

Binding of alkylsulfonate ligands to bovine β -lactoglobulin: Effects on protein thermal unfolding

Pablo Busti, Carlos A. Gatti, Néstor J. Delorenzi *

Area Fisicoquímica, Departamento de Química – Física, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LKR Rosario, Argentina

Abstract

The effects caused by alkylsulfonate ligands (AL_n), characterized by a similar sulfonate head and a progressively longer hydrocarbon tail, on the thermal unfolding of beta-lactoglobulin (β -LG) were examined by using UV-difference spectroscopy at 293 nm (ΔAbs_{293}) and by following the external quenching of the intrinsic protein fluorescence by acrylamide. At pH 6.8, the interaction with the different AL_n added promoted a stabilization of the protein structure in the following sense: $AL_8 < AL_{10} < AL_{12} \approx AL_{16} < AL_{14}$. At pH 2.5 AL_{12} and AL_{14} protected β -LG against thermal denaturation, since ΔAbs_{293} values did not appreciably vary in the temperature range 25–98 °C. In the presence of AL_{16} , ΔAbs_{293} values began to increase near 80 °C, evidencing an aggregation process possibly promoted by non-covalent interactions.

Our results suggest that the protective action toward thermal unfolding is exerted by a fraction of AL_n bound to β -LG monomer, not related to the dimer existence. Moreover, we conclude that AL_n stabilize mainly the monomer structure possibly strengthening the hydrophobic interactions and excluding water from the monomer binding sites by the hydrophobic moiety of the AL_n .

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Keywords: Beta-lactoglobulin thermal unfolding; Hydrophobic binding sites; UV spectroscopy; Fluorescence quenching; Two-state model; Dissociation coupled unfolding model (DCU)

1. Introduction

Beta-lactoglobulin (β -LG), the major protein in the whey of ruminant milk, is a globular protein with a monomer molecular weight of about 18,300. This small globular protein has a three-dimensional structure consisting of eight strands of antiparallel β -sheet twisted into a cone-shaped barrel that constitutes a hydrophobic pocket (Sawyer & Kontopidis, 2000).

β -LG is known to bind a great variety of hydrophobic ligands, such as fatty acids, retinoids and sodium dodecyl sulfate (SDS) (Pérez & Calvo, 1995). As reviewed by Sawyer et al. (2002), it has been determined from crystallographic studies (X-ray diffraction) that the major-

ity of these hydrophobic ligands binds within the central cavity of β -LG in the pH range 6.0–8.1. Moreover, the nuclear magnetic resonance (NMR) spectra of β -LG solutions obtained by Ragona et al. (2000), revealed that at neutral pH palmitate (PA) is bound within the central cavity of the protein, while at pH lower than 6.0 PA starts to be released. The release of PA is nearly complete at pH 2.1.

The interaction between β -LG and PA and SDS enhances the tryptophan fluorescence of β -LG (Frapin, Dufour, & Haertlé, 1993; Pérez et al., 1989). In a previous work (Busti, Scarpeci, Gatti, & Delorenzi, 1999), we applied this technique to the study of the interaction of β -LG with alkylsulfonate ligands (AL_n) characterized by a similar sulfonate head and a progressively longer hydrocarbon tail. The aim of the cited work was to elucidate the interactive properties of β -LG in order to generate ideas for applications of this protein, produced in large amounts by the dairy industry, as a carrier of a

* Corresponding author. Tel./fax: +54 341 480 4598.

E-mail address: ndeloren@fbioyf.unr.edu.ar (N.J. Delorenzi).

wide range of hydrophobic molecules. The apparent molar ratios of AL_n/β -LG at saturation ranged from 0.70 to 1.70, suggesting the presence of one binding site on the β -LG monomer. AL_{16} showed the highest affinity for β -LG among the studied ligands (AL_{10} , AL_{12} , AL_{14} and AL_{16}). At $pH < 3.0$, no binding was detected by fluorescence measurements for any of those AL_n . These results are in accordance with the results obtained by Frapin et al. (1993), which studied the interaction of β -LG with fatty acids varying in aliphatic chain lengths. At neutral pH, bovine β -LG is mainly dimeric, whereas the monomer is the predominant state at $pH < 3.0$. Since porcine β -LG is monomeric at neutral pH and does not bind PA, Frapin et al. (1993) suggested the possibility that the fatty acid binding site of bovine β -LG is close to the region involved in dimer formation and not inside the main hydrophobic pocket. Our results also suggested that the AL_n binding site is formed when β -LG monomers associates into dimers and that it is close to the junction region (Busti et al., 1999, Busti, Scarpeci, Gatti, & Delorenzi, 2005).

Some authors reported that the binding of PA and SDS increases the stability of the protein toward urea unfolding (Creamer, 1995; Narayan & Berliner, 1997). AL_n also showed a protective effect against chemical denaturation (Busti et al., 1999; Giroux & Britten, 2004). We suggested that the protective action toward urea unfolding is exerted by a fraction of AL_n bound to β -LG monomer, not related to the dimer existence and not detected by fluorescence measurements.

In a recent work (Giroux & Britten, 2004), it has been demonstrated that the presence of anionic surfactants such as SDS, increase the denaturation temperature of whey proteins. Kelley and McClements (2003), adding SDS prior to heating, improved the thermal stability of oil-in-water emulsions stabilized by globular proteins. Between other possibilities, the authors have supposed that the anionic surfactants could improve the thermal stability of emulsions by increasing the unfolding temperature of the adsorbed globular proteins. In that sense, in the present work we examine how the interaction of AL_n with β -LG promotes a protective action toward thermal denaturation of the protein. The results obtained in this work may have important implications for understanding the influence of hydrophobic ligands on globular protein functionality.

2. Materials and methods

2.1. Materials

Bovine milk β -LG AB, a mixture of A and B variants, was obtained from Sigma (St. Louis, MO) as a three-times crystallized and lyophilized powder that was used without further purification. Polyacrylamide gel electrophoresis was carried out on β -LG AB samples as described by

Laemmli (1970). The β -LG AB showed two major bands and no other significant bands.

The different AL_n used in this work were also purchased from Sigma (St. Louis, MO) and used without further purification. The AL_n used were the sodium salts ($CH_3(CH_2)_{n-1}SO_3Na$) of 1-octanesulfonic acid (AL_8), 1-decanesulfonic acid (AL_{10}), 1-dodecanesulfonic acid (AL_{12}), 1-tetradecanesulfonic acid (AL_{14}), and 1-hexadecanesulfonic acid (AL_{16}). All other chemicals were of analytical grade.

2.2. Thermal unfolding curves of β -LG

Thermal unfolding of β -LG was monitored by UV spectroscopy. The UV-difference absorbance at 293 nm (ΔAbs_{293}) of each solution was measured using a Jasco V-550 double-beam spectrophotometer equipped with a thermostated cell (Peltier effect) controlled by a programmable unity. The sample cuvette was filled with the protein solution whereas the reference cuvette was filled with buffer that was maintained at 25 °C. The experiments were performed in 50 mM phosphate buffer (pH 6.8) or in 50 mM citrate buffer (pH 2.5). A 2.5 mL aliquot of the protein (0.2%) was placed into the sample cuvette which was sealed with a teflon stopper to avoid evaporation during each experiment. ΔAbs_{293} was recorded as a function of temperature over a range of 25–98 °C. The heating rate in the experiments was 0.5 °C min⁻¹. The spectrophotometer compartment was continuously purged with nitrogen to avoid the condensation of water vapor on the sample cuvette.

To study the chemical unfolding of β -LG in the presence of AL_n , the different ligands used were immediately added after protein addition to give final AL_n : β -LG molar ratios of 3, that is to say at protein saturation [6]. In other experiments, AL_{14} was injected to give final AL_{14} : β -LG molar ratio of 3 when the temperature reached by the protein sample was 55 °C.

The final ligand concentrations used in the present work were always lower than the critical micelle concentration (CMC) of each ligand alone.

2.3. Quenching of the intrinsic fluorescence of β -LG by acrylamide

The thermal unfolding curves of β -LG, the mixture of β -LG and AL_{14} and β -LG with the addition of AL_{14} at 55 °C were stopped at the minimum value of ΔAbs_{293} of the corresponding denaturation profile. Besides this, a thermal unfolding curve of β -LG was stopped at 55 °C. All these samples were cooled immediately (up to 25 °C) and an aliquot of them was taken and diluted to make 20 μ M β -LG solutions in 50 mM phosphate buffer (pH 6.8). A 3 mL of each dilution was placed in the 10 mm square quartz cell of a Jasco FP-770 spectrofluorimeter. The emission spectrum (excitation at 295 nm) of each solution was run about 10 min after protein addition, and then it was scanned

across the peak and $\lambda_{\max}$ determined. At $\lambda_{\max}$, aliquots of 5 M acrylamide in 50 mM phosphate buffer (pH 6.8) were mixed into the cell content, and the fluorescence intensity was re-determined (F). Final acrylamide concentrations ranged from 0 to 0.55 M. Stern–Volmer plots, F_0/F (F_0 and F are the fluorescence in the absence and presence of quencher) versus acrylamide concentration, were then represented.

The thermal unfolding curves of β -LG in the presence of AL_{14} were carried out at AL_{14} : β -LG saturation ratio of 3.

The fluorescence quenching of β -LG prepared in buffered 8 M urea was also obtained.

All experiments were repeated three times. Statistical analysis were performed using Sigma-Plot (Version 4.0 for Windows 95).

3. Results and discussion

Ultraviolet spectrophotometry is a fast, accurate, quantitative and nondestructive technique used in studies of protein structure and function when more specialized instrumentation is not readily available. A number of studies have described in detail the experimental considerations for β -LG thermal unfolding followed by this optical method (Apenten, Khokhar, & Galani, 2002; Galani & Apenten, 2000; Kella & Kinsella, 1988; Poklar, Vesnaver, & Lapanje, 1993).

An essential component of any thermodynamic study is the denaturation model which must indicate clearly the reference (native and denatured) states. For a small globular protein, thermal denaturation is usually described by a two-state process:



where N and U refer to the native and unfolded states (random coil) of the protein. Only the N-and U-states will be significantly populated at equilibrium. This point of view was adopted by Kella and Kinsella (1988) and Poklar et al. (1993).

At room temperature and at physiological pH β -LG exists mainly as a dimer, but it dissociates into monomers at elevated temperatures (Wong, Camirand, & Pavlath, 1996). A dissociation coupled unfolding (DCU) model is more suitable for proteins with multiple subunits (D_n) (Neet & Timms, 1994). Studies using high sensitive differential scanning calorimetry (DSC) (Qi, Brownlow, Holt, & Sellers, 1995) and static and dynamic light scattering (Aymard, Durand, & Nicolai, 1996) point to the dissociation as the first stage in the thermal denaturation of β -LG, being dissociation of dimers and unfolding of monomers separate events of the denaturation process. Further evidence for a three-state reaction was also provided by measurements using small angle X-ray diffraction and Fourier transform infra red (FTIR) spectroscopy (Panick, Malessa, & Winter, 1999). In view of these results, Galani and Apenten (2000) and Apenten et al. (2002)

considered that β -LG dimer denature via a three-state DCU process involving the dissociation into monomers (N) which then unfold in accordance with Eq. (2):



Recently, we have proposed that at concentrations below 4 mg mL⁻¹, at low ionic strength (<0.1 M) and near neutral pH, the two-state model and the dissociation coupled unfolding (DCU) model fail to describe correctly the β -LG thermal unfolding profiles (Busti, Gatti, & Delorenzi, 2005). The probable causes of this failure are:

- (1) below 55 °C, β -LG solutions are a mixture of monomers and dimers (Apenten & Galani, 2000; Aymard et al., 1996; Galani & Apenten, 1999; Qi et al., 1995),
- (2) between 55 and 90 °C, β -LG heating induces irreversible changes in the monomer structure: at least one molten globule-like state and possibly aggregates of low molecular weight were produced (Apenten et al., 2002; Busti et al., 2005; Croguennec et al., 2004; Fessas et al., 2001; Manderson et al., 1988; Palazolo et al., 2000; Relkin, 1996),
- (3) above 90 °C, β -LG aggregates of high molecular weight are formed irreversibly.

For these reasons, it should not be forgotten that the two models can give only apparent (non-thermodynamic) quantities of conformational transitions (Busti et al., 2005; Poklar et al., 1993).

Figs. 1 and 2 show changes in ΔAbs_{293} of β -LG as a function of temperature in absence and presence of AL_n at pH 6.8 and at pH 2.5, respectively. The dissociation of

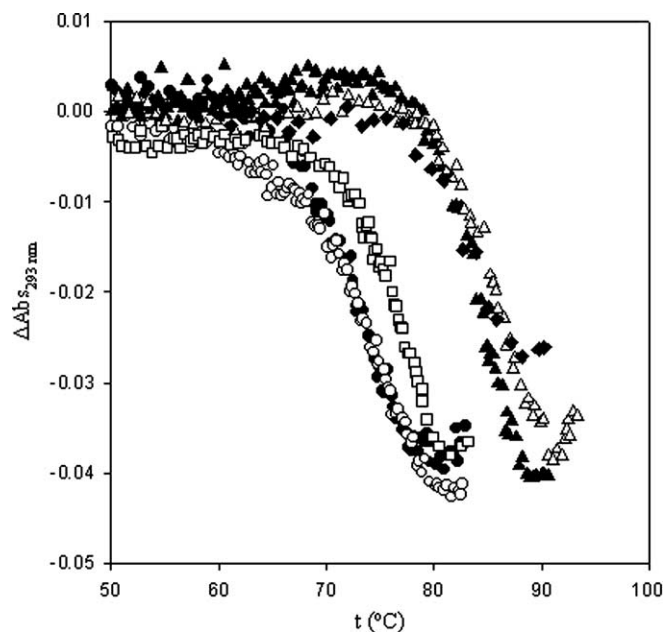


Fig. 1. Thermal denaturation curves of β -LG at pH 6.8, in absence (●) and in presence of alkyl sulfonate ligands: (○) AL_8 , (□) AL_{10} , (▲) AL_{12} , (△) AL_{14} and (◆) AL_{16} .

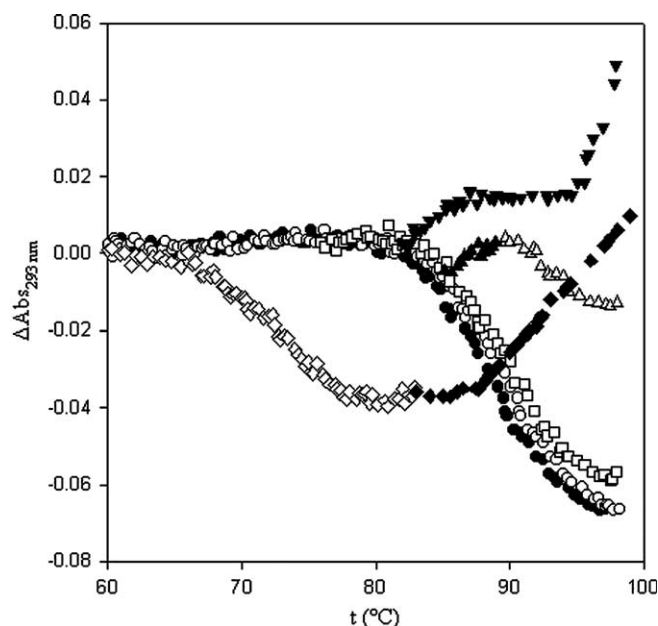


Fig. 2. Thermal denaturation curves of β -LG at pH 2.5, in absence (●) and in presence of alkyl sulfonate ligands: (○) AL₈, (□) AL₁₀, (▲) AL₁₂, (△) AL₁₄ and (▼) AL₁₆. For comparison, this figure also shows the thermal denaturation curve of β -LG at pH 6.8, in absence (◇) of alkyl sulfonate ligands. The last part of the curve (◆) was eliminated in Fig. 1 (see text for details).

β -LG dimer did not produce changes in UV-difference spectra at 293 nm. This is because the degree of solvent exposure of tryptophan residues is the same for β -LG dimer and monomer (Townend, Herskovits, Timasheff, & Gorbunoff, 1969). By contrast, unfolding of β -LG monomer produced S-shaped denaturation profiles at temperatures above 55–60 °C at pH 6.8 and at temperatures above 80 °C at pH 2.5. In these cases, the apparent temperature for 50% protein denaturation ($T_{m(\text{app})}$) calculated in accordance with the two-state model are the same to $T_{m(\text{app})}$ values for DCU model (Galani & Apenten, 2000). Therefore, $T_{m(\text{app})}$ was calculated using the van't Hoff equation as two-state model requires (Galani & Apenten, 2000). Tables 1a and 1b show the values of $T_{m(\text{app})}$ for each

Table 1a

$T_{m(\text{app})}$ ^a (mean $\pm$ standard error) for β -LG for different AL_n added, at pH 6.8

n^b	$T_{m(\text{app})}$
–	72.6 $\pm$ 0.5
8	73.4 $\pm$ 0.6
10	76.0 $\pm$ 0.6
12	83.5 $\pm$ 0.6
14	85.5 $\pm$ 0.7
14 ^c	84.8 $\pm$ 0.8
16	82.9 $\pm$ 0.8

^a $T_{m(\text{app})}$, the apparent temperature for 50% protein denaturation, calculated by supposing a two-state model. Each one of the unfolding curves assayed was repeated three times.

^b n , number of C atoms in the hydrophobic moiety of AL_n.

^c AL₁₄ was added when the temperature of the sample reached 55 °C.

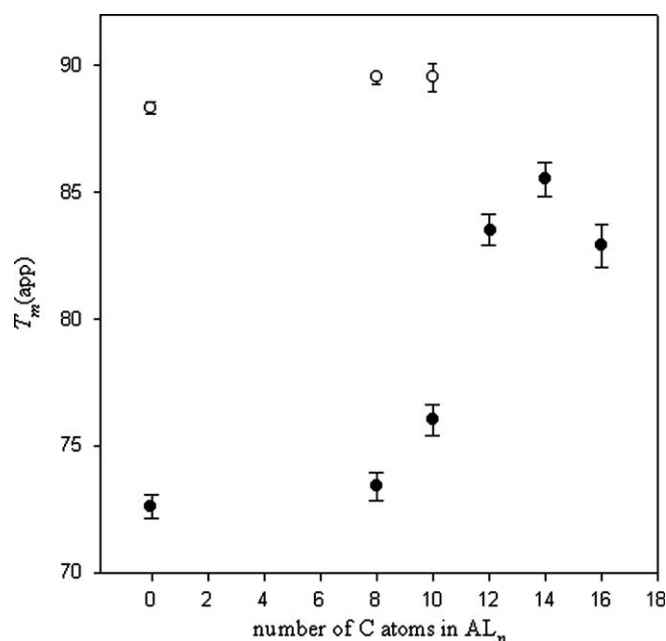


Fig. 3. The apparent temperature for 50% protein denaturation, $T_{m(\text{app})}$ in function of the number of C atoms in AL_n, (●) at pH 6.8 and (○) at pH 2.5. The bars show the standard error.

one of the S-shaped denaturation curves obtained at pH 6.8 and at pH 2.5, respectively.

The interaction with the different AL_n added promoted a stabilization of the protein structure, at pH 6.8 (Fig. 1 and Table 1a). Fig. 3 shows the change in $T_{m(\text{app})}$ versus the number of carbons in the ligand. The efficiency of AL_n in stabilizing the structure of β -LG near neutral pH was related to the length of the hydrocarbon tail between $n = 8$ and $n = 14$. In this range, significant differences between $T_{m(\text{app})}$ values were found. The higher the number of C atoms in AL_n structure, the higher was the protective action toward thermal denaturation. AL₁₄ produced the major increase in the $T_{m(\text{app})}$ of β -LG near neutral pH. The behavior observed suggests that hydrophobic interactions play a major role in the protective action toward β -LG thermal unfolding.

The difference in β -LG structural stability promoted by AL_n may be due to a stabilization of tertiary (3°) or quaternary (4°) structure or an action over both levels of protein conformation. Fig. 4 shows that the addition of AL₁₄, the ligand which the major protective action, produced the same effect on β -LG denaturation either if the ligand was injected at 25 °C or at 55 °C, at pH 6.8. Since the presence of dimers are insignificant at 55 °C, we supposed that the different AL_n added affects principally the stability of β -LG monomers (3°-structure) and not the protein dimerization (4°-structure). Therefore, our results suggest that the protective action of AL_n was promoted by a fraction of AL_n bound to the monomer, not related to the dimer existence. This fraction is not detectable by the fluorescence method (Busti et al., 1999), but evidenced by other methods. For

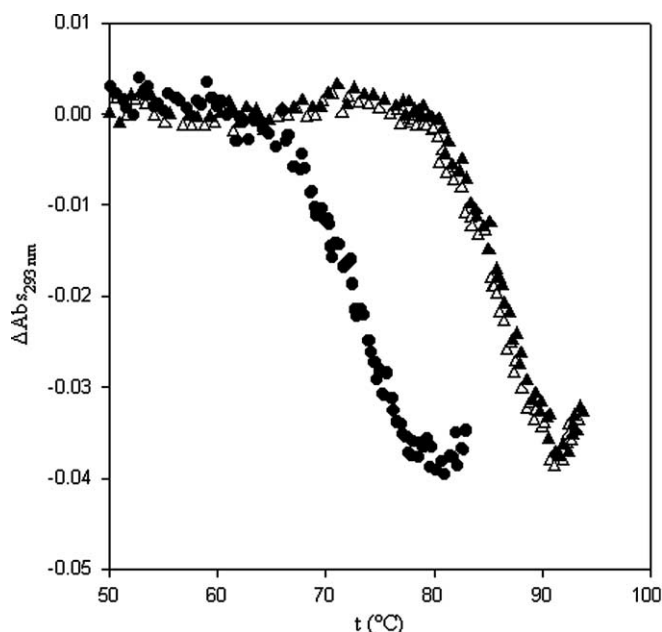


Fig. 4. Thermal denaturation curves of β -LG at pH 6.8, in absence (●) and in presence of alkyl sulfonate ligands: (△) AL₁₄ aggregated at 25 °C and (▲) AL₁₄ aggregated at 55 °C.

example, Wang, Allen, and Swaisgood (1998), using an ultrafiltration method, determined that 2 mol of PA are bound per mol of dimer with a binding constant of 10^5 M^{-1} and a larger number of PA molecules (>20) are bound per mol of monomer with a smaller binding constant of the order of 10^4 M^{-1} (weak sites), at pH 7.0. It may be concluded that the stabilization of β -LG could be the result from ligand binding to these weak sites and not to the dimerization sites.

Near neutral pH, ΔAbs_{293} measurements abruptly increased when β -LG solutions were heated above 80 °C (Fig. 2), evidencing the formation of aggregates of different species of β -LG (Hambling, McAlphine, & Sawyer, 1992; Mulvihill & Donovan, 1987). This fact also occurred in the presence of the different AL_{*n*} assayed. As it can be seen in Fig. 1, this last part of thermal denaturation curves was eliminated to calculate $T_{m(\text{app})}$ when the two-state model was applied. β -LG does not unfold to a random coil before aggregating in the final stage of the thermal denaturation (Cassal, Kohler, & Mantsch, 1988; Croguennec et al., 2004). Notwithstanding this, the buried SH group of Cys121 is exposed with the partial unfolding of β -LG, initiating sulfhydryl/disulfide (SH/S–S) interchange reactions that lead to irreversible aggregation/polymerization (Hoffmann & van Mil, 1997). However, there is evidence that in this aggregation reaction non-covalent interactions must also be involved (Manderson et al., 1988). The extent of their relative contribution to the overall aggregation process, however, is still unclear.

β -LG is more stable at acidic pH than neutral pH (Fig. 2, Table 1b and Fig. 3). $T_{m(\text{app})}$ increased approxi-

Table 1b

$T_{m(\text{app})}$ ^a (mean $\pm$ standard error) for β -LG for different AL_{*n*} added, at pH 2.5

<i>n</i> ^b	$T_{m(\text{app})}$
–	88.3 ± 0.2
8	89.5 ± 0.2
10	89.5 ± 0.6

^a $T_{m(\text{app})}$, the apparent temperature for 50% protein denaturation, calculated by supposing a two-state model. Each one of the unfolding curves assayed were repeated three times.

^b *n*, number of C atoms in the hydrophobic moiety of AL_{*n*}.

mately 16 °C from pH 6.8 to pH 2.5. At acidic pH the free thiol group (Cys 121) is not reactive but hydrophobic residues of the folded state, which were initially buried, come into contact with aqueous medium and therefore the extra hydrogen bonding of the structural water (which was initially buried around apolar groups) may be disrupted. This phenomenon is thought to be responsible for the increase in heat stability at acidic pH (Relkin, 1996).

At pH 2.5, the thermal denaturation curve of β -LG in the absence of AL_{*n*} did not show any aggregation process at temperatures up to 98 °C (Fig. 2). At acid pH, certain degree of reversibility of heat-induced conformational changes was observed, behavior explained by the non-reactivity of free SH groups in this pH range (Relkin, 1996).

The presence of AL₈ and AL₁₀ produced slight effects on thermal denaturation of β -LG, at pH 2.5 (Fig. 3). In contrary, the presence of AL₁₂, AL₁₄ and AL₁₆ modified the unfolding pattern of the protein. AL₁₂ and AL₁₄ protected β -LG against thermal denaturation, since ΔAbs_{293} values did not appreciably varied in the temperature range 25–98 °C. In the presence of AL₁₆, ΔAbs_{293} values began to increased near 80 °C, evidencing an aggregation process possibly promoted by non-covalent interactions (Manderson et al., 1988).

At acid pH the presence of dimer is clearly lower than near neutral pH (Apenten et al., 2002; Galani & Apenten, 2000). Therefore, the particular behavior of β -LG in presence of AL_{*n*} may be also attributed to an action of the ligands over the monomer structure, possibly strengthening the hydrophobic interactions and excluding water from the monomer binding sites by the hydrophobic moiety of the AL_{*n*}.

On the other hand, fluorescence is a useful technique to study the structure and dynamics of protein molecules in solution. The intrinsic fluorescence of Trp residues is particularly sensitive to their microenvironments and provides an appropriate method to perform this kind of study. The degree of exposure of Trp residues in the β -LG molecule (Trp-19 and Trp-61) can be evaluated by following the external quenching of the intrinsic protein fluorescence by added acrylamide (Busti et al., 2005; Palazolo et al., 2000). Protein unfolding cause a red shift on fluorescence emission spectrum due to the major exposition of Trp residues to the aqueous solvent. This

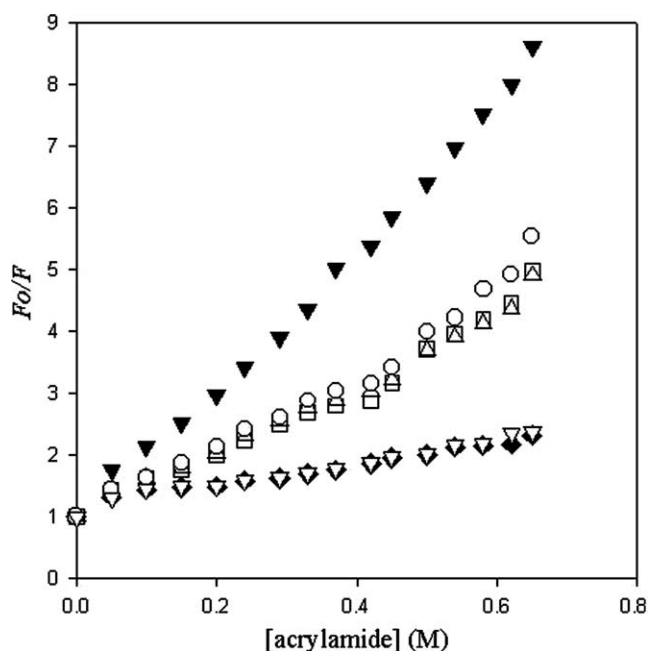


Fig. 5. Stern-Volmer plots of β -LG fluorescence quenching by acrylamide, at pH 6.8. F_o and F are the fluorescence in the absence and presence of acrylamide, respectively. (◆) β -LG without heat treatment; (▽) β -LG heated up to 55 °C; (□) heating of β -LG stopped at 80 °C; (△) heating of a mixture of β -LG with AL_{14} , stopped at 90 °C; (○) heating of β -LG with AL_{14} added at 55 °C, stopped at 90 °C; (▼) β -LG in 8 M urea without heating. See Section 2 for details.

major exposition of Trp residues to the aqueous solvent promotes an increase in the fluorescence quenching of denatured proteins by acrylamide. More flexible is the protein structure and/or the higher is the concentration of unfolded species, the more pronounced is the Stern–Volmer plot (Busti, Scarpeci, Gatti, & Delorenzi, 2002; Moro, Gatti, & Delorenzi, 2001). Based on these reasons, in a recent work we have demonstrated the presence of irreversible changes in β -LG after heat treatment below 90 °C (Busti et al., 2005).

Fig. 5 shows the Stern–Volmer plots for the different β -LG solutions carried out at pH 6.8. This figure shows that only the heating above 55 °C, temperature at which the presence of monomers is null, produced irreversible modifications on β -LG structure. This fact reinforces the idea that AL_n acts over the monomer structure, not affecting the dimer existence. For all the conditions assayed at pH 6.8, Fig. 5 shows a similar degree of irreversibility for each one of thermal unfolding curve stopped at the temperature corresponding to the minimum value of ΔAbs_{293} . However, the temperature of the minimum was increased approximately in 10 °C by the presence of AL_{14} , evidencing the protective action of alkylsulfonate ligands toward β -LG thermal denaturation.

In conclusion, the data obtained in this study contribute to understanding the influence of protein–ligand interactions on the functionality of globular proteins β -LG in foods.

Acknowledgements

This investigation was partially supported by the Universidad Nacional de Rosario (U.N.R.), the Agencia Nacional de Promoción Científica y Tecnológica (Argentina), and by the PIP 0362/98 of CONICET (Argentina).

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