J Therm Anal Calorim (2011) 104:37–44
DOI 10.1007/s10973-010-1156-3

Influence of calcium on the thermal stabilization of bovine α-lactalbumin by selected polyols

Alberto Albis · José Manuel Lozano · Javier Sancho · Carmen M. Romero

ISBCXVI Special Issue

Abstract Thermal stability of bovine α-lactalbumin in the presence of three different calcium concentrations in aqueous solutions of several concentrations of erythritol, xylitol, sorbitol, and inositol at pH 6.5 was evaluated by UV absorbance, fluorescence spectroscopy, and circular dichroism spectroscopy. At the selected conditions, the thermal denaturation process is reversible and is well described by a two-state model. Results show a higher stability for the holo form of the protein in the presence of calcium, followed by the holo- and the apo-lactalbumin, respectively. The stabilizing effect of the polyols increases with polyol concentration and it is higher for the apo-lactalbumin than holo-lactalbumin and is very small for the protein in the presence of a calcium excess.

Keywords Bovine lactalbumin · Glucose · Polyols thermal stability · Thermal stability

A. Albis (✉) · C. M. Romero
Departamento de Química, Universidad Nacional de Colombia, Cra 45 26-85 Oficina 409, Bogotá, Colombia
e-mail: aralbisa@unal.edu.co

A. Albis
Departamento de Ingeniería Química, Universidad del Atlántico, Barranquilla, Colombia

J. M. Lozano
Departamento de Farmacia, Universidad Nacional de Colombia, Bogotá, Colombia

J. M. Lozano
Fundación Instituto de Inmunología de Colombia FIDIC, Bogotá, Colombia

J. Sancho
Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias Universidad de Zaragoza, Zaragoza, Spain

Introduction

Bovine α-lactalbumin (LA) is the second more abundant protein of whey. It is a small globular protein (123 amino acids, 14.2 kDa) that can bind several metals, including calcium. The bound calcium protein is called holo-LA and the free calcium form is known as apo-LA. The calcium ion increases the thermal stability of the protein, producing only minor changes in its structure [1, 2]. In solution it is found as a monomer at pH 5.1–8.1 [3]. The denaturation process of this protein is very sensitive to the properties of the buffer in which it is dissolved, such as, pH, ionic strength, and buffer composition and in some cases the presence of intermediate states has been reported [4–9]. It has been shown that the presence of other substances can affect the LA stability positively, increasing stability, or negatively, decreasing protein stability [10–15].

Polyols have been used extensively to improve stability of the native structure of several proteins including LA; however, results obtained in a previous work in our laboratory shows that erythritol, xylitol, sorbitol, and inositol stabilize the holo-α-LA in a significant lesser extent than the reported for others proteins and this stabilizing effect of polyols depends on the number of OH-groups and concentration [4, 5]. The stabilizing effect of polyols depends on the nature of the stabilizing molecule as well as on the protein properties. Several theories have been proposed to explain the experimental observations, among them, the preferential interaction theory, however, the stabilization mechanism has not been elucidated [16–19].

In this study, we studied the thermal denaturation of both forms of the protein (apo and holo) in the presence of several concentrations of erythritol, xylitol, sorbitol, and inositol at pH 6.5. The denaturation process was followed

using several techniques such as fluorescence, far and near UV circular dichroism, and absorbance.

Materials and methods

Erythritol 99.9%, xylitol 99%, sorbitol 99.9%, inositol 99%, and bovine LA type I (99% by SDS-PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. Water was distilled and deionized, obtaining water with conductivity below 2 μS/cm. EDTA and other reagents were analytical grade.

The holo form of the protein was obtained dissolving the protein in a 1 mM $CaCl_2$ buffer solution of PIPES 5 mM. For the studies with the apo form a 1 mM EDTA buffer solution of PIPES 5 mM was employed. The thermal denaturation was also studied for the protein in phosphate buffer 5 mM. Protein concentration was about 30 μmol/L and was measured by UV spectroscopy using a $E_{1\%} = 20.9\ g^{-1}\ L\ cm^{-1}$ at 280 nm [20].

Thermal denaturation of bovine LA was studied in buffer and in aqueous solutions of erythritol, xylitol, and sorbitol at concentrations of 0.3000–2.0000 mol/kg of solvent and in aqueous solutions of inositol at concentrations of 0.1500–0.9000 mol/kg of solvent.

Fluorescence intensity measurements were carried out in 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 the 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. Baseline was obtained by scanning the buffer, at the correspondent heating rate to subtract it from experimental runs. The employed buffers were 5 mM PIPES 1 mM $CaCl_2$ buffer at pH 6.5 and 5 mM PIPES 1 mM EDTA buffer at pH 6.5. The EDTA was added to chelate any Ca^{2+} present in solution.

Circular Dichroism spectra were obtained in the far and near UV region using a CHIRASCAN spectrophotometer from Applied Photophysics Ltd. (Surrey, United Kingdom). 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.

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

Thermodynamic denaturation parameters were obtained from the thermal denaturation curves [21]. For a reversible denaturation process through a two-state transition between native and unfolded states, assuming a linear dependence with temperature for the spectroscopy signal of the native and denatured states, the measured spectroscopy signal, Y, can be related to the denaturation parameters according to the relation [22, 23]:

$$Y = \frac{Y_{N0} + m_N T - (Y_{D0} + m_D T) e^{-\frac{\Delta G^0}{RT}}}{1 + e^{-\frac{\Delta G^0}{RT}}} \quad (1)$$

where,

$$-\frac{\Delta G^0}{RT} = -\frac{\Delta H^0(Tm)\left(1 - \frac{T}{Tm}\right) + \Delta Cp\left(T - Tm - T\ln\left(\frac{T}{Tm}\right)\right)}{RT} \quad (2)$$

In the above equations Y is the value of the property at temperature T, R is the universal gas constant, ΔG^0 is the standard free energy, Y_{N0} and Y_{D0} are the properties of the native and denatured protein at 0 K, respectively, and m_N and m_D are the slopes of the linear dependence of the spectroscopic signal with temperature. Using Eqs. 1 and 2, experimental data are fitted to obtain the thermodynamic parameters that describe the thermal denaturation process.

The percent of stabilization of the protein by polyols was calculated as:

$$\%\Delta\Delta G_D^0 = \frac{\Delta G_D^0(m) - \Delta G_D^0(m=0)}{\Delta G_D^0(m=0)} * 100, \quad (3)$$

where $\Delta G_D^0(m)$is the Gibbs free energy of denaturation of the protein at the concentration m, and $\Delta G_D^0(m=0)$ is the Gibbs free energy of denaturation in the absence of polyol.

Results and discussion

Reversibility studies show that for solutions of LA with concentrations below 0.32 mg/mL at pH 6.5, the unfolding/refolding process is highly reversible even at temperatures as high as a 360 K. No dependence 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. The experimental

evidence allows affirming that the denaturation process of LA, is thermodynamically and no kinetically controlled, under the conditions used in this work thus the thermodynamics parameters are obtained when they are calculated using the appropriate relations.

Figure 1 shows the obtained DSC scans for the holo and apo form of the protein in buffer up to 360 K. Results show that at the conditions of this study, reversibility of the thermal denaturation of bovine LA is higher than 90% and no evidence of aggregation at these conditions is observed.

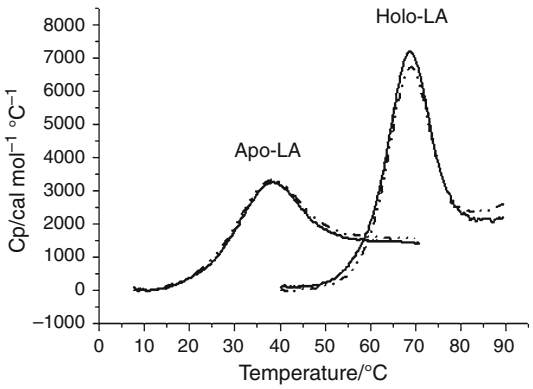

Fig. 1 Differential Scanning Calorimetry for the apo-bovine-LA in buffer at pH 6.5. *Solid lines* correspond to the first heating and *dashed lines* correspond to a second heating cycle of the same sample. The apo-LA was dissolved in PIPES 5 mM pH 6.5 + EDTA 1 mM and the holo protein was dissolved in PIPES 5 mM pH 6.5 + $CaCl_2$ 1 mM

In a previous work the thermal profiles for holo-LA obtained using different techniques, showed that the denaturation process can be modeled as a two-state process without the presence of intermediate states [8]. The calorimetric and van't Hoff enthalpies for both forms of the protein are very close indicating a high cooperativity of the denaturation process and confirming the hypothesis of a two-state transition. From Fig. 1 the stabilizing effect of calcium on the protein is notorious, as has been widely reported in the literature [6, 24].

In order to examine the effect of polyols on the stability of apo-LA, thermal unfolding curves were determined by emission fluorescence spectroscopy for the protein in buffer and in aqueous solutions of the considered polyols. The curves in Fig. 2 show that at the conditions of the study, the thermal denaturation of the protein in the presence of the selected polyols can also be described as a two-state transition between native and unfolded states without the presence of intermediate states. An increment in the denaturation temperature is observed at high concentrations of the selected polyols reflecting the increase in thermal stability of the protein. This behavior is similar to the reported by Sekhar and Prakash [10] for solutions of this protein and sorbitol, sucrose and glycerol who observe a stabilizing effect at concentrations higher than 30%.

The thermodynamic parameters that describe the thermal denaturation process of apo- and holo-LA with calcium in the presence of erythritol, xylitol, sorbitol, and inositol are presented in Tables 1, 2, 3, 4. The denaturation parameters

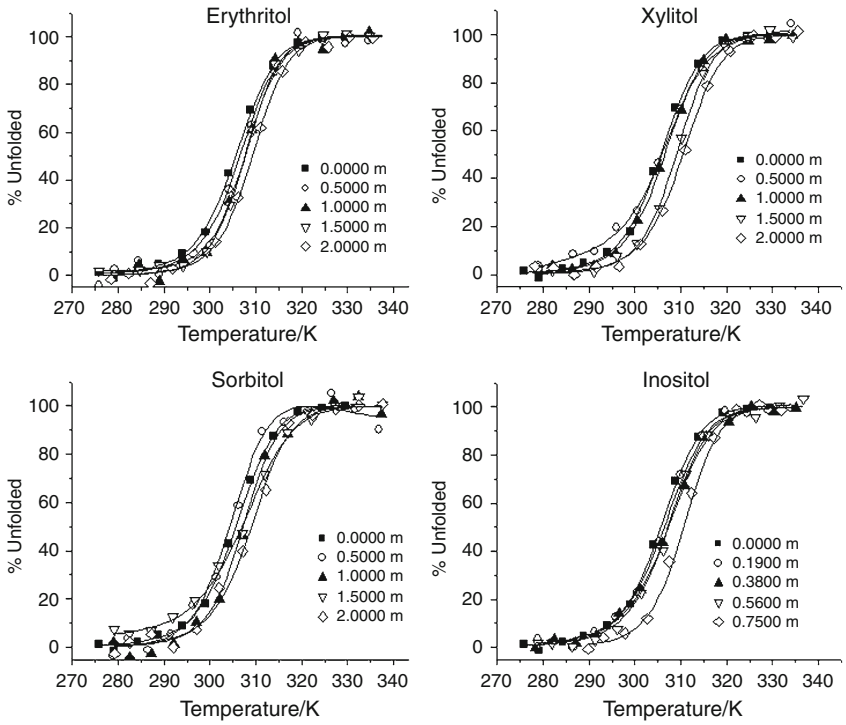

Fig. 2 Thermal unfolding curves for apo-LA in aqueous solutions of buffer PIPES 5 mM + EDTA 1 mM pH 6.5 and several concentrations of the selected polyols. Most experimental points are not showed for the sake of clarity

Table 1 Denaturation temperatures of apo-LA in buffer PIPES 5 mM + EDTA 1 mM pH 6.50 and holo-LA in aqueous solutions in buffer PIPES 5 mM + $CaCl_2$ 1 mM pH 6.5 of buffer, erythritol, xylitol, sorbitol, and inositol

Cosolute molality	*Tm*/K apo-LA				Cosolute molality [22]	*Tm*/K holo-LA + Ca^{2+}			
	Erythritol	Xylitol	Sorbitol	Inositol		Erythritol	Xylitol	Sorbitol	Inositol
0.0000	305.7	305.7	305.7	305.7	0.0000	341.0	341.0	341.0	341.0
0.1900				306.7	0.1500				341.0
0.3800				307.2	0.3000	339.7	340.7	340.9	341.3
0.5000	306.5	306.1	305.4		0.6000	341.0	341.1	341.4	342.0
0.5600				307.8	0.7500				342.2
0.7500				310.1	0.9000	341.0	341.2	341.7	342.4
1.0000	306.8	307.6	307.6		1.2000	341.3	341.5	341.8	
1.5000	307.6	308.3	307.7		1.5000	341.8	341.8	342.7	
2.0000	309.1	309.1	310.0						

Table 2 Denaturation enthalpies of apo-LA in buffer PIPES 5 mM + EDTA 1 mM pH 6.50 and holo-LA in aqueous solutions in buffer PIPES 5 mM + $CaCl_2$ 1 mM pH 6.5 of buffer, erythritol, xylitol, sorbitol and inositol

ΔH/kJ mol^{-1}

Cosolute molality	Apo-LA				Cosolute molality [22]	Holo-LA + Ca^{2+}			
	Erythritol	Xylitol	Sorbitol	Inositol		Erythritol	Xylitol	Sorbitol	Inositol
0.0000	166	166	166	166	0.0000	291	291	291	291
0.1900				170	0.1500				295
0.3800				169	0.3000	300	304	291	298
0.5000	168	155	177		0.6000	317	283	303	295
0.5600				175	0.7500				298
0.7500				187	0.9000	288	302	307	305
1.0000	176	173	176		1.2000	312	306	298	
1.5000	211	183	166		1.5000	290	285	299	
2.0000	196	177	187						

Table 3 Denaturation entropies of apo-LA in buffer PIPES 5 mM + EDTA 1 mM pH 6.50 and holo-LA in aqueous solutions in buffer PIPES 5 mM + $CaCl_2$ 1 mM pH 6.5 of buffer, erythritol, xylitol, sorbitol, and inositol

ΔS/kJ mol^{-1} K^{-1}

Cosolute molality	Apo-LA				Cosolute molality	Holo-LA + Ca^{2+}			
	Erythritol	Xylitol	Sorbitol	Inositol		Erythritol	Xylitol	Sorbitol	Inositol
0.0000	0.54	0.54	0.54	0.54	0.0000	0.85	0.85	0.85	0.85
0.1900				0.55	0.1500				0.86
0.3800				0.55	0.3000	0.88	0.89	0.85	0.87
0.5000	0.55	0.51	0.58		0.6000	0.93	0.83	0.89	0.86
0.5600				0.57	0.7500				0.87
0.7500				0.60	0.9000	0.85	0.88	0.90	0.89
1.0000	0.57	0.56	0.57	0.54	1.2000	0.91	0.89	0.87	
1.5000	0.69	0.59	0.54	0.55	1.5000	0.85	0.83	0.87	
2.0000	0.63	0.57	0.60	0.55					

for apo-LA are in accordance with other previously reported using similar conditions. Table 2 gives the unfolding enthalpy of the process at the denaturation temperature, Table 3 presents the unfolding entropy of the process at the denaturation temperature, and Table 4 presents the denaturation free energy in the presence of erythritol, xylitol,

Table 4 Denaturation free energy of apo-LA in buffer PIPES 5 mM + EDTA 1 mM pH 6.50 and holo-LA in aqueous solutions in buffer PIPES 5 mM + $CaCl_2$ 1 mM pH 6.5 of buffer, erythritol, xylitol, sorbitol, and inositol

ΔG/kJ mol^{-1}									
Molality	Apo-LA				Molality	Holo-LA + Ca^{2+}			
	Erythritol	Xylitol	Sorbitol	Inositol		Erythritol	Xylitol	Sorbitol	Inositol
0.0000	3.67	3.67	3.67	3.67	0.0000	24.5	24.5	24.5	24.5
0.1900				4.20	0.1500				25.0
0.3800				4.42	0.3000	25.3	26.0	24.4	25.4
0.5000	4.06	3.59	3.81		0.6000	27.7	23.5	26.1	25.2
0.5600				4.83	0.7500				25.7
0.7500				6.18	0.9000	24.1	25.9	26.7	26.6
1.0000	4.43	4.69	4.76		1.2000	27.2	26.4	25.6	
1.5000	5.85	5.30	4.53		1.5000	24.5	23.7	25.8	
2.0000	6.11	5.43	6.15						

sorbitol, and inositol. They were obtained assuming a two-state process, and were determined in the temperature range where the process is reversible.

The enthalpy changes are very small and do not follow a clear trend with cosolute concentration. The typical standard deviation estimated is ± 18 kJ mol^{-1} for the apo form of the protein and ± 8 kJ mol^{-1} for the holo-protein, respectively. As can be seen, the differences between the values obtained are around the estimated relative standard deviation for the holo-LA, and a small increase in the denaturation enthalpy is observed for the apo-LA. In all cases the enthalpy changes are higher for holo-LA with calcium excess, indicating a stronger stabilizing effect of calcium than the studied poyols. A similar behavior is observed for the denaturation entropies. In this case the typical standard deviation estimated is ± 0.06 and ± 0.02 kJ mol^{-1} K^{-1} for the apo- and holo-LA, respectively. No conclusive results were obtained about if the stabilization exerted by polyols is predominantly enthalpic or entropic.

Figure 3 shows the effect of erythritol, xylitol, sorbitol, and inositol on the denaturation temperatures of aqueous solutions of apo- , holo- [5] and holo-LA in the presence of calcium. The uncertainty in temperature is not higher than 1.0 K. For the holo form of the protein, the effect of the polyols employed is small and at low concentration there is no significant effect. At the same concentrations, a higher stabilizing effect of the polyols is observed for the apo form. However, the presence of the calcium ion in the molecule has a much higher stabilizing effect than polyols. The stabilizing effect of the polyols is higher for the apo-LA than holo-LA with a calcium excess. This suggests that the stabilizing effect of the selected polyols on the LA depends on the initial stability of the protein dissolved in the buffer without the cosolvent. This stabilizing effect is

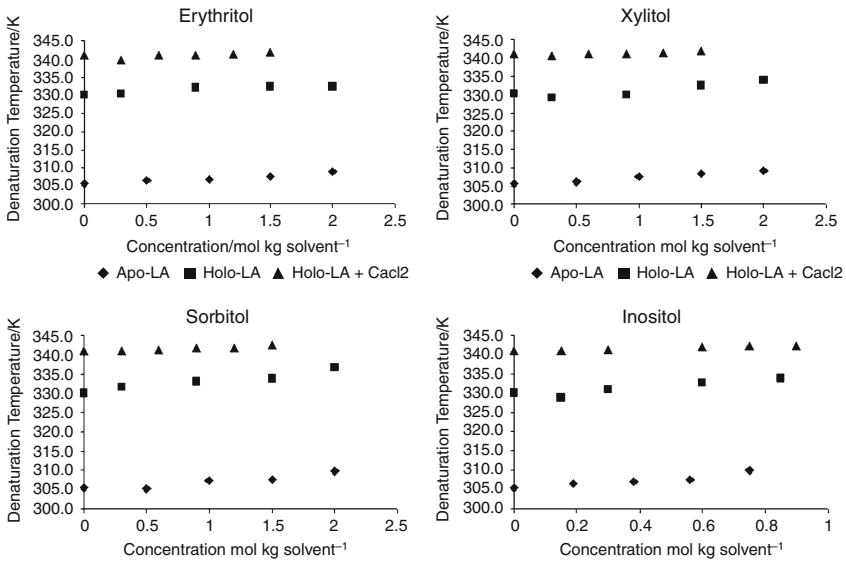

Fig. 3 Effect of erythritol, xylitol, sorbitol, and inositol on the denaturation temperatures of aqueous solutions of apo- and holo- and holo-LA in the presence of calcium

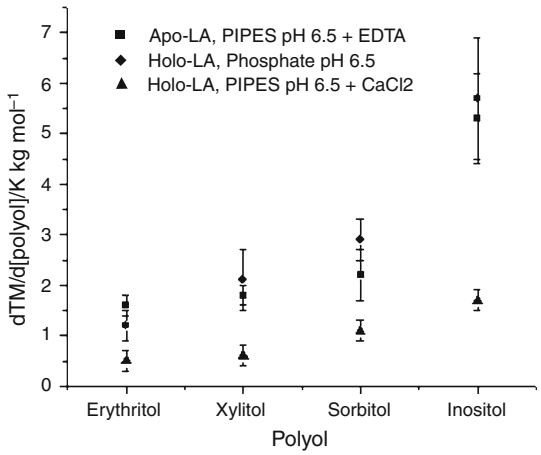

Fig. 4 Slopes of the dependence of *Tm* with polyol concentration for LA in the presence of three different calcium concentrations

function of the numbers of hydroxyl groups of the polyol: when the number of OH groups increases the stabilizing effect becomes larger, being the effect higher for the cyclic polyol inositol. The dependence of the stabilizing effect with the form of the protein and thus on its initial stability can be observed in Fig. 4, where the increase in denaturation temperature resulting from the addition of 1 mol of the selected solute is plotted for the apo- , holo- , and holo-LA in presence of an excess of calcium. The data of Fig. 3 were fitted to a straight line and by least squares the ordinate values in Fig. 4 were obtained. For all the selected polyols the effect exerted on apo- and holo-LA is similar, but it is significantly lower for the holo-protein in the presence of a calcium excess.

Table 4 shows the denaturation free energy of apo- and holo-LA with a calcium excess in the presence of erythritol, xylitol, sorbitol, and inositol. Typical standard deviation estimated is ± 2.0 kJ mol^{-1}, for apo- and holo-LA, respectively. Even though, the changes are small, results show an increase with cosolute concentration which is more significant for apo-LA. The results confirm that the stabilizing effect of the polyols is higher for apo-LA than for holo-LA and is very small for the protein in the presence of calcium. Again, a smaller stabilizing effect is exerted on the more stable form of the protein. The percent of stabilization of the protein by polyols, expressed as the relative change in the free Gibbs energy of denaturation at 298.15 K, is shown in Fig. 5. The stabilizing action of the polyols depends on the free Gibbs energy of the protein as was seen previously with the effect on the denaturation temperature: the most stable protein is less stabilized by the selected polyols. Haque et al. [19] found a similar behavior for lysozyme and ribonuclease A with several polyols at various pHs, being both proteins less stable at lower than at neutral pH. They interpreted their results in terms of the effect of the protonation of the carboxylic groups that occur at low pH and hence the degree of exclusion (repulsion) of polyol from the protein domain (the stabilizing effect) becomes larger with increasing protonation of COO^- groups. However, this interpretation doesn't apply in our case because our experiments were all made at the same pH. It appears that differences in the stabilizing effects are related with changes in the enthalpic/entropic behavior of these thermodynamic parameters for the different forms of the protein, as the same authors pointed out. These observations have been poorly discussed in literature from a theoretical point of view.

When compared with other proteins as lysozyme [11] it can be observed that the stabilizing effect exerted on LA by the selected polyols is lower than for other proteins [17, 18, 25–28]. This is interpreted in terms of the compact denatured state found for LA at the conditions employed in this work. Figure 6 shows the CD spectra in the far and near

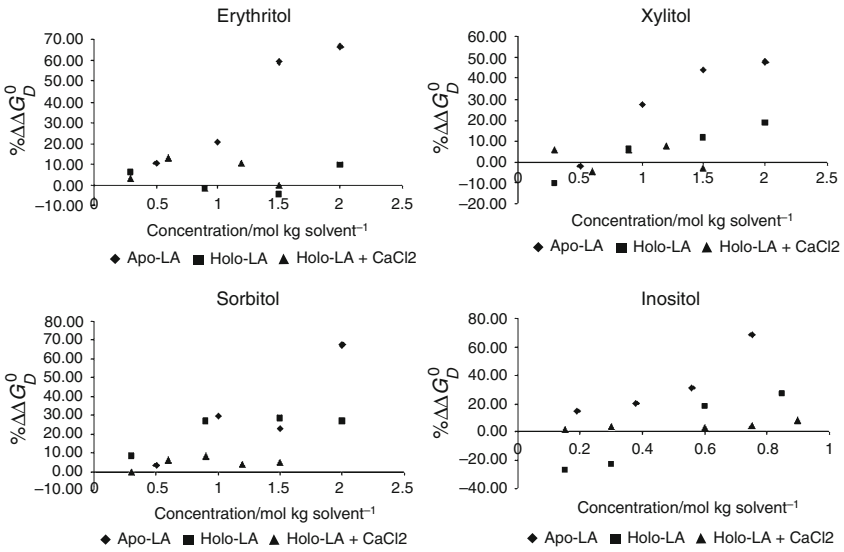

Fig. 5 Percent of stabilization of the different forms of bovine LA by the selected polyols

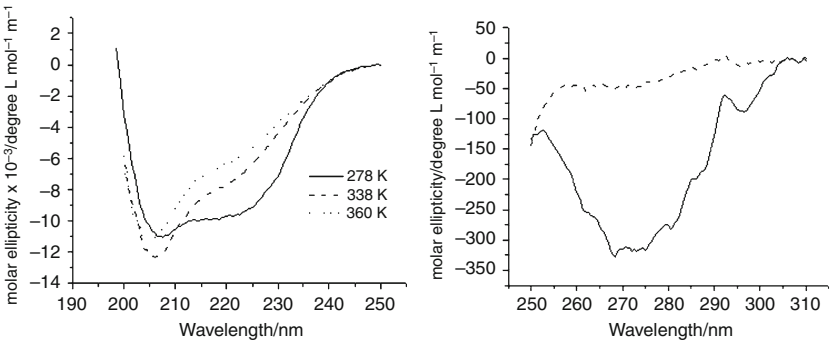

Fig. 6 Near and far UV–CD spectra of apo-LA at several temperatures

UV for the apo-LA at different temperatures. It can be seen a high content of secondary native-like structure at high temperatures where no tertiary structure is present. This suggests a partially unfolded state and a denaturation process which does not induces large changes in volume and exposed area when compared with other proteins. This could explain the lower stabilization exerted by the selected polyols in the LA case: core residues are less exposed in the LA denatured state than in other proteins, thus the change in area and volume in the denaturation process could be smaller for this protein than for others [16, 29, 30].

Conclusions

Results show that considered polyols stabilize bovine LA; however, their effect on the thermal denaturation parameters is lower than the effect observed with other proteins. The small extent of stabilization produced in this case by polyols, suggests that LA is not fully unfolded at high temperatures as has been shown in previous works [5, 12]. It is noteworthy the dependence of the stabilization effect exerted by the selected polyol on the stability of the protein in the absence of these cosolutes, an observation that hasn't been previously reported for a protein.

Acknowledgements This work was supported by Universidad Nacional de Colombia, COLCIENCIAS and by grant BFU2007-61476/BMC (Spain).

References

1. Permyakov EA, Berliner LJ. α-Lactalbumin: structure and function. FEBS Lett. 2000;473:269–74.
2. Chrysina ED, Brew K, Acharya KR. Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2-Å resolution reveal an effect of calcium on inter-lobe interactions. J Biol Chem. 2000; 257:37021–9.
3. Kronman MJ, Andreotti RE. Inter- and intramolecular interactions of α-lactalbumin. I. The apparent heterogeneity at acid pH. Biochemistry. 1964;3:1145–51.
4. Apenten RKO. A three-state heat-denaturation of bovine α-lactalbumin. Food Chem. 1995;52:131–3.
5. Zhong H, Gilmanshin R, Callender R. An FTIR study of the complex melting behavior of α-lactalbumin. J Phys Chem B. 1999;103:3947–53.
6. Griko YV, Freire E, Privalov PL. Energetic of the α-lactalbumin states: a calorimetric and statistical thermodynamic study. Biochemistry. 1994;33:1889–99.
7. Apenten RKO. Thermodynamic parameters for 3-state thermal denaturation of human and bovine α-lactalbumin. Thermochim Acta. 1995;262:1–12.
8. Veprintsev DB, Permyakov SE, Permyakov EA, Rogov VV, Cawthern KM, Berliner LJ. Cooperative thermal transitions of bovine an human apo-α-lactalbumins: evidence for a new intermediate state. FEBS Lett. 1997;412:625–8.
9. Hendrix TM, Griko Y, Privalov P. Energetic of structural domains in α-lactalbumin. Protein Sci. 1996;5:923–31.
10. Sekhar G, Prakash V. Interaction of selected cosolvents with bovine α-lactalbumin. Int J Biol Macromol. 2008;42:348.
11. O'Connor TF, Debenedetti PG, Carbeck JD. Stability of proteins in the presence of carbohydrates; experiments and modeling using scaled particle theory. Biophys Chem. 2007;127:51–63.
12. France RM, Grossman SH. Acrylamide quenching of apo- and holo-α-lactalbumin in guanidine hydrochloride. Biochem Biophys Res Commun. 2000;269:709–12.
13. Banerjee T, Kishore N. Insights into the energetics and mechanism underlying the interaction of tetraethylammonium bromide with proteins. J Chem Therm. 2007 (in press).
14. Cawthern KM, Narayan M, Chaudhuri D, Permyakov EA, Berliner LJ. Interactions of α-lactalbumin with fatty acids and spin label analogs. J Biol Chem. 1997;272:30812–6.
15. Rishi V, Anjum F, Ahmad F, Pfeil W. Role of non-compatible osmolytes in the stabilization of proteins during heat stress. Biochem J. 1998;329:137–43.
16. Davis-Searles PR, Saunders AJ, Erie DA, Winzor DJ, Pielak GJ. Interpreting the effects of small uncharged solutes on protein folding equilibria. Annu Rev Biophys Biomol Struct. 2001;30: 271.
17. Kaushik J, Bhat R. Thermal stability of proteins in aqueous polyol solutions: role of the surface tension of water in the stabilizing effect of polyols. J Phys Chem B. 1998;102:7058–66.
18. Xie G, Timasheff SN. The thermodynamic mechanism of protein stabilization by trehalose. Biophys Chem. 1997;64:25–43.
19. Haque I, Singh R, Moosavi-Movahedi AA, Ahmed F. Effect of polyol osmolytes on ΔG_D, the Gibbs energy of stabilisation of proteins at different pH values. Biophys Chem. 2005;117:1–12.
20. Wetlaufer DB. Osmometry and general characterization of α-lactalbumin. CR Trav Lab Carlsberg. 1961;32:125–38.
21. Cooper A. Thermodynamics of protein folding and stability. In: Allen G, editor. Protein: a comprehensive treatise, vol. 2. Stamford: JAI Press Inc.; 1999. p. 217–70.

22. Romero CM, Albis A, Lozano JM, Sancho J. Thermodynamic study of the influence of polyols and glucose on the thermal stability of holo-bovine α-lactalbumin. J Therm Anal Calorim. 2009;98:165–71.
23. Albis A, Lozano JM, Romero CM. Estabilización de la holo-α-lactoalbúmina en presencia de polioles. Rev Colomb Quím. 2009;38:209–19.
24. Hendrix T, Griko YV, Privalov PL. A calorimetric study of the influence of calcium on the stability of bovine α-lactalbumin. Biophys Chem. 2000;84:27–34.
25. Xie G, Timasheff SN. Mechanism of the stabilization of ribonuclease A by sorbitol: preferential hydration is greater for the denatured than for the native protein. Protein Sci. 1997;6:211–21.
26. Wimmer R, Olsson M, Neves Petersen MT, Hatti-Kaul R, Petersen SB, Müller N. Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. J Biotechnol. 1997;55:85–100.
27. Tiwari A, Bhat R. Stabilization of yeast hexokinase A by polyol osmolytes: Correlation with the physicochemical properties of aqueous solutions. Biophys Chem. 2006;124:90–9.
28. Santoro MM, Liu Y, Khan SMA, Hou L-X, Bolen DW. Increased thermal stability of proteins in the presence os naturally occurring osmolytes. Biochemistry. 1992;31:5278–83.
29. Parsegian VA, Rand RP, Rau DC. Osmotic stress, crowding, preferential hydration, and binding: a comparación of perspectives. PNAS. 2000;97:3987–92.
30. Timasheff SN. Thermodynamic binding and site occupancy in the light of the Schellman exchange concept. Biophys Chem. 2002;101–102:99–111.