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Calorimetric and Spectroscopic investigations of β-lactoglobulin upon interaction with copper ion

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The effect of copper(II) ions (Cu^{+2}) on the structure of β-lactoglobulin (β-lg) was investigated spectroscopically using UV-visible, fluorescence and circular dichroism (CD) and calorimetrically using isothermal titration calorimetry (ITC), at different temperatures. Results of the UV-visible studies showed that adding Cu^{+2} to β-lg solution caused increasing turbidity, indicative of protein aggregation. It was noticeable that the rate of increasing turbidity was directly proportional to increasing temperature. The far-UV CD studies displayed that the Cu^{+2} cannot induce any significant changes in the secondary structures of β-lg at different temperatures. Also, the ITC data indicated that the binding process of Cu^{+2} to β-lg is mainly entropically driven. The results highlight that copper ions cause the tertiary structure of β-lg to change and induce a slightly open structure leading to the formation of supramolecular aggregates in β-lg which may result in the reduced allergenicity of β-lg and its increased use in industrial applications.

Keywords: β-Lactoglobulin, copper ion, fluorescence quenching, titration calorimetry, allergenicity, turbidity.

Abbreviations: β-lg, β-lactoglobulin; Cu^{+2} ion, copper ion; CD, circular dichroism; ΔH°, enthalpy; ΔS°, entropy; ΔG°, Gibbs free energy.

β-Lactoglobulin (β-lg), the major protein in bovine whey, is responsible for most of the bioactive properties of whey proteins. β-Lg belongs to the lipocalin protein family; the members are the most important group of mammalian allergens. The molecular mimicry between endogenous lipocalins and exogenous lipocalin allergens has been suggested as an explanation for the allergenicity of lipocalins (Suutari et al. 2006). Then, β-lg is considered a major milk allergen (Besler et al. 2002). Allergy to bovine milk protein occurs most often in children under 2 years of age (Chatchatee et al. 2001). Approximately 3% of infants and young children suffer from cow's milk allergy during their first years of life (Host et al. 1988). This allergy is characterised by a strong IgE response to milk proteins and clinical symptoms in skin and the gastrointestinal tract, such as atopic eczema, vomiting and diarrhoea (Vaarala et al. 1995). Bovine β-lg can induce allergies in infants because of the underdevelopment of their gastrointestinal tract and immune system, and it is significant that β-lg is absent in human milk (Pescuma et al. 2011). Therefore, with the purpose of increasing the degree of applicability of β-lg in the food industry, the reduction of its allergenicity has been tested over recent years by use of different methods such as chemical modification of the protein, proteolysis and physical treatments. Also, several reports show that glycation of food proteins could affect their allergenic activity at a structural level. Some reports have suggested that the interaction of β-lg with sugars could mask the allergen epitopes, reducing its allergenicity. In addition, Corzo-Martinez et al. (2010) show that a high degree of glycation of β-lg causes noticeable reduction in its IgG binding and formation of protein aggregates, which could mask the antigenic regions of β-lg.

Copper is an essential trace element for all biological organisms, from bacteria to humans. There are eight negative charges on the surface of β-lg in neutral pH conditions (Simons et al. 2002), which may be sites for binding positively charged metal ions. There is not any precise report on the binding of Cu^{+2} ion to β-lg. Here, we present the results of a systematic thermodynamic analysis of the effects of Cu^{+2} ion on the structural and conformational properties of bovine

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β-lg, as a carrier protein, in aqueous solution at two different temperatures (room temperature of 25 °C and physiological temperature of 37 °C) with special attention given to the nature of the interactions upon complex formation between β-lg and Cu^{+2} ion in order to obtain new insights into the formation of aggregates which might cause reduction in allergenicity of β-lg (Corzo-Martinez et al. 2010). Since the binding of Cu^{+2} ion on β-lg was expected to influence β-lg structure and conformation, and consequently its functional properties. Therefore, molecular and supramolecular characteristics of β-lg upon interaction with Cu^{+2} were evaluated.

Materials and Methods

Bovine β-lg (AB form, with 98% purity) was purchased from Sigma. ANS (1-anilino-8-naphthalene sulphonate) and copper nitrate were purchased from Merck. All other materials and reagents were of analytical grade. All solutions were made in double-distilled water. A 50 mM NaCl solution adjusted at pH 7 was prepared and used as the solvent for β-lg, ANS and cooper nitrate. The concentrations of β-lg was determined spectrophotometrically using a value of 17 600/M/cm for the molecular absorption coefficient at 278 nm (ε_{278}) (Dufour et al. 1992).

UV-visible measurements

The UV absorption spectra of β-lg solution at 278 nm were studied upon addition of different concentrations of copper ion. The UV–Visible absorption spectra were measured with a Cary spectrophotometer (Bio-model 100), with jacketed cell holders. Spectral changes of 13 μM β-lg were monitored after adding different concentrations of copper ion (0–93 μM) by recording the UV–Visible absorption (200–700 nm). All experiments were run in NaCl solution (50 mM) in a conventional quartz cell, thermostated to maintain the temperature at 25 or 37 °C.

Fluorescence measurements

Fluorescence intensity measurements were carried out using a Cary spectrofluorimeter at an excitation wavelength of 290 nm, and the emission spectra of all samples were recorded in the range of 300–470 nm at 25 and 37 °C. All measurements were made using a 1-cm path length fluorescence cuvette. The tryptophan fluorescence spectra of the β-lg (5 μM) was also measured in the absence and presence of different concentrations of copper ion solution from 0 up to 146·3 μM.

The binding of a hydrophobic fluorescent probe, ANS, to β-lg was monitored by exciting the ANS (135 μM) at 350 nm and recording the emission spectra in the range of 400–600 nm. All measurements were made using a 1-cm path length fluorescence cuvette. The extrinsic fluorescence spectra of the β-lg (5 μM) were measured in the absence and presence of 25, 50 and 100 μM of the Cu^{+2} ion solution at different temperatures of 25 and 37 °C.

Circular dichroism (CD) measurements

CD spectra were recorded using an Aviv spectropolarimeter (model 215). Changes in the secondary structure of β-lg at 25 and 37 °C in the absence and presence of different concentrations of Cu^{+2} ion solution (125, 250 and 375 μM) was monitored in the far-UV regions (200–260 nm) using 1-cm path length cells, at protein concentrations of 13·5 μM. The results were expressed in mean residue ellipticity [θ] (deg cm^2/dmol) based on a mean residue weight of 114 (MRW). The mean residue ellipticity at a wavelength, λ, was obtained using the relation, $[\theta]_\lambda = (100 \times MRW\theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength λ, c is the protein concentration in mg/ml and l is the path length in cm. The CD software was used to predict the secondary structure of the protein according to the statistical method (Marthasarathy & Johnson, 1987).

Isothermal titration calorimetric (ITC) measurements

The isothermal titration calorimetric experiments were carried out on a VP-ITC ultra sensitive titration calorimeter (MicroCal, LLC, Northampton, MA). The microcalorimeter consists of a reference cell and a sample cell of 1·8 ml in volume, with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with β-lg solution (47 μM) and the reference cell contained NaCl solution. The solution in the cell was stirred at 307 rpm by the syringe (equipped with micro propeller) filled with Cu^{+2} solution (1·5 mM) to ensure rapid mixing. The titration of protein with Cu^{+2} solution involved 30 consecutive injections of the copper solution, the first being 5 μl, and the remainder being 10 μl each. In all cases, each injection was done in 6 s at 3-min intervals. To correct the thermal effects due to Cu^{+2} dilution, control experiments were done in which identical aliquots were injected into the NaCl solution in the absence of β-lg. The measurements were performed at constant temperatures of 25·0 and 37·0 ± 0·02 °C and the temperature was controlled using a Poly-Science water bath. The data were collected automatically and subsequently best fitted to a binding model depending upon the least χ^2 values using the Origin 7.0 software package supplied by the manufacturer. After subtracting the heat of dilution, a nonlinear least-squares algorithm (minimisation of χ^2) along with the concentrations of the titrant and the sample were used to fit the heat flow per injection to an equilibrium binding equation, providing best fit for the values of stoichiometry (n), the change in enthalpy ($\Delta H°$), and the binding constant (K). Also, the change in free energy ($\Delta G°$) and the change in entropy ($\Delta S°$) were calculated.

Results and Discussion

Effects of the Cu^{+2} ion on the structure of β-lg were investigated using different spectroscopic techniques (UV-visible,

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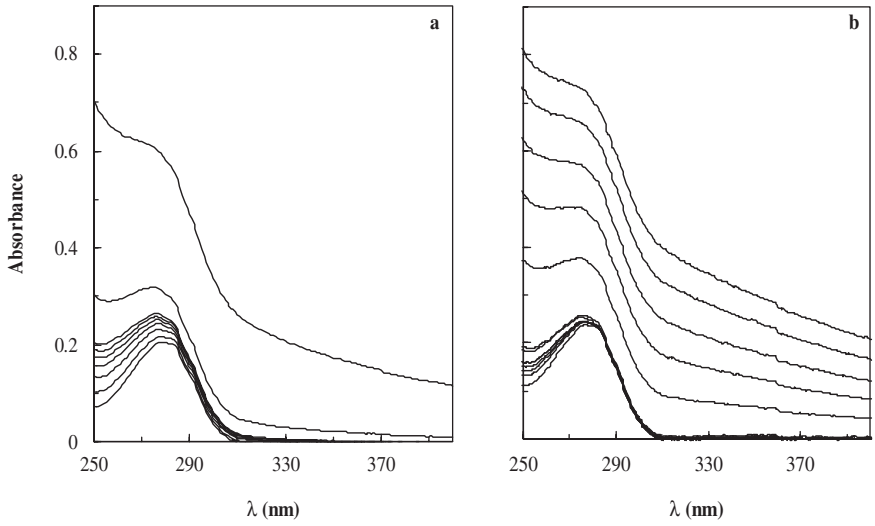

Fig. 1. Changes in absorption of β-lg (13 μM) upon interaction with different concentrations of Cu^{+2} ion (from bottom to up: 0–93 μM) at different temperatures of 25 °C (a) and 37 °C (b) in 50 mM sodium chloride solution.

intrinsic and extrinsic fluorescence and CD) and a calorimetric (ITC) method.

UV-visible studies

The amino acid responsible for the major absorbance of proteins in the near-UV region is tryptophan (Trp), with a maximum absorbance at ~ 280 nm (Viseu et al. 2004). Figure 1a, b shows the absorbance change of β-lg at 280 nm upon titration by different aliquots of a fixed stock concentration of Cu^{+2} at different intentionally chosen temperatures of 25 (room temperature) and 37 °C (physiological temperature). At both 25 and 37 °C, as the aliquots of Cu^{+2} were added in the titration mixture, the absorbance of the protein increased dramatically, revealing that the increase in Cu^{+2} caused increase in the absorbance of the protein. Furthermore, as shown in Fig. 1, addition of Cu^{+2} to β-lg solution caused an increase in the absorbance at 400 nm, indicative of turbidity in protein solution. It is noticeable that the rate of increasing turbidity is higher at 37 °C (changes in absorbance at 400 nm are 0 to 0·212) relative to 25 °C (changes in absorbance at 400 nm are 0 to 0·117). On the other hand, Cu^{+2} induce significant change in Abs_{280} at higher temperatures.

Intrinsic fluorescence studies

Fluorescence spectroscopy is a useful technique to study the structure, dynamics and binding properties of protein molecules in solution. The intrinsic fluorescence of tryptophanyl residues is a particularly sensitive method for this kind of study, as β-lg has two tryptophanyl residues. The intrinsic fluorescence of β-lg is almost exclusively attributed to Trp-19, positioned in a more apolar environment than Trp-61 (Shaikh et al. 2006). As shown in Fig. 2a, b, the Cu^{+2} reduces the intrinsic fluorescence emission of β-lg markedly at different concentrations (from molar ratio of Cu^{+2}/β-lg = 0–29·2) and then quenches it. Inset of Fig. 2a shows the intrinsic fluorescence of β-lg at 335 nm and at different temperatures and concentrations of Cu^{+2}. Reports have shown that the fluorescence intensity of a compound can be decreased by a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collision. The reason for this decrease in intensity, called fluorescence quenching (Shaikh et al. 2006) can be studied experimentally by determining quenching rate parameters using Stern–Volmer plots. The intrinsic fluorescence of β-lg is strongly quenched by the addition of Cu^{+2}. Furthermore, the fluorescence emission of the protein is quenched at lower concentrations of the Cu^{+2} when the temperature is increased (inset of Fig. 2a). In order to speculate on the fluorescence quenching mechanism, the fluorescence quenching data for β-lg was first analysed using the classic Stern-Volmer equation,

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of the β-lg in the absence and presence of the Cu^{+2} ion, respectively. K_{SV} is the Stern–Volmer dynamic quenching constant and [Q] is the total concentration of the quencher (in this case, the Cu^{+2} ion). Using Eq. (1), the fluorescence quenching data for β-lg shows good linearity for the plots of F_0/F *vs.* [Q] (see inset of Fig. 2b), where the values of K_{SV} at 25 and 37 °C calculated from slope of plots (inset of Fig. 2b) were 7·5 and 13·6/mM, respectively. By increasing the temperatures from 25 to 37 °C, the value of Stern-Volmer dynamic quenching constant increased approximately

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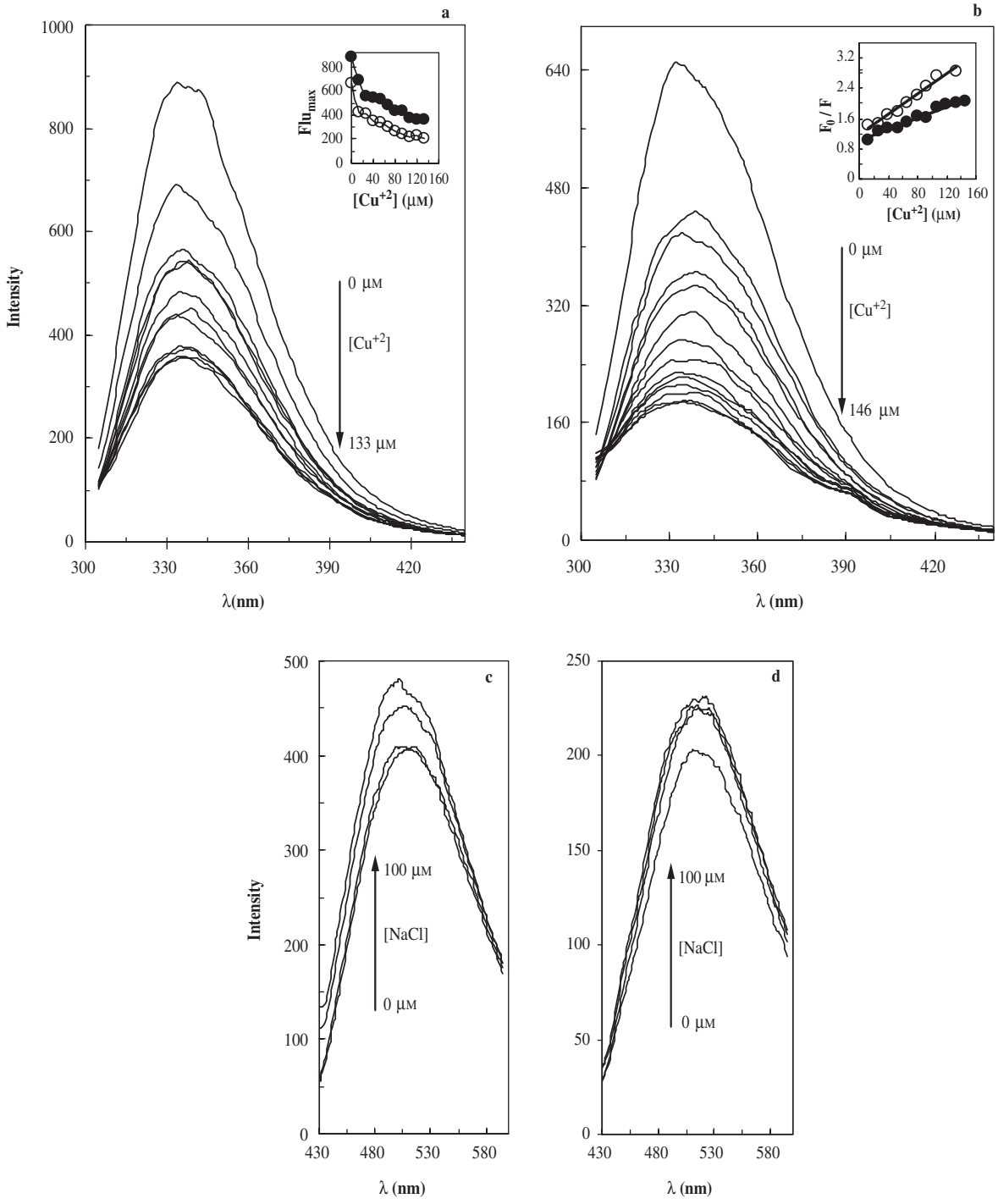

Fig. 2. The fluorescence titration curve of β-lg (5 μM) with different concentrations of Cu^{+2} ion; 0, 13·3, 26·6, 39·3, 53·2, 66·5, 79·8, 93·1, 106·4, 119·7, 133·0 and 146·3 μM (from top down) at different temperatures of 25 °C (a) and 37 °C (b) in 50 mM sodium chloride solution. Inset of Fig. 2(a) shows the changes in maximum intrinsic fluorescence of β-lg (5 μM) with different concentrations of Cu^{+2} ion in 50 mM NaCl solution at 25 °C (●) and 37 °C (○). Inset of Fig. 2(b) shows the Stern-Volmer curve for quenching of Cu^{+2} ion to β-lg in 50 mM NaCl solution at 25 °C (●) and 37 °C (○). The ANS fluorescence spectra of the β-lg (5 μM) were measured in the absence and presence of 25, 50 and 100 μM (from bottom up) of the copper solution at different temperatures of 25 °C (c) and 37 °C (d).

two times. Our results show that values of Stern–Volmer quenching constants (K_{SV}) are explicitly dependent on temperature. On the other hand, it can be seen that by increasing the temperatures from 25 to 37 °C the values of K_{SV} were increased indicating the highest contribution of dynamic mechanism of quenching.

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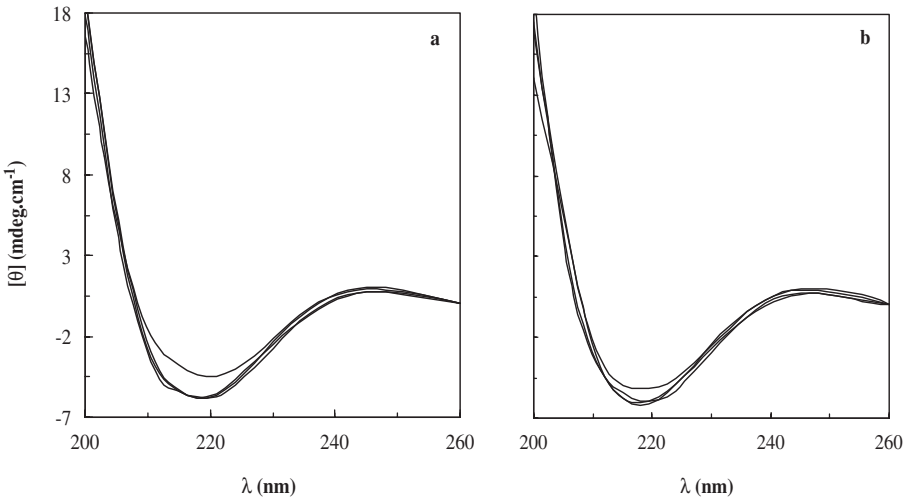

Fig. 3. Far-UV circular dichroism spectra of 13·5 μM β-lg measured in the absence and presence of different concentrations of Cu^{+2} from bottom up: 0, 125, 250 and 375 μM at different temperatures of 25 °C (a) and 37 °C (b) in 50 mM NaCl solution.

ANS fluorescence studies

As shown in Fig. 2a, b, the intrinsic fluorescence intensity of β-lg decreases on increasing the concentration of Cu^{+2}, perhaps due to the changing environment of Trp-19. The question is then asked; is the calyx structure of the native β-lg slightly loosened in the presence of Cu^{+2}? In order to answer this question, we used ANS fluorescence intensity measurements in our studies. ANS is a molecule that shares both hydrophilic and hydrophobic characters and whose fluorescence response provides a convenient and sensitive tool to investigate the nature of proteins binding regions. ANS is frequently used to demonstrate the presence of partially unfolded conformations of globular proteins. This is because ANS binds to solvent-exposed hydrophobic clusters, which results in a considerable increase in ANS fluorescence intensity and in a blue shift of the fluorescence emission maximum (Matulis et al. 1999). It can be seen from the ANS spectra shown in Fig. 2c, d that the presence of different concentrations of Cu^{+2} considerably enhances the ANS fluorescence intensity of the protein at the two different temperatures investigated in this study. Results of ANS fluorescence studies showed that interaction of the Cu^{+2} and increase of temperature from 25 to 37 °C, increase ANS fluorescence emission, meaning that Cu^{+2} and temperature cause conformational changes that lead to increased exposure of non-polar or hydrophobic surfaces of the protein to the solvent environment. It can therefore be concluded that Cu^{+2} induces a relatively open structure in β-lg.

CD studies

CD has proven to be an ideal technique for monitoring secondary structure conformational changes in proteins, which can occur as a result of changes in experimental parameters such as pH, temperature, and ligand binding (Zsila et al. 2002). The β-lg CD spectrum is typical of a protein that is composed of antiparallel β-structure and shows a minimum at 217 nm (Divsalar et al. 2009). The far-UV CD spectra of β-lg in the absence and presence of Cu^{+2} at different temperatures are shown in Fig. 3. The insets show the changes in $[\theta]_{217}$ for protein upon titration with the Cu^{+2} ions. Changes in secondary structure of β-lg upon interaction with different concentrations of Cu^{+2} at 25 and 37 °C are shown in Fig 3a, b. From the CD data, it can be seen that as the concentration of the Cu^{+2} increases, the content of regular structures of protein does not change significantly. It can, therefore, be concluded that Cu^{+2} cannot significantly alter the secondary structure of β-lg.

Thermodynamic analysis using ITC data

Figure 4 shows the ITC curves of binding of Cu^{+2} to β-lg. The raw ITC data are shown on the top panel, while on the bottom panel (a, b) are shown plots of the heat flow per mole of the titrant (Cu^{+2}) *vs*. the molar ratio of the titrant to protein at each injection, after subtraction of the background titration. Each peak in the raw data represents an injection of Cu^{+2} ions. Positive defections from the baseline upon addition of Cu^{+2} ions indicate an endothermic ligand binding event. The best fit for the integrated heat is obtained using the one ($n = 0{\cdot}93$) and four ($n = 4{\cdot}1$) independent binding sites with binding constants of 6·25 and 23·9/mM (at 25 and 37 °C, respectively). Since the ITC measurements of the Cu^{+2} binding to β-lg were obtained under identical conditions, a direct comparison of the thermodynamic parameters between them is possible. The ITC data demonstrates that the positive values of enthalpies (10·63 and 5·58 kJ/mol, at 25 and 37 °C, respectively) and entropies (53 and 38·7 J/mol K, at 25 and 37 °C, respectively) in binding process of β-lg to Cu^{+2} at two temperatures obey the direct order. These indicate that Cu^{+2} binds more tightly

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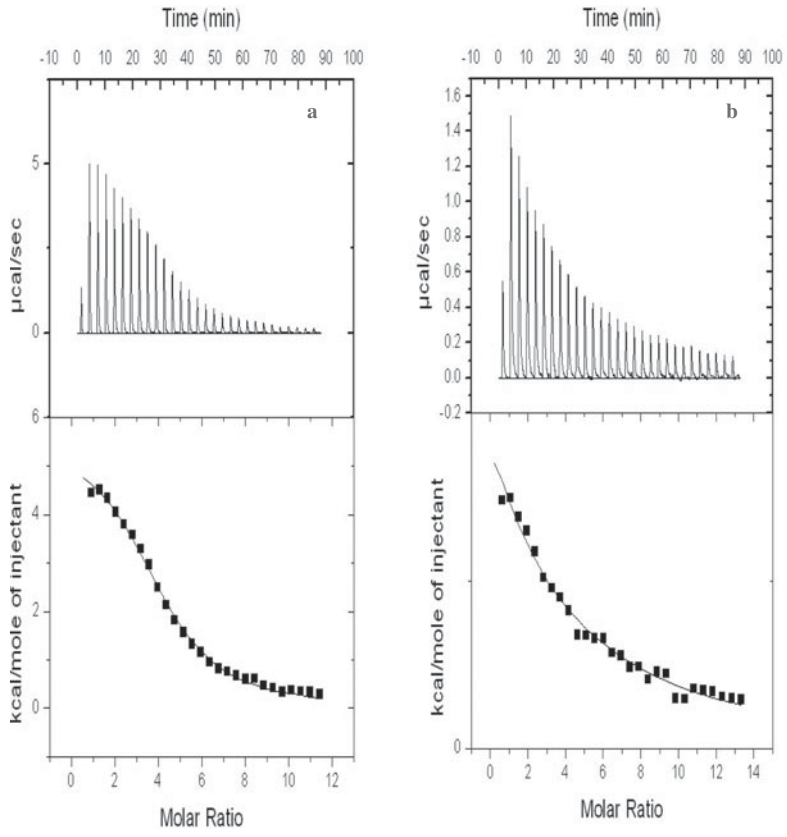

Fig. 4. ITC curves of binding of Cu^{+2} ion (1·5 mM) to β-lg (47 µM) at different temperatures of 25 °C (left, a) and 37 °C (right, b). The raw ITC data are shown on the top panel, while on the bottom panel are shown a plot of the heat flow per mole of the titrant (Cu^{+2} ion) *vs*. the molar ratio of the titrant to protein at each injection, after subtraction of the background titration.

to β-lg at 37 °C than 25 °C. Furthermore, the data indicate that although the binding reaction is mainly entropy driven, the results show a positive value of $\Delta H°$ for the Cu^{+2} ion–β-lg binding indicating that the binding is disfavoured by enthalpy (Divsalar et al. 2009, 2010).

Previous reports have shown that some metal ions such as calcium, copper and zinc ions induce protein aggregation by participating in intermolecular salt bridge formation between negatively charged amino acids (Navarra et al. 2007). Also, Muhammad et al. (2009) have reported that copper ions can induce oxidation of the free sulphhydryl group of β-lg resulting mainly in the formation of covalent dimmers (via disulphide bond formation), which were further associated into large non-covalent aggregates. In agreement to these reports, our UV-visible and calorimetry results demonstrate that Cu^{+2} ion could bind to β-lg and induce aggregation in protein solution.

Aggregation process of proteins is of interest both on a microscopic scale, involving structural and conformational changes that lead to the exposure of apolar residues, and on macroscopic scale, resulting in intramolecular interactions (Navarra et al. 2007). ANS intensities significantly increased in the presence of Cu^{+2}, indicative of exposure of nonpolar or hydrophobic surfaces of the protein to solvent environment. Then, β-lg is able to aggregate in the partially unfold state (as a result of binding of Cu^{+2} ion) as the hydrophobic and thiol groups are exposed, which were previously buried inside the protein and decrease its solubility. Since solubility is an important property for the functional behaviour of whey proteins, decreased solubility affects the β-lg functionality (Anandharamakrishnan et al. 2008).

Also, reports show that aggregation process appears to be strictly dependent on temperature and it starts only after rearrangement of the protein structure. Aggregate formation seems to proceed via a linear polymerisation mechanism by monomer addition (Vetri & Militello, 2005). From UV-visible spectra, it can be concluded that the rate of this polymerisation increases more steeply with increasing temperature.

In addition, several studies have demonstrated that emulsions based on β-lg are flocculated in the presence of calcium, sodium and other multivalent counterions. The mechanism involves both the screening of inter-droplet electrostatic repulsion forces and the reduction in surface charge density arising from direct ion binding to adsorbed protein and also due to associative interaction of hydrophobic patches on different droplet surfaces following protein surface denaturation. Then, it might be suggested that in neutral pH, the positively charged Cu^{+2} ion can bind to negative charged sites on the surface of β-lg (Simons et al. 2002) and cause a screening of the electrostatic repulsion

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and association of accessible hydrophobic patches, leading to flocculation.

The results presented and discussed in this paper, show that Cu^{+2} ion induce a slightly open tertiary structure and the formation of supramolecular aggregates in β-lg. Cu^{+2} ion might induce protein aggregation by participation in intermolecular salt bridges between negatively charged amino acids of β-lg.

β-Lg, the most abundant whey protein in milk, governs the overall process-induced aggregation and gelation or emulsification properties of whey protein products. In spite of its industrial applications, its use can be limited due to its high allergenicity; Food processing can alter the allergenic properties of proteins by hiding, destroying or disclosing allergenic epitopes through conformational changes, or by changing digestibility of proteins (Chicon et al. 2008). For example, high degree of glycation of β-lg causes noticeable reduction in its IgG binding and formation of protein aggregates, which could mask the antigenic regions of β-lg (Corzo-Martinez et al. 2010). Also, it seems that induction of aggregation in β-lg upon interaction with copper ions may be responsible for the disappearance and masking of conformational epitopes leading to reduced allergenicity of β-lg which can be most useful for the increased future industrial applications of β-lg and more studies in this field.

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