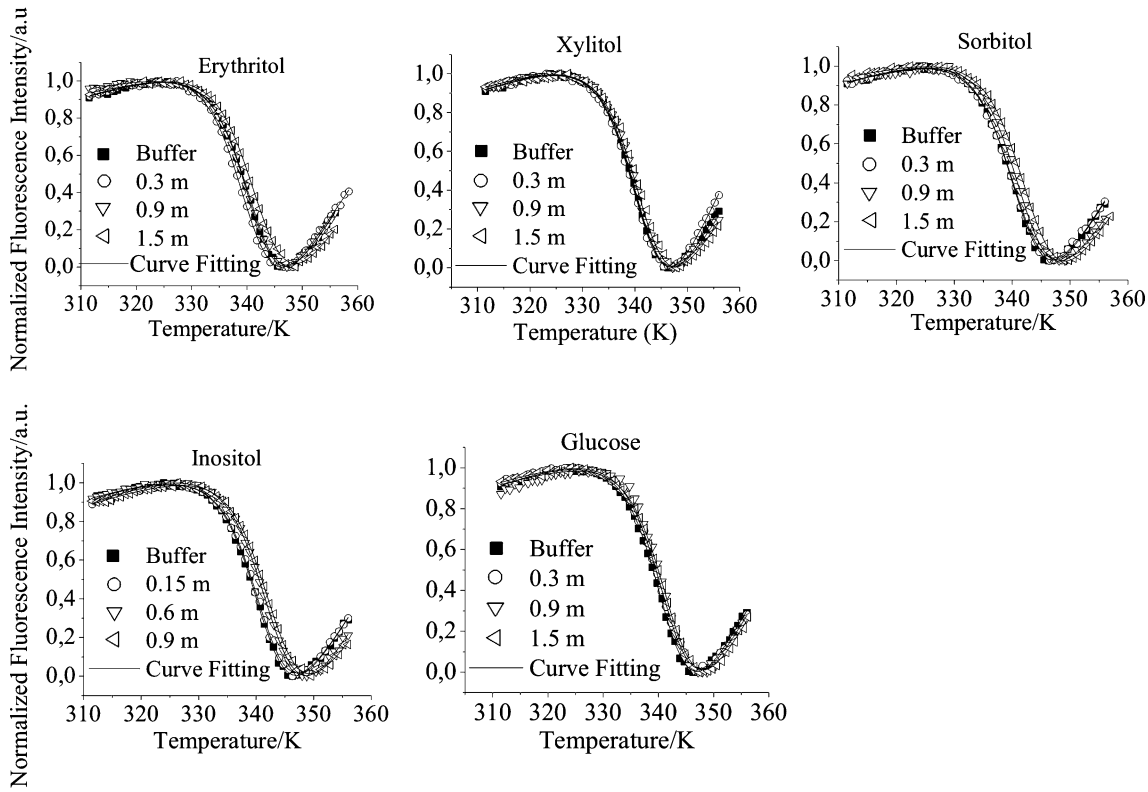

Fig. 4 Effect of concentration of polyols and glucose on the thermal denaturation profiles of holo-bovine-α-lactalbumin followed by fluorescence intensity measurements

Table 2 Denaturation temperature (T_m) of holo-bovine-lactalbumin in buffer and aqueous solutions of polyols and glucose

Solvent\concentration (m)	T_m^a (K)							
	Buffer	0.15	0.3	0.6	0.75	0.9	1.2	1.5
Erythritol	340.5		339.2	340.2		340.5	340.9	341.7
Xylitol	340.5		340.3	340.6		340.7	341.0	341.3
Sorbitol	340.5		340.4	340.9		341.3	341.3	342.1
Inositol	340.5	340.7	340.6	341.5	341.7	341.9		
Glucose	340.5		340.4	340.5		341.5	341.7	342.1

[a] Typical standard deviation is 0.3 K

Table 3 Enthalpy change at T_m for thermal denaturation of holo-bovine-lactalbumin

Solvent/molality (mol kg^{-1})	ΔH_D^a (kJ mol^{-1})							
	Buffer	0.15	0.3	0.6	0.75	0.9	1.2	1.5
Erythritol	296		302	318		286	311	290
Xylitol	296		302	281		301	305	284
Sorbitol	296		291	304		309	301	300
Inositol	296	298	294	296	300	309		
Glucose	296		305	301		304	305	283

[a] Typical standard deviation is 11 kJ mol^{-1}

Table 4 Entropy change at T_m for thermal denaturation of holo-bovine-lactalbumin

Solvent/molality (mol kg^{-1})	ΔS_D^a (kJ mol^{-1} K^{-1})							
	Buffer	0.15	0.3	0.6	0.75	0.9	1.2	1.5
Erythritol	0.87		0.89	0.94		0.84	0.91	0.85
Xylitol	0.87		0.89	0.82		0.88	0.90	0.83
Sorbitol	0.87		0.86	0.89		0.91	0.88	0.88
Inositol	0.87	0.87	0.86	0.87	0.88	0.90		
Glucose	0.87		0.90	0.88		0.89	0.89	0.83

[a] Typical standard deviation is 0.03 kJ.mol^{-1} K^{-1}

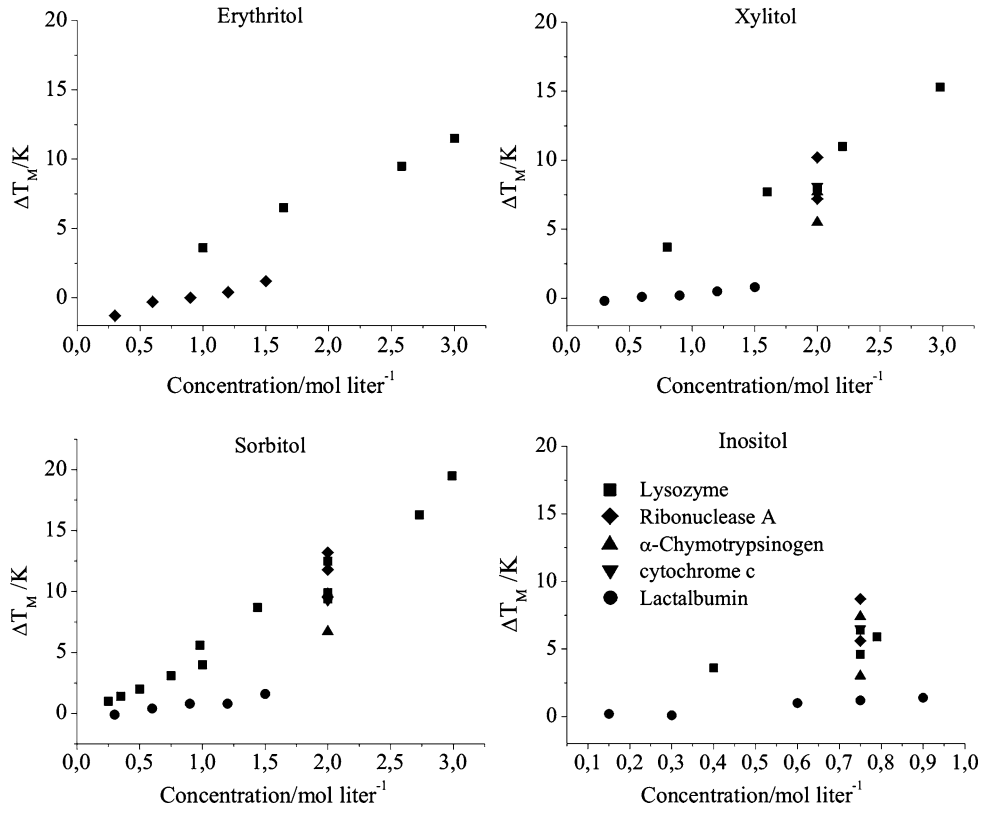

Fig. 5 Comparation of the change in *Tm* produced by polyols on the holo-bovine-lactalbumin and on other proteins [15, 29, 30]

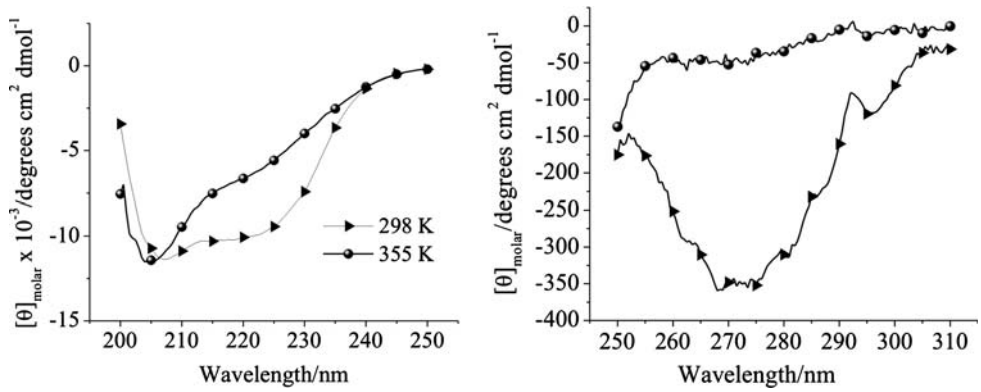

Fig. 6 Influence of the temperature on the far (**a**) and near (**b**) UV-CD spectra of bovine lactalbumin

The difference in the extent of stabilization produced in this case could be explained taking into account results that show that lactalbumin is not fully unfolded at high temperatures [8] and as a consequence, the change in area and volume after denaturation could be small when compared to others proteins. The differences in the extent of stabilization produced by these osmolytes on the bovine lactalbumin and on others proteins, could be related with the extent of unfolding for each protein denaturated state. The change in area and volume for the denaturation process can be related with the extent of stabilization through models such as molecular crowding or surface tension increment [14]: according to them, small changes in the spatial properties is related to small stabilizing effect produced by osmolytes. The high presence of secondary structural elements in the bovine lactalbumin at high temperatures (Fig. 6) indicates a small change in area and volume after denaturation which could explain the low stabilization achieved when the osmolytes studied are added to the protein solution. On the other hand, the preferential interaction parameter of native lactoalbumin with sorbitol, glycerol and sucrose [31] show that these cosolutes are excluded of the protein domain in a similar way that osmolytes are excluded from the domain of other proteins. However, the thermal stabilization exerted by these osmolytes on other proteins is much higher than the reported here with the lactalbumin. Our results suggest that the incomplete exposure of the core residues in the denaturaded state of bovine lactalbumin could also explain the lower protein stabilization produced by the osmolytes

at the light of theories such as the preferential interaction model [14].

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