The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2017), 41(2), 62-74

ORIGINAL RESEARCH PAPER

pH INDUCED STRUCTURAL CHANGES OF THE COMPLEX FORMED BETWEEN CAROTENOIDS FROM SEA BUCKTHORN (*Hippophae rhamnoides* L.) AND BOVINE B-LACTOGLOBULIN

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Received on 8th May 2017
Revised on 6th September 2017

Detailed information regarding the pH-dependent conformational state of β-lactoglobulin in a complex formed with sea buckthorn extract was attained by fluorescence spectroscopy investigations. β-lactoglobulin is a small globular protein (MW ≈18 kDa) with a very well characterized structure that reveals several possible binding sites for ligands, whereas sea buckthorn has gained importance as a versatile nutraceutical, due to its high nutritive value in terms of carotenoids. The stability of the complex was tested at pH 4.5, 5.2, 6.5 and 8.8. Higher fluorescence intensity was observed at acidic conditions compared to neutral and alkaline pH, regardless of the excitation wavelength, whereas the ANS fluorescence intensity was slightly higher at pH 8.8. Based on the maximum emission wavelengths values, it was suggested that Trp residues are buried while Tyr residues are partially exposed to the solvent at every tested pH. The synchronous spectra and the excitation-emission matrices did not reveal any significant pH induced conformational changes. It seems that β-lactoglobulin is stable within the pH range considered in our experiments, with no significant conformational changes within the complex.

Keywords: β-lactoglobulin, carotenoids, fluorescence, sea buckthorn

Introduction

Natural products for food and nutritional supplements have gained increased attention in recent years, leading to an expanding interest in the beneficial health effects of plant derived compounds (Korekar *et al*., 2014). High amounts of vitamin C, flavonoids, oils and oil-soluble compounds, as well as minerals, are present in the sea buckthorn (SBT) berry (Kallio *et al*. 2000), which also contains many bioactive substances and can be used to treat several diseases, such as cardiovascular disease, cancer, and acute mountain sickness (Negi *et al*., 2005). Different experimental studies of alcoholic and hydroalcoholic extracts of fruits, seeds and leaves of SBT have confirmed its medicinal and nutritional value (Geetha & Gupta, 2011; Upendra *et al*., 2008). All the parts of this plant are

considered to be a good source of a large number of bioactive compounds, including carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, minerals, etc. (Upendra *et al*., 2008, Kumar *et al*., 2011) which contribute to its wide usage as a natural antioxidant. The berries color may differ from orange-yellow to red fruits, being a rich source of valuable compounds such as multiple vitamins (C and E), carotenoids (β-carotene, lycopene, lutein and zeaxanthin), flavonoids (isorhamnetin, quercetin, isorhamnetin-3-beta-D-glucoside; isorhamnetin-3-beta-D-glucosaminide; kaempferol, etc.) organic acids, amino acids, micro and macronutrients (Yang & Kallio, 2001; Kallio *et al*., 2002). Rich carotenoid lipoprotein complexes have been extracted from SBT berry pulp and studies showed that carotenoids and fatty acid esters are more stable in their supra-molecular lipoproteic complexes, stored into oleosome vesicles where their physiological functions are better kept (Socaciu & Noke, 2003).

β-carotene (BC) is widely used as a colorant in foods and beverages. However, the use of BC as functional food in food industry is currently limited because of its poor water solubility, high chemical instability, and low *in vivo* bioavailability. The encapsulation of BC can be used to improve the aqueous solubility, physico-chemical stability, and bioavailability (McClements *et al*., 2015).

β-lactogobulin (β-LG), the major whey protein in bovine milk, is an extensively studied protein and is known to bind hydrophobic ligands such as fatty acids or vitamins (Le Maux *et al*., 2014). The protein is included in the lipocalin-protein family because of its high affinity to small hydrophobic ligands (Li *et al*., 2013). The protein contains 16 free amino groups that can act as a binding site for potential covalent ligands (Morgan *et al*., 1999). Based on this good biocompatibility and biodegradability, β-LG could be considered a natural carrier for lipid-soluble compounds. Hence, β-LG would be a suitable carrier for β-carotene and related derivatives and it should also improve the solubility and bioavailability of these compounds (Gholami & Bordbar, 2014). However, the binding ability depends on the pH value. At a pH higher than 7.0, the EF loop of the protein is open, allowing ligands to enter the hydrophobic core (Li *et al*., 2013).

Therefore, the aim of the present study was to investigate the effect of pH on the β-LG- carotenoids from the sea buckthorn extract (CSB) complex, in relation with the protein structural changes as mainly followed by fluorescence spectroscopy. The fluorescence spectroscopy methods involved the use of intrinsic and extrinsic fluorescence, phase diagram, synchronous spectra, three-dimensional fluorescence spectroscopy and quenching experiments.

Materials and methods

Materials

SBT berries were purchased from the local market (Galați, Romania) in October 2015 and immediately stored at -70˚C until use. β-LG (90 purity %, A and B genetic variants) from bovine milk, β-carotene, 1-anilino-8-naphtalenesulphonic acid (ANS), acrylamide and potassium iodide (KI) were purchased from Sigma

(Sigma–Aldrich Co., St. Louis, MO). Unless otherwise stated, all other reagents were of analytical grade.

Carotenoids extraction

The extraction of carotenoids from SBT was performed according to a slightly modified method of Taungbodhitham *et al*., (1998). Briefly, five grams of sea-buckthorn berries were extracted in 35 mL of ethanol:hexane solutions (4:3, v/v) containing 0.05 g magnesium carbonate, on an orbital shaker for 1 h at room temperature. After extraction, the supernatant was separated and the residue was re-extracted with 70 mL ethanol:hexane solutions (4:3, v/v). The resulted residue was washed with 25 mL ethanol and afterwards with 12.5 mL hexane, while the extract was washed with 100 mL 10% NaCl and 150 mL of water. The carotenoids extract (CSB) was concentrated at 40°C to dryness, dissolved in 10 mL ethanol (70%) and filtered through 0.45 μm membranes. CSB was quantified using a colorimetric method. Shortly, 1 mL of CSB ethanolic extract was added to 0.5 ml 0.05 g/L NaCl, vortexed for 30 s, and centrifuged at 1500 g for 10 min. The supernatant was diluted, and the absorbance at 460 nm was measured. The amount of CSB was calculated by plotting a calibration curve with β-carotene as standard (0-0.5 mg/mL).

Preparation of β-LG-CSB complex

To obtain the protein solution, β-LG was weighed and dissolved in 0.01 M Tris-HCl buffer solution at different pH values (4.5, 5.2, 6.5 and 8.8), at a concentration of 3.0 mg/mL. Fresh CSB solution was prepared by dissolving the CSB extract in ethanol to achieve a concentration of 4 μM. β-LG-CSB complex was prepared by simply mixing the two components. The CSB extract was added to the protein solution to reach a final protein/CSB molar ratio of 1:1. The resulting ethanol concentration never exceeded 5% (v/v), which had no appreciable effect on the protein structure (Liang *et al*., 2007).

Fluorescence spectroscopy

All fluorescence spectra were performed on a LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA) equipped with the Perkin Elmer FL Winlab software. The fluorescence spectroscopy investigations, which consisted of intrinsic and extrinsic fluorescence, phase diagram, synchronous spectra, excitation-emission matrix spectroscopy and fluorescence quenching experiments, were performed as described earlier by Dumitraşcu *et al*., (2015). Different excitation wavelengths were used for the intrinsic fluorescence studies, namely 274 nm, 280 nm and 292 nm, while the emission spectra were collected from 310 nm to 420 nm. Both the excitation and emission slit widths were set at 10 nm.

Statistical analysis

All experiments were performed in triplicates with duplicate samples. The results were expressed in terms of average and standard deviation values. The statistical analysis of data was performed using the Data analysis tool pack of the Microsoft Excel software.

Results and discussion

Intrinsic fluorescence

In proteins, three aromatic amino acids, namely Trp, Tyr and Phe, are fluorescent. However, Mocz & Ross (2015) suggested that Trp dominates over the other two aromatic amino acids as a relatively stronger fluorescence emitter due to its higher molar absorption coefficient, higher quantum yield, and the possibility of more efficient energy transfer mechanisms compared to Tyr and Phe. Therefore, in our study, in order to obtain a complete picture of the pH-induced conformational changes of β-LG in the complex, three different excitation wavelengths were used in terms of intrinsic fluorescence intensity and maximum emission wavelengths (Figure 1).

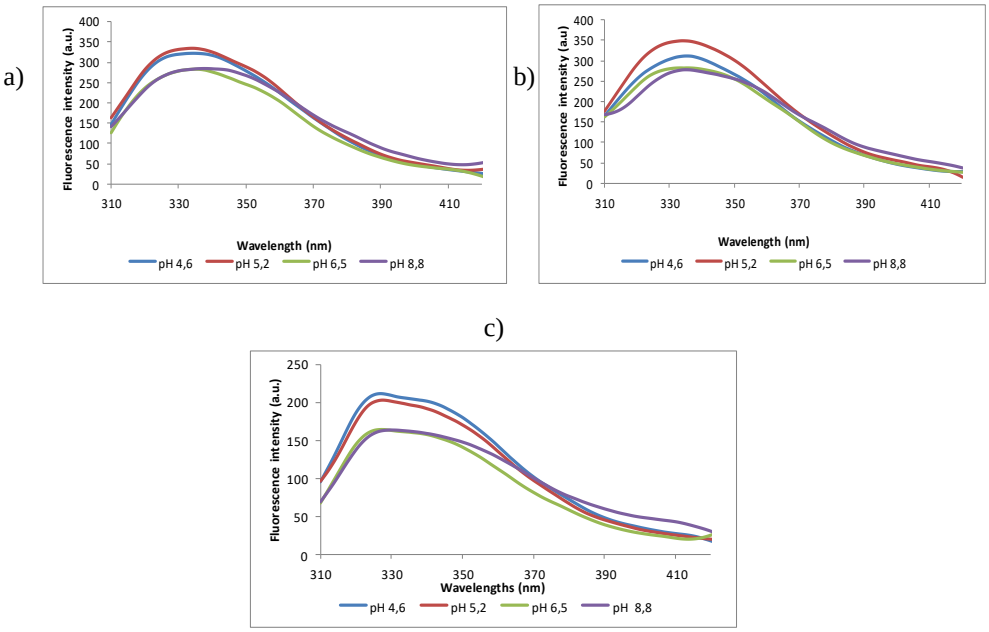


Figure 1. pH-induced structural changes of β-LG-CSB complex monitored by emission spectrum. The excitation wavelength was 274 nm (a), 280 nm (b) and 292 nm (c) and the spectra were collected between 310 and 420 nm. Three independent tests were carried out in each case and SD was lower than 2.5%.

When excited at 292 nm, the emission peaks were located at 327 nm for pH 4.6, 5.2 and 6.5 and at 329 nm for pH 8.8. At the excitation wavelength of 280 nm, the emission peaks were positioned at 335 nm for pH 4.5 and 8.8, at 334 nm for pH 5.2 and at 333 nm for pH 6.5, whereas when exciting at 274 nm the emission peaks were located at 334 nm which corresponds to pH 4.5 and 5.2, at 335 nm for pH 6.5 and at 337 nm for pH 8.8. At the acidic pH, higher fluorescence intensity was observed when compared to neutral and alkali pH, regardless of the excitation wavelength. From our results, it can be concluded that in the complex, the Trp residues are assigned to be buried in the protein core, since the maximum

fluorescence emission (λ_{max}) at excitation wavelength of 292 nm is lower than 330 nm. The Tyr residues are partially exposed to the solvent since λ_{max} is higher than 330 nm.

It is well known that each monomer of β-LG contains two Trp (Trp^{19} and Trp^{61}) and four Tyr residues (Tyr^{20}, Tyr^{42}, Tyr^{99} and Tyr^{102}) (Liang & Subirade, 2012). In the structure of β-LG, Trp^{19} that is located in the first strand of the β-sheet is buried, whereas Trp^{61} is being exposed to the solvent (Kuwata *et al*., 2001). Albani *et al.* (2014) suggested that only Trp^{19} residue that is buried in the hydrophobic pocket of β-LG emits, whereas the Trp^{61} residue present at the protein surface near the aperture of the pocket does not emit. The increase in fluorescence suggests that one or both residues are buried in a nonpolar environment during the initial stages of folding whereas the subsequent decrease can be attributed to the quenching of Trp^{61} in its native environment (Kuwata *et al*., 2001). Tyr^{42} and Tyr^{102} residues are buried, while Tyr^{20} and Tyr^{99} are exposed (Renard *et al*., 1998).

Phase diagram

As it has been mentioned earlier (Stănciuc *et al.*, 2012), the phase diagram method allows the building of a diagram by plotting $I_{\lambda 1}$ versus $I_{\lambda 2}$ (where I_1 and I_2 are the spectral intensity values measured at the λ_1 and λ_2 wavelengths); in order to identify the partially unfolded and hidden intermediate species that may occur in the unfolding mechanism of protein molecules (Kuznetsova *et al*., 2004). This correlation may be linear or non-linear due to the protein environment changes. A linear plot involves an *all-or-none* transition between the two conformations, while a non-linear plot reflects the sequential character of structural transformations (Kataeva *et al*. 2004). This method was used in the present study to analyze the unfolding/refolding mechanism of β-LG when in complex with CSB, by identifying the intermediate states upon changing the pH of the complex.

Figure 2 shows the plot of I_{320} versus I_{365} of the pH induced structural changes of β-LG-CSB complex.

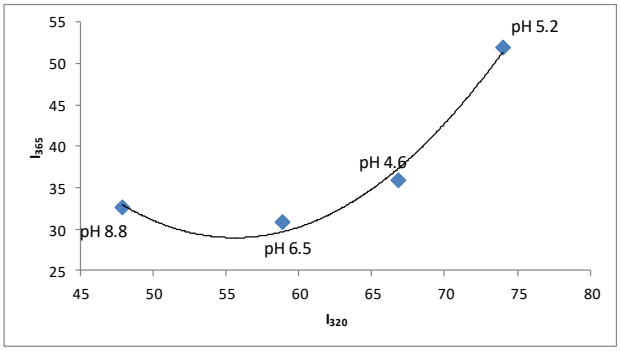


Figure 2. Phase diagram analysis of pH-induced conformational changes of β-LG-CSB complex based on the intrinsic fluorescence intensity values measured at wavelengths 320 and 365 nm. The pH values are indicated in the vicinity of the corresponding symbol. Three independent tests were carried out in each case and SD was lower than 3.5%.

The non-linearity of the correlation suggests a *three-state transition* between the several structurally distinct conformations. Depending on the solution conditions, β-LG can exist in one of its six pH-dependent structural states, as suggested by Taulier *et al*. (2001). The first transition of β-LG starts at pH 2.0 and it is followed by the second one, namely by the dimer-to-monomer transition between pH 2.5 and pH 4.0. The third transition, known as N-to-Q (*native to acidic state*) transition, occurs between pH 4.5 and pH 6.0 and is accompanied by increases in volume, whereas the so called *Tanford transition* is centered at pH 7.5 and is accompanied by a decrease in volume. The main structural change of the protein is related to the displacement of the EF loop which opens the interior of the calyx above pH 7.5 (Qin *et al*., 1998). The previously buried carboxyl group of Glu^{89} becomes exposed. Taulier *et al*. (2001) suggested also an increase of the surface area and intrinsic volume when the pH increases from 6.2 to 8.2.

ANS fluorescence intensity

ANS is extensively used for the identification of the external binding sites of proteins. The mechanism of interaction was explained by Gasymov & Glasgow (2007), this involving an increase in the ANS fluorescence with a hypsochromic shift due to the interaction of the charged group of Lys and Arg with the sulfonate group of ANS. A positive charge near the -NH group of the ANS changes the rate of the intramolecular charge transfer process by producing a blue shift of fluorescence.

It has been suggested that β-LG has two binding sites of ANS: one externally located close to the hydrophobic cluster at the surface of protein and an internal one, located inside the hydrophobic core of the protein (Vetri & Militello, 2005).

The external site is responsible for a nonspecific interaction with ANS, unlike the internal one which contains one disulphide bridge (Cys^{106}-Cys^{119}) (Collini *et al*., 2000). The ANS binding to β-LG-CSB complex at different pH values is given in Figure 3.

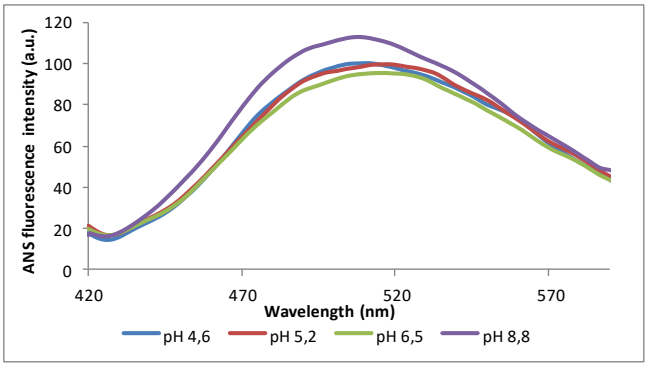
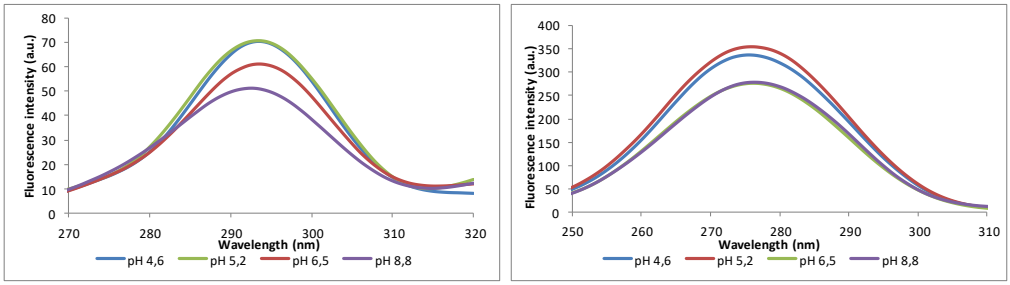


Figure 3. pH-induced structural changes of β-LG-CSB complex monitored by ANS fluorescence intensity. The excitation wavelength was 365 nm and the spectra were collected between 400 and 600 nm. Three independent tests were carried out in each case and SD was lower than 2.5%.

An increase in the ANS fluorescence intensity at pH 8.8 was observed, whereas the maximum emission wavelength varied from 511 nm at pH 4.6 to 517 nm at pH 5.2. Blue-shifts of 3 nm and 9.5 nm were registered at pH 6.5 and 8.8 when compared to pH 5.2, suggesting that the hydrophobic residues were exposed to a non-polar environment.

Synchronous spectra

Synchronous fluorescence spectroscopy (SFS) plays an important role in the simultaneous analysis of multicomponent samples due to the remarkable advantages of spectral simplification, light scattering reduction, and selectivity improvement over the conventional fluorescence spectroscopy (Li *et al*., 2011). The characteristic features of the Tyr residues and Trp residues were obtained after setting the $\Delta\lambda$ at 15 nm and 60 nm, respectively. SFS of the complex at different pH values at $\Delta\lambda$ of 15 and 60 nm are shown in Figure 4 a and b.



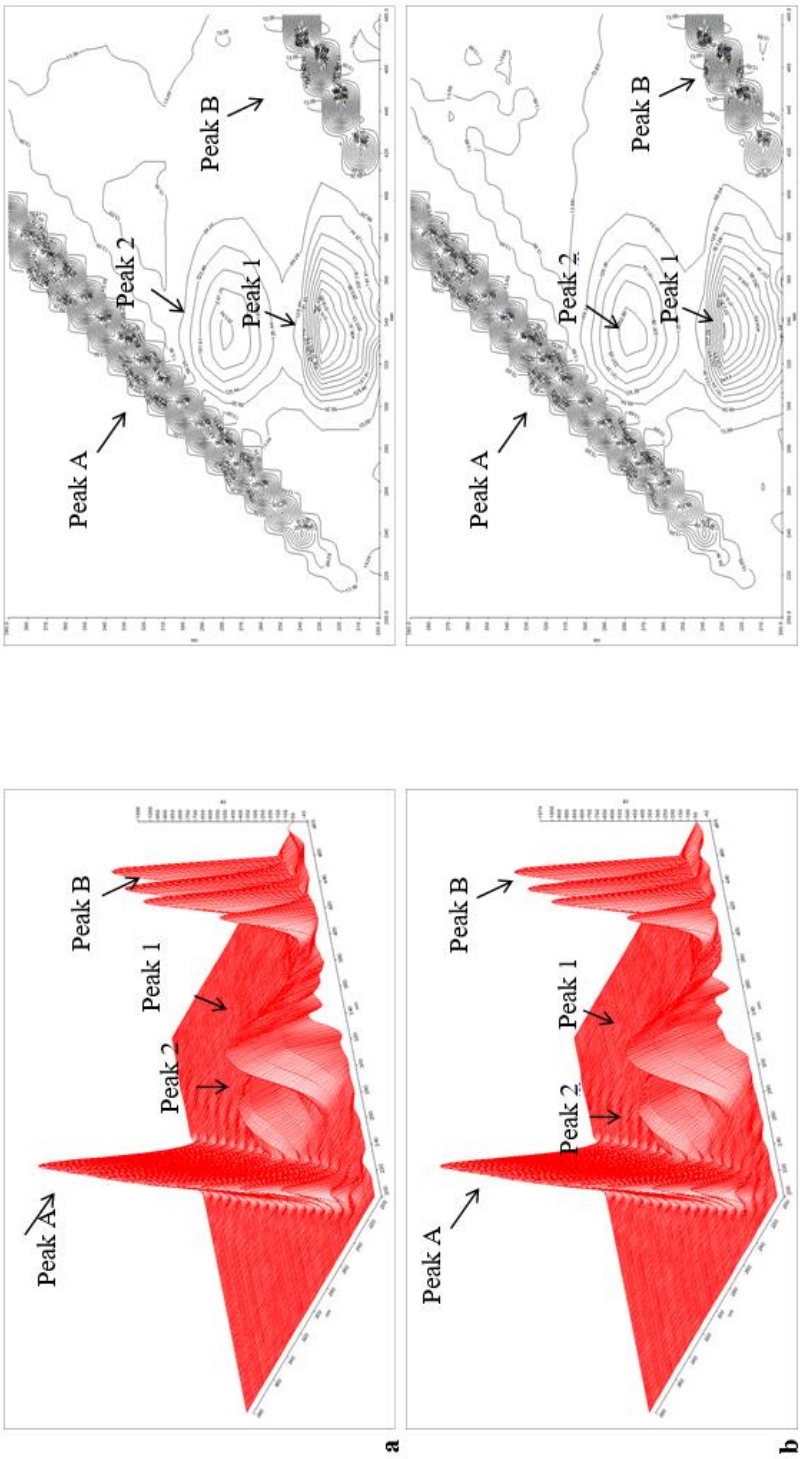
a) b)

Figure 4. Synchronous fluorescence spectra of β-LG-CSB complex at Δλ = 15 nm (a) and Δλ = 60 (b) nm at different pH values. Three independent tests were carried out in each case and SD was lower than 3.5%.

The shifts in the position of λ_{max} correspond to changes of polarity around the chromophore molecules. In Figure 4 a, it can be seen that the spectrum had a maximum at 293 nm at all the pH values tested, with a small 1 nm blue-shift at pH 8.8. In Figure 4 b, it can be observed that the maximum emission was located at 275 nm at pH 4.5, whereas a small red-shift of 1 nm was found at higher pH values. Therefore, it can be concluded that the pH induced conformational changes that led to a partial burial of Tyr residues at alkali condition and exposure of Trp residues at pH higher than 5.2.

Three-dimensional fluorescence spectroscopy (EEMs)

The last method for complex multi-compounds mixture detection is to record the fluorescence signal as excitation-emission matrices (EEMs). EEMs represent the fluorescence contour maps, in which repeated emission scans are collected at numerous excitation wavelengths providing highly detailed information. Moreover, the contour spectra can provide information to understand the structure and conformational variation of the new complex (Dumitraşcu *et al*., 2015). The 3D fluorescence spectra of β-LG-CSB complex at different pH values are given in Figure 5.


Peak A
Peak 2
Peak 1
Peak B
Peak A
Peak 2
Peak 1
Peak B
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Peak 1
Peak 2
Peak B
a
Peak A
Peak 2
Peak 1
Peak B
b

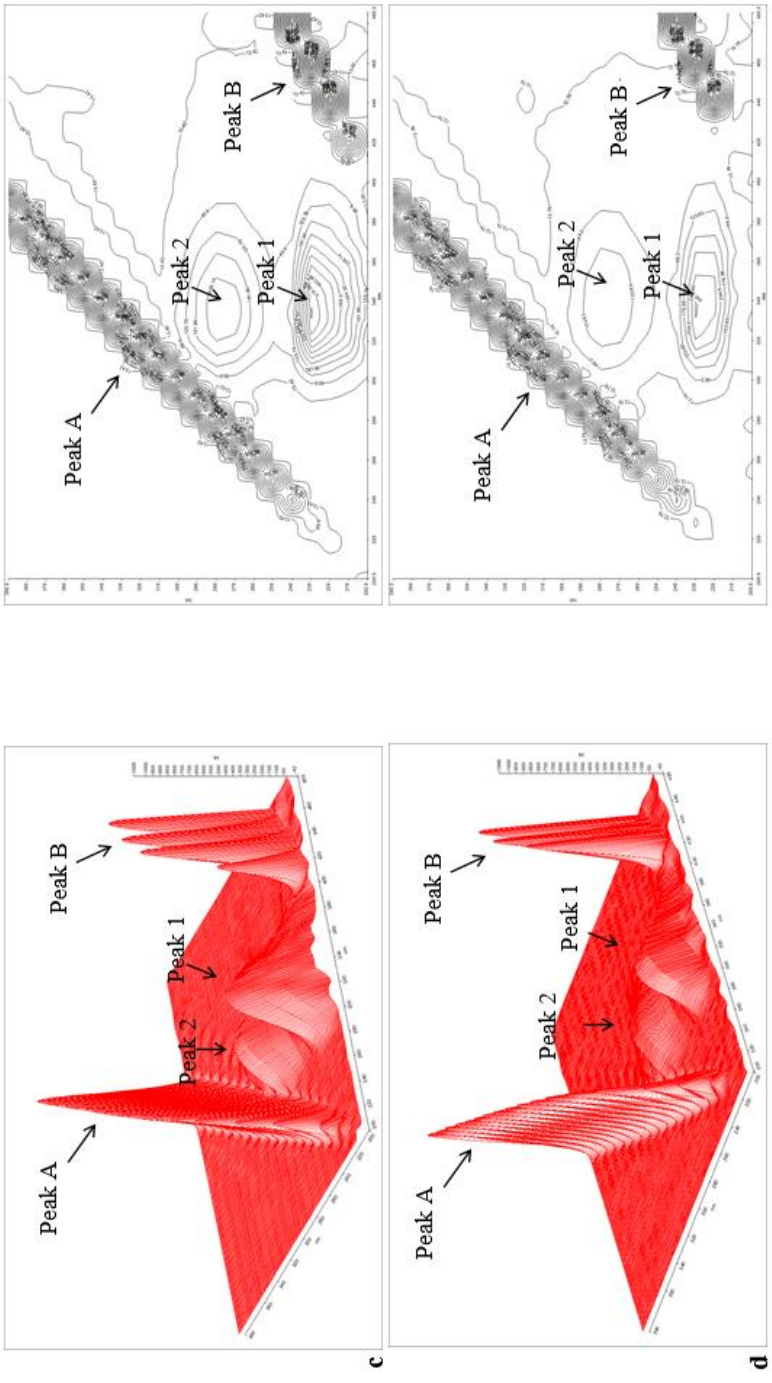

Figure 5. The EEMs spectra of β-LG-CSB complex at pH 4.6 (a), 5.2 (b), 6.5 (c) and 8.8 (d)

In addition, the contour map provides a bird's eye view of 3D fluorescence spectra. Peak A denotes the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$), peak 1 mainly reveals the spectral characteristics of Trp and Tyr residues, peak 2 reflects the fluorescence characteristic of polypeptide backbone structures, whereas peak B is the second-order scattering peak ($\lambda_{em} = 2\ \lambda_{ex}$) (Figure 5). The changes of the three dimensional fluorescence spectra of β-LG-CSB complex are listed in Table 1.

From the data presented in Table 1 it can be observed that the changes in the intensity of both peaks follow the same pattern, regardless of the pH value.

Table 1. Characteristic parameters of the three-dimensional fluorescence spectra of β-LG-CSB complex at different pH values

		pH values			
		4.5	**5.2**	**6.5**	**8.8**
Peak 1	Peak position (λ_{ex}/nm, λ_{em}/nm)	280/336	280/335	280/336	280/335
	Stokes shift Δλ (nm)	56	55	56	55
	FI (a.u.)	309.48±25.47	330.84±47.57	288.54±37.84	174.95±54.67
Peak 2	Peak position (λ_{ex}/nm, λ_{em}/nm)	230/335	230/335	230/334	230/335
	Stokes shift Δλ (nm)	105	105	104	105
	FI (a.u.)	628.38±12.54	637.01±11.47	578.81±24.78	349.03±45.45

The increase of peak **2** intensity at pH 5.2 reveals a decrease in the polarity surrounding the Trp and Tyr residues, by increasing the exposure of some hydrophobic regions. The fluorescence intensity of peak **2** increases at pH 5.2, followed by a decrease at higher pH values.

Quenching experiments

Quenching experiments were performed for monitoring the pH induced conformational changes of β-LG-CSB complex. The K_{sv} values are given in Table 2.

Table 2. The Stern Volmer quenching constant (K_{SV}) of β-LG-CSB complex at different pH values

pH values	K_{SV} **(10^{-3}L·mol^{-1})**	
	Acrylamide	KI
4.5	11.96±0.33	18.08±2.31
5.2	9.50±0.13	23.18±2.63
6.5	8.50±0.88	23.93±0.96
8.8	10.50±0.59	2.81±0.15

For the quenching experiments with acrylamide, the K_{SV} values showed a sharp decrease by increasing the pH, which can be attributed to the fact that Trp residues become less accessible to the acrylamide. Thus, the maximum K_{SV} value ($11.96\pm0.33\cdot10^{-3}$ mol^{-1} L) was obtained at pH 4.5 and the minimum value

($8.50\pm0.88\cdot10^{-3}$ mol^{-1} L) at pH 6.5, suggesting that Trp residues presented the highest exposure at the acidic pH, being less accessible to the quencher at neutral pH.

In the case of quenching with KI, which quenches only the fluorescence of exposed Trp, the lowest K_{SV} value was calculated at pH 8.8 ($2.81\pm0.15\cdot10^{-3}$ mol^{-1} L) and the highest at pH 6.5 ($23.93\pm0.96\cdot10^{-3}$ mol^{-1} L) (Table 2). The amplitude of the increase between pH 4.6 and 6.5 is not large (K_{SV} increases from $18.08\pm2.31\cdot10^{-3}$ mol^{-1} L to $23.93\pm0.96\cdot10^{-3}$ mol^{-1} L) ($p > 0.05$), but the extent of the pH interval of change implies that the ionization of more than one group should be responsible for the effect. It can be assumed that the Trp residues underwent structural changes that increase the accessibility of these residues to the quencher up to pH 6.5 and decrease at higher pH. It can be concluded that the accessibility of Trp residues to the quencher was significantly modified by changing the pH.

Conclusions

In this study, the pH-dependent conformational transitions of β-LG-CSB complex were investigated based on fluorescence spectroscopy techniques. Detailed information regarding the position of hydrophobic residues of β-LG-CSB complex was obtained by using three excitation wavelengths. The Trp residues were assigned to be buried in the protein core, since the maximum fluorescence emission at the excitation wavelength of 292 nm was lower than 330 nm. The Tyr residues are partially exposed to solvent since λ_{max} was higher than 330 nm. A *three-state transition* between several structurally distinct conformations was suggested based on the phase diagram technique. The ANS fluorescence investigation suggested an exposure of the hydrophobic residues to a non-polar environment at neutral and alkaline pH. The synchronous fluorescence spectroscopy investigations revealed the partial burial of Tyr residues at alkali condition and the exposure of Trp residues at a pH higher than 5.2. The quenching experiments with acrylamide showed a higher molecular flexibility at the acidic pH, while those with KI allowed obtaining maximum values of Stern-Volmer constants at pH 6.5. The data obtained in this study can be considered valuable in terms of obtaining new functional ingredients or by adding these ingredients in different food matrices.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS-UEFISCDI, project number PN-II-RU-TE-2014-4-0115.

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