

Thermally driven interactions between β -lactoglobulin and retinol acetate investigated by fluorescence spectroscopy and molecular modeling methods

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Abstract Whey proteins have important functional properties as it can bind a wide range of bioactive molecules. In this study, the complexation of β -lactoglobulin with retinol at alkaline pH was investigated. The fluorescence spectroscopy was used to determine the temperature dependent behavior of the β -lactoglobulin-retinol acetate (β LG-RET) ensemble. The pattern of protein unfolding in the temperature range of 25–85 °C was followed mainly considering the exposure of tryptophan (Trp) residues. The β LG-RET complex appeared rather stable in the 25–60 °C range, while further increase of the temperature caused partial unfolding. The anisotropy measurements indicated a more flexible conformation at temperature increase up to 80 °C. In addition, the *in silico* approach was used to complement the experimental results. Important changes in the interaction surface were observed after performing molecular dynamics simulations. The temperature increase caused important rearrangements of the amino acids of the EF loop involved in the interaction with the retinol molecule, which got twisted. These atomic level events induced a significant increase of the affinity between β LG and retinol. This study offers useful information on the potential use of β LG as a carrier for biologically active compounds in order to obtain food products with desired functionalities.

Keywords β -lactoglobulin · Retinol · Thermodynamic parameters · Fluorescence spectroscopy

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1 Introduction

Certain proteins can ensure the encapsulation, protection, and delivery of bioactive components in functional foods, due to their ability to form protein-ligand complexes (Frits et al. 2000).

β -lactoglobulin (β LG) is the major whey protein found in ruminant and some non-ruminant species, being also one of the most studied ligand-binding food protein. At normal milk pH of 6.5, it occurs as a dimer, with molecular weight of 18 kDa per monomer. Below pH of 3.5 and above 7.5, β LG dimers dissociate into monomers, the process being accompanied by a structural transition. β LG belongs to the lipocalin family (Kontopidis et al. 2004), having the ability to bind small hydrophobic compounds such as fatty acids (Collini et al. 2003), retinol and other fat soluble vitamins (Muresan et al. 2001), isothiocyanates (Keppler et al. 2014), and various polyphenols (Wu et al. 2013). Therefore, several ligand-binding sites on protein molecules have been characterized. Some similarities between β LG and retinol binding proteins (RBP) have been suggested regarding the role of retinol binding and transport (Sahihi et al. 2011).

Retinoids are fat-soluble micronutrients, essential for several biological processes such as growth, cell proliferation and differentiation, reproduction, and vision. Retinoids have poor solubility in water, and consequently, retinoids in the *in vivo* aqueous environment are usually bound to proteins (N'soukpoé-Kossi et al. 2007). Several papers describe the particularities of retinoids binding to β LG preliminary subjected to chemical modifications, pH-induced structural changes, and pressure treatment (Mousavi et al. 2008; Sahihi et al. 2011). The functionality of β LG can be also improved through controlled thermal treatment. The thermal-induced β LG conformational changes may help to design delivery systems for bioactive compounds with better binding affinities and releasing mechanisms (Fioramonti et al. 2014).

Fluorescence spectroscopy is a useful technique for studying conformational changes of proteins containing tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues. The intrinsic fluorescence of aromatic amino acids in proteins is particularly sensitive to their microenvironments and has long been used for monitoring the unfolding/refolding transitions induced by chemical and physical denaturants (Wang et al. 1997).

Being highly unsaturated, retinols are prone to isomerization and oxidation during food processing and storage, thus influencing its bioavailability. Due to its structural particularities, β LG can bind retinol, which consequently increases the resistance of the protein to thermal denaturation (Considine et al. 2005), thus offering the possibility of ligand protection from early degradation and transporting. Given the potential applications of bioactive compounds- β LG complexes in various foods and nutraceuticals, the aim of the present study was to investigate the binding of retinol acetate (RET) to the β LG on a thermodynamic basis, and to evaluate the thermal stability of the complex. The fluorescence spectroscopy was used to study the structural particularities of β -lactoglobulin-retinol acetate (β LG-RET), and information regarding retinoid binding sites was reported after running molecular dynamic simulations. Moreover, for the ligand binding and thermodynamics studies, the thermally treated β LG solutions were sequentially titrated with retinol acetate.

2 Materials and methods

2.1 Sample preparation

Bovine β LG (99% purity) and ethanol (96% purity) were provided by Sigma (Sigma-Aldrich Co., St. Louis, MO), and RET (20 mg/mL) was purchased from a local pharmacy (S.C. Biofarm S.A., Bucharest, Romania). β LG was used without further purification. All other chemicals used were of analytical grade.

β LG solutions with concentration of 20 mg.mL⁻¹ were prepared in Tris-HCl buffer at pH 8.0. Fresh stock solution of RET (10 mg.mL⁻¹) was prepared in 5% ethanol at room temperature for each experiment. RET was added to β LG stock solution in order to achieve a ligand to protein molar ratio of 1:1. Further, the solutions were mixed and analyzed by means of fluorescence spectroscopy. Although the RET solution was prepared using ethanol, the final ethanol concentration in the mixture used for fluorescence measurements was lower than 3%, the denaturation effect of the solvent being therefore negligible. The background contribution of buffer was subtracted from each spectrum.

2.2 Heat treatment

In order to estimate the thermal behavior of β LG-RET solutions and RET fresh stock solution, the mixtures (25 μ L) were filled in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and were subjected to thermal treatment in a thermostatic water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain) at temperatures ranging from 25 to 85 °C for 10 min. A heating up time of 10 s was considered for all the heating experiments. The holding time was long enough to ensure structural rearrangements within the protein. After the thermal treatment, the samples were immediately cooled in ice water to prevent further thermal denaturation of the protein.

In order to evaluate the thermodynamics of RET binding to β LG, 25 μ L of protein solutions was heated and cooled in the same conditions as previously described. The binding of RET to β LG was estimated by following the quenching of Trp fluorescence at 292 nm, by sequential addition of RET solutions (0 to 1.5 mg.mL⁻¹). All spectroscopic studies were carried out on the heated-cooled complex in order to detect only irreversible/permanent structural changes. Before fluorescence spectroscopy analysis, the (un)-heated protein solutions were diluted in Tris-HCl pH 8.0 buffer.

2.3 Fluorescence spectroscopy

Fluorescence spectroscopy measurements were performed using LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT, USA) with a quartz cell of 10-mm path length.

2.3.1 Phase diagram

Conformational changes of proteins can be distinguished by using spectroscopic data. The *phase diagram* method is based on Eq. (1) (Kuznetsova et al. 2004):

$$I(\lambda_1) = a + bI(\lambda_2) \quad (1)$$

where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensity values measured at wavelengths λ_1 of 320 nm and λ_2 of 365 nm under different experimental conditions for a protein undergoing structural changes; a and b are the intercept and the slope, respectively, of the $I(\lambda_1)$ versus $I(\lambda_2)$ plot. The excitation wavelength was set at 292 nm, whereas the fluorescence intensity was measured at emission wavelengths of 320 and 365 nm, respectively.

2.3.2 Intrinsic fluorescence

Intrinsic fluorescence measurements were performed at excitation wavelengths of 292 and 274 nm, whereas the emission spectra were collected between 320 to 420 nm, using a 10-nm bandwidth, excitation and emission slits of 10 and 10 nm, respectively, and a scan speed of 500 nm.min⁻¹. In order to investigate the effect of heating on RET, the (un)-treated β LG-RET complex and RET solutions were excited at 325 nm, and the emission was collected between 400 and 600 nm.

The raw fluorescence intensity was corrected for inner filter effects (i.e., for light absorption of the compounds in the mixture) by measuring the absorbance of β LG-RET complex between 240 and 600 nm, using quartz cuvettes of 1-cm path length. A correction factor of $10^{(A_{exc} + A_{em}) \times 0.5}$ was applied, where A_{exc} was the absorbance of β LG-RET complex at the excitation wavelength (280 nm for β LG or 330 nm for retinol), and A_{em} was the absorbance at each fluorescence emission wavelength of spectra; 0.5 (cm) as accounts for half of the cuvette path length (Blayo et al. 2014).

2.3.3 Synchronous fluorescence spectroscopy

The synchronous fluorescence spectra of β LG-RET solutions were collected by simultaneously scanning the excitation and emission monochromator in the excitation wavelength range from 250 to 340 nm. The spectra were obtained by measuring the excitation wavelength in the same spectral region and varying the wavelength interval at 15 and 60 nm, corresponding to the changes of the polarity around Tyr and Trp residues, respectively (Guo et al. 2009).

2.4 Ligand binding studies

The number of binding sites and the corresponding binding constant were calculated according to Eq. (2) (Lakowicz 2006):

$$\log_{10} \left[\frac{F_0 - F}{F} \right] = \log_{10} K_A + n \log_{10} [RET] \quad (2)$$

where F and F_0 are the fluorescence intensity in the presence or absence of different concentration of retinol acetate; $[RET]$ is the retinol acetate concentration; K_A is the binding constant; and n is the number of binding sites per β LG molecule.

2.5 Thermodynamics of β LG-RET interactions

Temperature-dependent thermodynamic parameters were studied in order to elucidate the interactions between β LG and RET. The thermodynamic parameters were determined by carrying out binding studies in the temperature range of 25 to 85 °C. The values of ΔH and ΔS for the β LG-RET interactions were estimated through the Van't Hoff equation (Eq. 3):

$$\ln K_A = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where ΔH and ΔS are enthalpy and entropy change, respectively, R is the gas constant, and T is the temperature. The values of ΔH and ΔS were calculated from the slope and intercept of the $\ln(K_A)$ versus $1/T$ plot.

ΔG value was obtained according to Eq. 4:

$$\Delta G = \Delta H - T\Delta S. \quad (4)$$

2.6 In silico investigation of β LG-retinol interactions

The atomic structure of the β LG-retinol complex was obtained from RCSB Protein Data Bank-PDB entry 1GX8.pdb (Kontopidis et al. 2002). The 3D model to be used in the simulation procedure was solved through X-ray diffraction and was chosen based on the good resolution value of 2.40 Å and R value of 0.210. After a preliminary refinement, the complex was minimized in vacuum by Steepest Descent algorithm, was placed in a cubic box, was solvated by adding explicit water molecules, and was minimized again to remove any large forces acting between proteins or retinol molecule and water. Further molecular dynamics simulations in explicitly solvated environment were carried out first to heat the complex at 25 and 75 °C, and then to equilibrate the molecular models in the canonical ensemble (NVT). A weak coupling Berendsen thermostat was used to control the temperature. The simulations were performed by means of GROMACS 4.6 package (Hess et al. 2008) on an Intel® Core™ 2 CPU 6300 1.86 GHz processor-based machine running Linux. The gromos43a1 force field was used to define the topology, and periodic boundary conditions were applied along the xyz coordinates. The electrostatic interactions were treated with the particle mesh Ewald method with a Coulomb cutoff of 1.7 nm; the van der Waals interactions were treated using Lennard-Jones potential and a switching function using cutoff and switching distances of 1.1 and 0.9 nm, respectively.

The equilibrated systems were used as starting models for further molecular investigations by means of PDBsum tool (Laskowski 2009). Dedicated GROMACS tools were used for quantitative analysis, and protein residues mainly responsible for the interaction with retinol molecule have been identified by means of PDBEPIA tools (Krissinel 2010) and LigPlot+ (Laskowski and Swindells 2011).

2.7 Statistical analysis of data

Each fluorescence experiment was carried out at least in triplicate. All standard deviations were calculated using Microsoft Excel. The standard deviations associated with each experiment were lower than 3.5%.

3 Results and discussion

3.1 Phase diagram

To analyze the mechanism by which thermal treatment induced conformational changes in β LG-RET complex, the *phase diagram* was constructed, based on the I_{320} versus I_{365} correlation. This method is very sensitive and was used for highlighting the presence of various molecular species. When the abovementioned correlation is linear, the presence of only two different conformations, without other intermediate states, was suggested (Kuznetsova et al. 2004). The non-linearity of the correlation reflects the sequential character of structural changes (*three-state* transition), which indicates the presence of partially folded species and intermediates.

The results presented in Fig. 1 revealed an *all or none* transition, indicating that the thermal treatment of β LG-RET solutions induced the protein transition from the native state to the unfolded one. A two-state denaturation model for β LG was previously discussed by several authors (e.g., Manderson et al. 1999; Tolkach and Kulozik 2007; Stănciuc et al. 2012). These authors suggested a denaturation mechanism involving the dissociation of dimers into monomers, followed by partial unfolding with exposure of the free thiol and some of the hydrophobic groups at temperatures higher than 70 °C.

Heat treatment caused significant changes in the fluorescence intensity of β LG-RET complex in the whole temperature range studied. Binding of RET caused conformational changes that stabilized the protein during the thermal treatment, the complex being more stable when compared with the protein heated alone. The most important rearrangements of the polypeptide chains occurred at temperatures higher than 60 °C, leading to fluorescence intensity increase as a consequence of increased exposure of

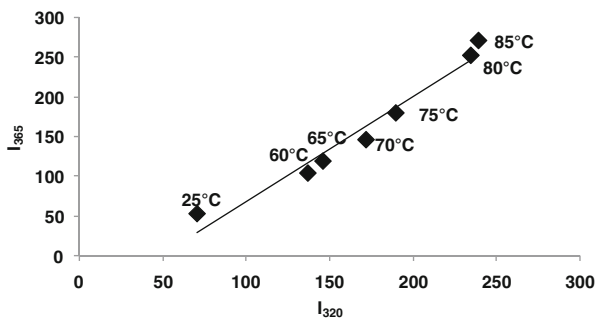


Fig. 1 Phase diagram analysis of heat-induced conformational changes of β LG-RET complex based on intrinsic fluorescence intensity values measured at wavelengths 320 and 365 nm. The temperature values are indicated in the vicinity of the corresponding symbol. Three independent tests were carried out in each case, and SD was lower than 2.5%

buried Trp moieties to the aqueous environment (Simion (Ciuciu) et al. 2015). Tolkach and Kulozik (2007) suggested that up to 60 °C, the transition of β LG is mainly due to the complete unfolding of the α -helix structure and to the exposure of the free Cys¹²¹ and some parts of the hydrophobic core. Intramolecular interchange reactions in the partially unfolded β LG leading to the exposure of Cys¹¹⁹ were also suggested. This form is not able to refold to the native state, resulting in the formation of irreversible non-native β LG monomers upon cooling (Croguennec et al. 2003). Similar results were reported by Simion (Ciuciu) et al. (2015), who indicated the presence of two different conformations induced by thermal treatment of β LG solutions. However, Stănciuc et al. (2012) obtained two linear parts corresponding to the temperature range of 25–65 and 70–80 °C, respectively, for β LG solutions heated alone. These authors concluded that in the lower temperature range, the dissociation of the dimer predominates, whereas in the higher temperature range, the unfolding is a single-phase reaction. Manderson et al. (1999) suggested a two-state model for the denaturation of β LG A, B, and C in temperatures range between 40 and 94 °C for 10 min. These authors reported that the Trp emission intensity arose from consequential disulfide bond reorganization and not from the initial unfolding reaction.

3.2 Intrinsic fluorescence

The intrinsic fluorescence of aromatic amino acids in proteins has been used as a mean of monitoring the conformational transitions induced by chemical denaturants, temperature, pH changes, and pressure. Therefore, it is possible to use fluorescence spectra of the intrinsic Tyr (Tyr²⁰, Tyr⁴², Tyr¹⁰², and Tyr⁹⁹) and Trp (Trp¹⁹ and Trp⁶¹) residues in β LG as a tool to study the alteration of the polarity around the chromophore microenvironment. The Trp⁶¹ residue is located at the surface of the protein, whereas Trp¹⁹ is situated in the hydrophobic core of the protein. The indole ring of Trp¹⁹ from strand A is located in an apolar environment within the cavity of β LG, whereas Trp⁶¹ is adjacent to strand I, which is involved in interactions at dimer interface (Croguennec et al. 2003). Therefore, the intrinsic fluorescence of β LG might be almost exclusively due to Trp¹⁹ (Mousavi et al. 2008). Tyr²⁰, Tyr⁴², and Tyr¹⁰² are located in a β -sheet, and Tyr⁹⁹ is positioned in a random coil. Tyr²⁰ is located on the surface of the protein and Tyr¹⁰² in a hydrophobic environment.

Based on the fact that the excitation of Trp residues is accomplished at wavelengths range of 285–305 nm, and excitation of Tyr residues is accomplished at 275 nm, Fig. 2 shows the effect of heat treatment on the intrinsic fluorescence intensity of β LG-RET complex when excited at 292 nm (Fig. 2a) and at 274 nm (Fig. 2b), respectively.

A significant increase in fluorescence intensity was registered in the whole temperature range studied (Fig. 3). The gradual increase in the fluorescence of β LG-RET as function of temperature suggested the protein unfolding, leading to the exposure of hydrophobic regions to the aqueous environment. The Trp¹⁹ residue, situated at the bottom of the calyx formed by eight antiparallel β strands, contributes with about 80% to the total fluorescence, whereas the Cys⁶⁶–Cys¹⁶⁰ disulfide bond quenches Trp⁶¹ emission (Taheri-Kafrani et al. 2008).

When raising the temperature from 25 to 85 °C, an increase in the fluorescence intensity of β LG was also observed by Simion (Ciuciu) et al. (2015); the fluorescence intensity increased by 58 and 53%, when excited at 292 and 274 nm, respectively.

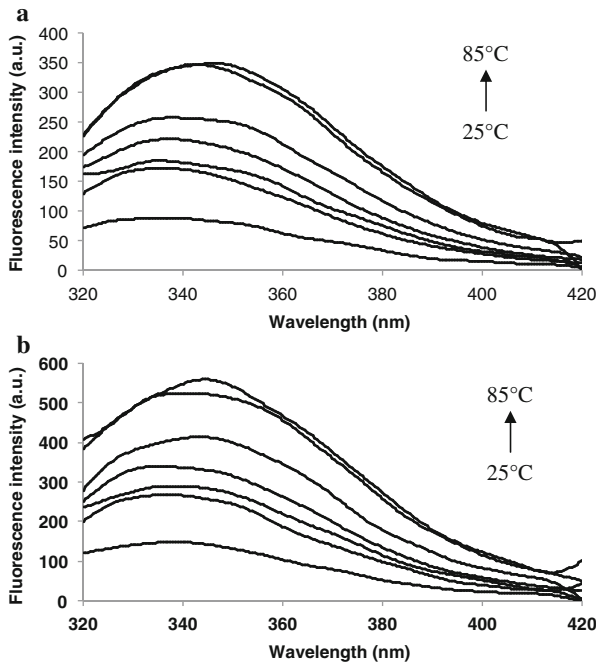


Fig. 2 The emission spectra of Trp (a) and Tyr (b) fluorescence of β LG-RET complex as a function of temperature. The excitation wavelength was 292 and 274 nm, respectively. Three independent tests were carried out in each case, and SD was lower than 3.5%

This difference in fluorescence intensity as a function of temperature implies that, in the native conformation, the Trp residues are surrounded by amino acids that might quench its fluorescence.

Wang et al. (1999) reported two lipophilic ligand binding sites for β LG: a central hydrophobic β -barrel and a superficial pocket. The fluorescence increase with increasing temperature suggested that β LG binds the RET molecule, and the binding site is not located in the vicinity of any Trp residue. Moreover, the denaturation of the original RET binding site, as well as defining a new binding site or restructuring of the old

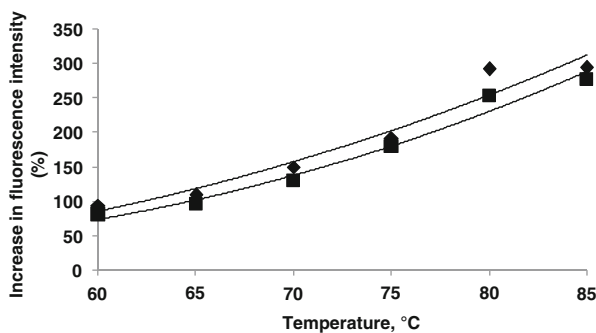


Fig. 3 The increase in the fluorescence intensity of Trp (black diamond) and Tyr (black square) fluorescence in the temperature range 25–85 °C. The excitation wavelength was 292 and 274 nm, respectively. Three independent tests were carried out in each case, and SD was lower than 3.5%

one, was suggested by Mousavi et al. (2008). Similarly, a significant increase in fluorescence intensity at 75 and 80 °C (110–124%), suggesting unfolding of the molecule with a partial exposure of Trp¹⁹, and reduction of intramolecular quenching of Trp were suggested by Stănciuc et al. (2012).

The maximum emission wavelength of the intrinsic fluorescence spectrum (λ_{max}) was used to follow the structural changes of the β LG-RET induced by temperature. When λ_{max} varies from 330 to 332 nm, the fluorophores are buried, whereas between 340 and 342 nm, the fluorophores are exposed to the water environment. When excited at 292 nm, λ_{max} remained constant at 336–337 nm in the temperature range of 25 to 75 °C, whereas red shifts of 6.5 nm at 80 °C and 10 nm at 85 °C were observed with respect to the thermally untreated protein (Fig. 2a). When the complex solutions were excited at 274 nm, sequential changes in λ_{max} were recorded. A maximum red shift of 6 nm was obtained when heating at 85 °C (Fig. 2b), suggesting higher exposure of the hydrophobic regions to the aqueous environment. The red shifts were associated with the unfolding of polypeptide chains affecting the tertiary structure of β LG. The exposure of hydrophobic regions was also suggested by Fang et al. (2014), when testing the oleic acid binding to bovine lactoferrin and α -lactalbumin. Red shifts of 5 nm in the λ_{max} were reported, indicating the increase of the exposure of hydrophobic regions upon ligand binding.

At room temperature, retinol exhibited a weak fluorescence intensity in aqueous solutions when excited at 325 nm, but a significant increase of the fluorescence was obtained when binding to the β LG molecule (Fig. 4a). This behavior might be caused by the increased conformational rigidity of retinol molecule within the complex, especially due to the β -ionone ring rather than the polyene chain moiety (Cho 2000).

However, when considering the thermal behavior of the complex, the heat-induced changes on retinol should be considered, as the degradation of these compounds may be caused by thermal isomerization, dehydration, or decomposition in the presence of oxygen. Therefore, in order to evaluate the influence of heating on RET, fluorescence spectra were generated by exciting the solutions at 325 nm and collecting the spectrum between 400 and 600 nm. A significant increase in fluorescence intensity can be observed when increasing the temperature from 25 to 85 °C, whereas a 16-nm red shift in maximum emission wavelengths was observed, suggesting degradation of RET (Fig. 4c). Regarding the thermal degradation of retinols, a geometric isomerization from the *trans* to *cis* form is expected. In particular, a slight constant increase in 13-*cis* retinol was observed, while the amount of the 9-*cis* retinol isomer remained constant during processing (Fратиани et al. 2012).

However, the protective effect of protein was observed when the complex was heated and cooled, followed by excitation at 325 nm. The maximum fluorescence intensity was registered at 25 °C, whereas λ_{max} was observed at 478 nm. As the temperature increases, significant decrease in fluorescence intensity can be observed in Fig. 4b, together with significant blue shifts of λ_{max} from 9 to 42.5 nm. The heating might alter the protein ability to bind the retinol molecule in a chiral environment and might also cause the loss of the chiral environment of the Trp¹⁹. Cho (2000) suggested that the retinol-binding ability of β LG can be restored upon cooling if the protein does not completely lose its native structure.

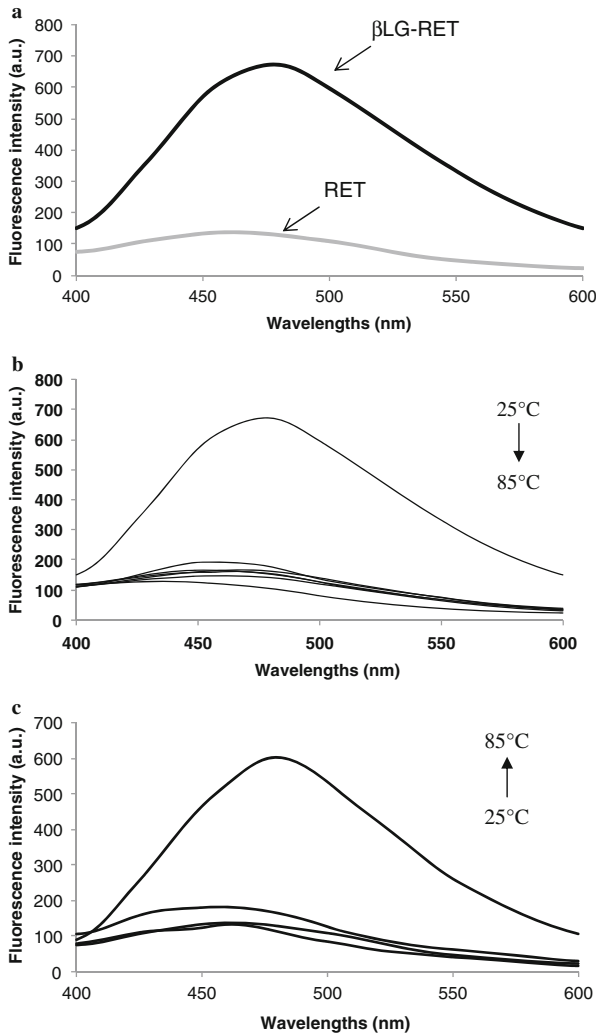


Fig. 4 The emission spectrum of β LG-RET complex and RET solutions at 25 °C (a). Temperature-dependent emission spectrum of β LG-RET complex (b) and RET solutions (c). The emission spectra were recorded between 400 and 600 nm at excitation wavelength of 325 nm, using excitation and emission slit widths of 10 nm at a scan speed of 500 nm.min⁻¹. Experimental details are given in Section 2

3.3 Synchronous fluorescence spectroscopy

Synchronous fluorescence spectroscopy is a very useful method for detailed investigation of the protein conformations, depending on the particularities of the chromophore microenvironment (Klajnert and Bryszewska 2002), and offers many advantages, such as sensitivity, spectral simplification, and avoiding different perturbing effects. Therefore, when $\Delta\lambda$ (the difference between excitation wavelength and emission wavelength) is fixed at 15 or 60 nm, the synchronous fluorescence spectra evidence the characteristic of Tyr and Trp residues environment, respectively. Figure 5 shows the

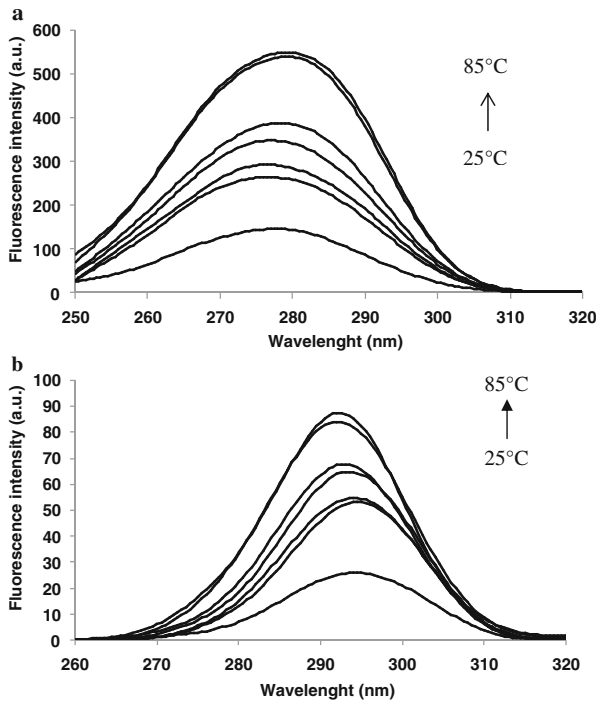


Fig. 5 Synchronous fluorescence spectra at $\Delta\lambda$ of 60 nm (a) and $\Delta\lambda$ of 15 nm (b) for β LG-retinol acetate complex. Three independent tests were carried out in each case, and SD was lower than 3.0%

synchronous fluorescence spectra of β LG-RET complex at $\Delta\lambda$ of 60 nm (a) and of 15 nm (b). Regardless of $\Delta\lambda$, when increasing the temperature, a small red shift (2 nm and about 5 nm) in λ_{max} was observed (Fig. 5a, b, respectively).

The heat treatment at 85 °C induced unfolding of polypeptide chains with an increase in fluorescence intensity of approximately 29% at $\Delta\lambda$ of 60 nm, and with 27% at $\Delta\lambda$ of 15 nm. In agreement with the abovementioned results, the synchronous fluorescence spectra indicated that the microenvironment around Tyr residues was altered, causing an increase in the polarity along with temperature increase. Moreover, the polarity around Trp residues increases, these residues being located in a more hydrophilic environment (Shen et al. 2014). It is more likely that the conjugated effect of both heat treatment and RET addition changed the microenvironment and polarity around Trp and Tyr residues.

3.4 Fluorescence quenching

Quenching of fluorescence is due to a variety of molecular interactions with quencher molecules that include excited-state reactions, molecular conformations, energy transfer, ground-state complex formation, and collisional quenching processes (Meti et al. 2014). The different mechanisms of quenching are usually classified as dynamic or static. In the quenching experiments, the protein solutions were first heat treated, followed by the sequential addition of different concentrations of RET. In order to

predict the probable quenching mechanism, the fluorescence quenching data were subjected to Stern-Volmer analysis.

The fluorescence intensity gradually decreased when increasing the concentration of RET. Addition of RET, up to $1.5 \text{ mg}\cdot\text{mL}^{-1}$, combined with the heat treatment induced a slight red shift (2–6 nm) in λ_{max} , reaching a value of 336 nm. With the increasing concentration of RET, the maximum emission wavelength of β LG was 2–6 nm red shifted, indicating that the ligand caused the lost of the protein compact structure in the hydrophobic subdomain where Trp is placed. Assuming that the observed changes in fluorescence come from the interaction between RET and β LG, the quenching constant can be taken as the binding constant of the complex formation. The corresponding values of K_{SV} are presented in Table 1. When quenching with RET, the Stern-Volmer constant for β LG solutions at 25°C was $0.171 \pm 0.00 \text{ mol}\cdot\text{L}^{-1}$, whereas the heat treatment caused a significant increase of approximately 61% at 75°C .

The results may suggest that thermal denaturation significantly affected the native structure of β LG, without influencing its binding affinity for RET.

Figure 6 shows the temperature-dependent plots of F_0/F versus $[RET]$ ranging from 0 to $1.5 \text{ mg}\cdot\text{mL}^{-1}$. Given the linearity of the correlation within the ligand concentration studied, it seems that a single static quenching mechanism occurred; therefore, the quencher formed a non-fluorescent complex with the fluorophore molecule (Lakowicz 2006).

The corresponding K_{SV} values reflect lower accessibility of RET to the β LG up to 70°C , probably due to the decrease in Trp residues exposure, followed by a higher accessibility due to the unfolding of polypeptide chains at higher temperature. These values highlight a sequential denaturation process involving the folding of molecules in the temperature range $25\text{--}75^\circ\text{C}$, followed by unfolding at higher temperatures.

Fluorescence quenching mechanisms depend on temperature. In a dynamic quenching process, during the lifetime of excited state, the fluorophore is in contact with the quencher whereas in a static quenching, the quencher interacts with the fluorophore through a combination in the ground state (Lakowicz 2006). From Table 1, it can be observed that the K_{SV} decreased with the temperature increase up to 70°C , indicating a static quenching mechanism, followed by an increase at 75°C , which is an indicative of the dynamic process. However, at higher temperature, due to the decrease in K_{SV} values, it can be concluded that the quenching mechanism is static. A static quenching mechanism for retinoid-bovine serum albumin complexes was also reported Belatik et al. (2012).

Table 1 Stern-Volmer quenching constants in different conformational stages for the β LG-RET complex

Temperature ($^\circ\text{C}$)	K_{SV} ($\text{L}\cdot\text{mol}^{-1}$)
25	0.171 ± 0.00^a
60	0.102 ± 0.01
65	0.112 ± 0.02
70	0.077 ± 0.02
75	0.275 ± 0.08
80	0.110 ± 0.02
85	0.168 ± 0.03

^a Standard deviation

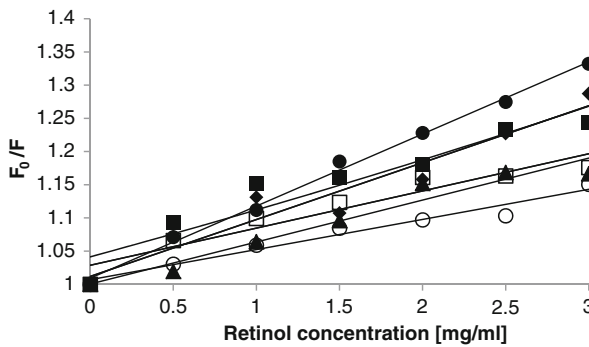


Fig. 6 Plot of F_0/F for β LG against $[Q]$ of retinol acetate ranging from 0 to 1.5 $\text{mg}\cdot\text{mL}^{-1}$ at different temperatures, 25 (black diamond), 60 (white square), 65 (black triangle), 70 (white circle), 75 (black circle), 80, 85 °C (black square)

3.5 Ligand binding studies

The binding constant (K_A) and the number of binding sites (n) were calculated based on Eq. (2). A plot of $\log [(F_0-F)/F]$ versus $\log [RET]$ gives straight lines (Fig. 7), whose slope equals to n and the intercept on Y -axis equals to $\log K_A$.

The corresponding values of K_A and n at different temperatures are given in Table 2. In the native state, the interaction between β LG and RET displayed an apparent dissociation constant of $2.1 \text{ L}\cdot\text{mol}^{-1}$ and a number of binding sites of 0.703. Taheri-Kafrani et al. (2008) suggested a K_A value of $5.42 \times 10^{-8} \text{ M}$ and a similar n value of 0.74 for the association of retinol with β LG at pH 8.0. The results suggest that there is a strong binding force between β LG and RET. The n was higher in the temperature range 65–75 °C.

Further temperature increase caused a decrease of n , which may be due to protein unfolding (Mousavi et al. 2008) (Table 2).

Similar results for the number of binding sites were obtained by Mousavi et al. (2008), while Belatik et al. (2012) reported higher values for retinoids binding to serum albumins. Cho et al. (1994) showed that, upon binding of all-trans retinol, the wild-type

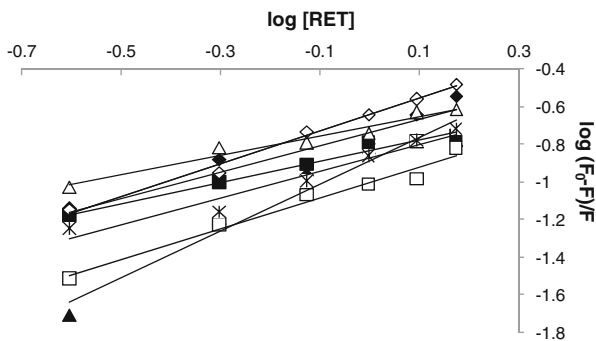


Fig. 7 The plot of $\log (F_0-F)/F$ versus $\log [RET]$ for quenching of β LG by RET at different temperatures, 25 (black diamond), 60 (black square), 65 (black triangle), 70 (white circle), 75 (white diamond), 80 (black circle), 85 °C (white square)

Table 2 Thermodynamic parameters of β LG-RET complex

Temperature, (K)	Binding constant (K_A) L.mol ⁻¹	No. of binding sites (n)	ΔH (kJ.mol ⁻¹)	ΔS (JK ⁻¹ .mol ⁻¹)	ΔG (kJ.mol ⁻¹)
298	2.100	0.703	-19.34	66.33	-19.78
333	2.302	0.565	-19.34	66.33	-22.11
338	2.442	1.238	-19.34	66.33	-22.44
343	2.726	0.824	22.96	-56.77	19.49
348	1.900	0.875	22.96	-56.77	19.78
353	2.393	0.717	22.96	-56.77	20.06
358	2.028	0.515	22.96	-56.77	20.35

β LG fluorescence was quenched by 32%. High affinity binding of retinoids occurred at pH 7.0, and the apparent dissociation constant ranged from 1.7 to 3.6×10^{-8} M. Furthermore, a stoichiometry of 1.0 mol.mol⁻¹ of protein was obtained, indicating that all sites of the protein were available (Wang et al. 1997).

The K_A values first increased with the temperature up to 70 °C, decreased afterwards significantly at 75 °C, and finally slightly increased at 80 °C. Therefore, increasing temperature may cause the decrease of the binding strength.

The only α -helical structure with three turns is at the COOH-terminus (residues 130–141) of β LG, which is followed by β -strand I, lying on the outer surface of the calyx (Uhrinova et al. 2000). It seems that in the whole temperature range studied, the binding of RET takes place within the central calyx of the protein. However, at 65 °C, a second ligand binding site beyond the calyx can be considered, since n value was higher than 1. This surface pocket, limited by Phe¹³⁶ and followed by residues 139–143 (Ala-Leu-Lys-Ala-Leu), has been suspected to potentially bind retinol. Song et al. (2005) showed that thermally denatured β LG (heated at 100 °C for 5 min) was unable to bind retinol because of the unfolding of the calyx. Moreover, these authors suggested a second binding site for ligands by thermally removing of the calyx, whereas a thermally stable site in β LG was revealed.

3.6 Thermodynamics of β LG-RET interactions

The forces generally acting between small molecules and biomacromolecules mainly include van der Waals forces, electrostatic interactions, hydrogen bonds, and hydrophobic forces. Thermodynamic parameters are important descriptors of the acting forces between β LG and RET. The values for ΔH and ΔS indicate that the binding process was driven by the change in entropy (ΔS) (Table 2). At higher temperatures (70–85 °C), the process seems to be enthalpy driven (ΔH). Negative values of ΔG indicate that the binding process was spontaneous in the temperature range 25–65 °C. According to Ross and Subramanian (1981), when $\Delta H < 0$ or $\Delta H \sim 0$, and $\Delta S > 0$, the main binding forces are electrostatic, while when $\Delta H < 0$ and $\Delta S < 0$, the main binding forces are van der Waals or hydrogen bond. Therefore, the results indicated that in the range 25–65 °C, the electrostatic forces were the binding force that stabilized the β LG-

RET. In the temperature range of 70–85 °C, it was difficult to appreciate which were the binding forces involved. When $\Delta S > 0$, the hydrophobic interaction should not be excluded (Bi et al. 2014).

3.7 In silico analysis of thermally induced changes in β LG-retinol complex

A detailed atomic level analysis of the β LG-retinol was performed by means of the in silico approach, after running molecular dynamics steps to equilibrate the models at 25 and 75 °C. The reliability and stability of the equilibrated models were verified based on the Ramachandran plot statistics (in case of both temperatures, the number of amino acids placed in the allowed regions of the plot was higher than 98%). Moreover, the standard deviation of the potential energy was reduced to less than 0.1% in the last 400 ps of the equilibration dynamics. An increase of the total energy value was obtained at high temperature due to the increased thermal motion (Table 3). No significant change in hydrophobic area exposed to the solvent has been obtained with the temperature increase ($48.32 \pm 0.79 \text{ nm}^2$ at 25 °C and $48.25 \pm 0.95 \text{ nm}^2$ at 75 °C), although a 10% reduction of the hydrogen bonds stabilizing the protein structure was obtained at 75 °C with respect to 25 °C.

The main conformational differences between protein models equilibrated at 25 and 75 °C were checked by means of PROMOTIF v 3.0. program through PDBsum tool (Laskowski 2009). Regardless of the thermal treatment, the β LG structure is dominated by the strand-based motifs. The temperature increase caused the reduction of both strands and helical contents (Table 3). Although the same number of helices (four) was observed in the case of both structures, a close check indicated important changes: the Ile¹²-Val¹⁵ α -helix identified in the protein structure at 25 °C turned into a beta-turn motif at 75 °C, and a new α -helix was defined at higher temperature (Ala⁸⁶-Glu⁸⁹).

Table 3 Molecular details on β LG-retinol complex at thermal treatment

	Temperature, °C	
	25	75
Secondary structure descriptors		
Strands, %	42.2	39.1
α -helix, %	13.7	11.8
3–10 helix, %	1.9	1.9
Energy descriptors		
β LG-retinol total energy, $\text{kJ}\cdot\text{mol}^{-1}$	-371385.96 ± 669.89	-309492.38 ± 627.80
β LG-retinol interaction energy ^a , $\text{kJ}\cdot\text{mol}^{-1}$	-118.89 ± 10.86	-133.13 ± 12.58
Surface descriptor		
β LG-retinol interface area, \AA^2	347	384
Hydrogen bonding		
Hb within protein	124 ± 5	112 ± 5
Hb water protein	304 ± 9	298 ± 11

^a Estimated as the sum of the short-range Lennard-Jones and Coulomb energy terms

Concerning the Ala⁸⁶-Glu⁸⁹, it was shown to play a key role in the interaction of the β LG with the ligand; as part of the EF loop, they act as a flap over the retinol binding site (Kontopidis et al. 2002), being therefore in close contact with the retinol molecule. In fact, due to the important side-chain rearrangements at higher temperature, a threefold decrease of the contact between retinol molecule and Leu⁸⁷ was observed. Moreover, a significant repositioning of the Ala⁸⁶-Asn⁹⁰ residues was noticed, such as to make room for the retinol molecule which got twisted at high temperature (Fig. 8).

Overall, the temperature increase from 25 to 75 °C caused the increase of the total number of interacting residues of β LG molecule with retinol from 19 to 22. Only the relative position of the Leu³⁹, Ile⁸⁴, and Asn⁹⁰ side chains with respect to retinol atoms was conserved in the β LG-retinol equilibrated at 25 and 75 °C (Fig. 8). As a result of the rearrangements occurring at the level of the ligand binding site, a significant increase of the affinity between β LG and retinol was observed. The interface area increased with the temperature; the total solvent-accessible surface area buried upon β LG-retinol formation increased from 695.1 to 768 Å². Moreover, the β LG-retinol

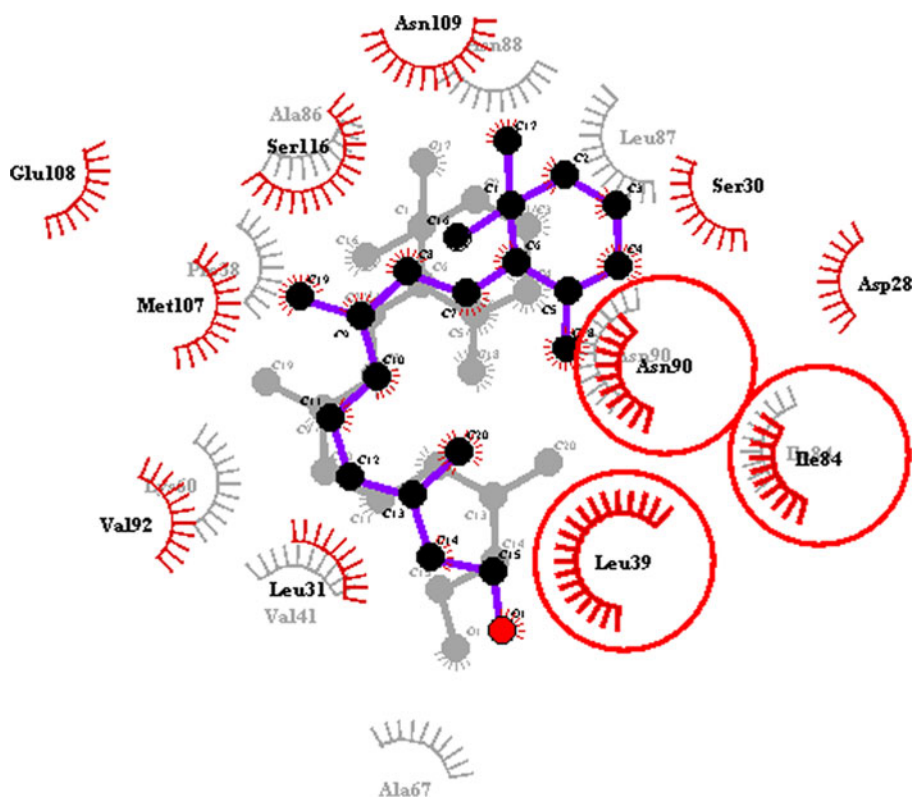


Fig. 8 Atomic level details on the interaction between β LG and RET molecules. The differences were observed by superimposing the models equilibrated at 25 °C (gray) and at 75 °C (colored). Only the β LG amino acids placed in close hydrophobic contacts with RET atoms are represented by an *arc with spokes* radiating towards the ligand atoms they contact. The equivalent side chains involved in the interaction with RET molecule at both temperatures are *circled*. The figure was drawn using LigPlot+ (Laskowski & Swindells 2011)

interaction energy calculated as the sum of the short range Lennard-Jones and Coulomb terms decreased (Table 3).

4 Conclusions

Our data provide insight into the mechanism of complex formation and the heat-dependent changes of β LG-retinol acetate complex. The complex was thermally treated at different temperatures ranging from 25 to 85 °C. Fluorescence spectroscopy revealed that the complexes were stable in 25–60 °C, whereas at higher temperatures, the polypeptide chain unfolds. The *in silico* approach allowed deeper understanding of the main conformational changes occurring in the models equilibrated at 25 and 75 °C. The thermal treatment led to changes in the tertiary structure of protein, and the retinol binding site became more accessible and a higher affinity was observed. These results can help better understanding of the heat-induced conformational changes of β LG-retinol acetate complex, providing useful information for potential use of the complex in different food matrices.

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