



## Interactions of $\beta$ -lactoglobulin with Cationic Surfactants: Spectroscopy Study

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### Abstract

The interactions of  $\beta$ -lactoglobulin AB in the presence of cationic surfactants such as Cetyltrimethylammonium bromide and Cetyltrimethylammonium p-Toluenesulfonate have been investigated using a variety of experimental techniques such as conductivity, UV-Vis spectrophotometry and fluorimetry. The conductivity of surfactants aqueous solutions with  $\beta$ -lactoglobulin shows that the cmc of cationic surfactants decreases with increasing of counterion size. The results of UV-Vis and fluorescence studies show a red shift in wavelength and an increase in absorbance and intensity of the emission maximum of protein during the interactions of surfactants with  $\beta$ -lactoglobulin. The results of UV-Vis also show two distinct conformational changes at pHs 6.7 and 8.0 and the cooperative character of binding at pH 2.0. The results of fluorescence studies show that the binding strength of  $\beta$ -lactoglobulin / surfactant complex decreases with increasing of the pH.

**Keywords:**  $\beta$ -lactoglobulin; Cetyltrimethyl ammonium bromide; Cetyltrimethyl ammonium p-toluen sulfonate  
Conductivity, UV-Vis spectrophotometry; Fluorimetry.

### 1. Introduction

The interaction of proteins with surfactants has been a subject of extensive study for many years as it is of great importance in a wide variety of industrial, biological, pharmaceutical and cosmetic systems. Protein-surfactant interaction has been studied for many years with the aim to understand how surfactant binding affects protein structure and function [1]. It is known that the molecular structure of surfactant plays a substantial role in the protein-surfactant interaction [1-4]. The protein  $\beta$ -lactoglobulin (BLG) is the major protein of whey ruminant milk [5] and it is a member of the family so-called lipo calins.

BLG which is a small globular protein with well known structure [6,7]. It exists at neutral pH as a dimer. Each monomer is constituted by 162 amino acids with molar mass of about 18,400 Da. This small globular protein has a three-dimensional structure consisting of one  $\alpha$ -helix and nine anti-parallel  $\beta$ -strands, with eight  $\beta$ -sheets folded into a cone-shaped barrel forming a hydrophobic pocket [8].

It has been shown that BLG in the presence of similar concentration of sodium dodecyl sulfate (SDS) is established by a significant increase in the unfolding temperature while such increase of the SDS concentration causes unfolding of the protein. As opposed to these effects of the anionic surfactant, a minor decrease in the unfolding temperature was observed in the presence of cationic

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surfactant dodecyltrimethyl ammonium chloride under similar conditions [9]. A study of the interactions of the mixtures of cationic–anionic surfactants with BLG was reported by Lu et al. [10]. They concluded that the extent of interaction is dependent of the ratio of surfactants in mixture. Recently, the interaction of BLG with some ionic and non-ionic surfactants was studied by Taheri-Kafrani et al. [11,12]. Results have shown that BLG has conformational changes during its interaction with dodecyltrimethyl ammonium bromide (DTAB), SDS and Triton X-100 while retinol binding properties of BLG do not show any significant change in the presence of such surfactants. However, the variation of structural index parameters of BLG versus these surfactants concentration did not show sigmoidal feature, in order that no cooperative unfolding transitions have been detected.

In the present study, the interactions of cationic surfactants CTAB and CTAT with BLG have been studied using conductivity, UV–Vis and fluorescence spectroscopy to monitor the structural changes of protein when interacting with surfactants.

## 2. Materials and Methods

The  $\beta$ -lactoglobulin AB, CTAB and CTAT were purchased from Sigma Chemical Co., and used without further purification. All salts used for buffer preparation were analytical grade and dissolved in double distilled water. The 50 mM glycine buffer pH 2.0, the 50 mM phosphate buffer pH 6.7, and 8.0 were used as buffers. The BLG concentration was determined from the optical density of appropriate solution using the extinction coefficient at 280 nm of 17600 M<sup>-1</sup> cm<sup>-1</sup>.

### 2.1. Conductivity

The conductivity of the aqueous solutions of surfactants and the mixtures of surfactant and BLG was measured using Metrohm Conductivity. The critical micelle concentration (cmc) of surfactant was taken as the concentration corresponding to the break

point in the plot representing the variation of the specific conductivity  $K$  with the surfactant concentration [13]. The binding ratio of surfactants to BLG could be obtained by the equation [13]:

$$R = \Delta cmc / C_{BLG} \quad (1)$$

where  $\Delta cmc$  is the difference between cmc values of surfactant with and without BLG, and  $C_{BLG}$  is the concentration of BLG. The binding degree ( $\beta$ ) of the counterions, which reflects degree of surfactant counterion binding to the micelles, could be obtained by the equation below [14].

$$\beta = 1 - (\text{slope above } C_{cmc}) / (\text{slope below } C_{cmc}) \quad (2)$$

Where slope above  $C_{cmc}$  and slope below  $C_{cmc}$  are the slopes of conductivity versus surfactant concentration in solutions with surfactant concentration above and below cmc, respectively.

### 2.2. UV-Vis spectroscopy measurements

The absorbance measurements were carried out using Lambda 35 UV-Vis double beam spectrophotometer, which is well equipped with a thermostated cell compartment at 298 K. In UV-Vis spectroscopy measurements, the samples were put in quartz cuvettes of 1 cm optical path. In typical experiments 800  $\mu$ L of BLG solution were placed into the cuvette. The absorbance spectra were recorded between 250 to 350 nm after each addition of CTAB and CTAT stock solutions (10 mM) at 298 K. The observed absorbances were corrected for dilution. The BLG solutions were freshly prepared just before the measurements.

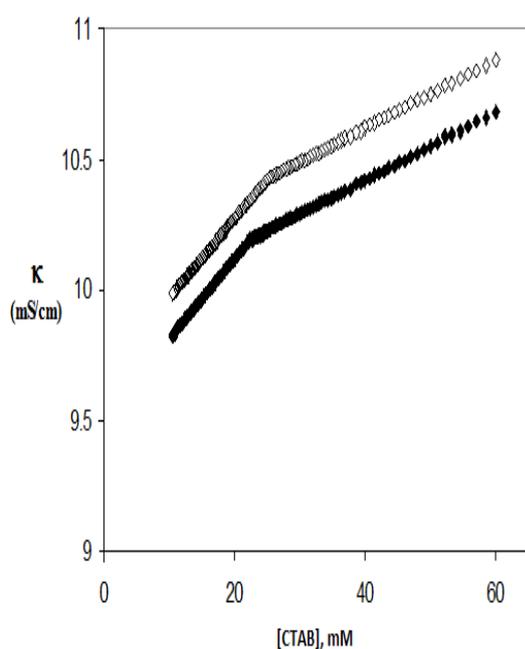
### 2.3. Fluorescence spectroscopy measurements

Fluorescence measurements were performed using Carry Eclipse Fluorescence Spectrophotometer at 298 K. Fluorescence spectroscopy was used to study the conformational changes of proteins when binding ligands occurred, because the intrinsic fluorescence of tryptophan (Trp) residues is particularly sensitive to their microenvironments [15]. In fluorescence measurements, the samples were put in quartz

cuvettes of 1 cm optical path. In typical experiments 3.0 mL of BLG solution were placed into the cuvette. Emission spectra were recorded after each addition of CTAB and CTAT stock solutions at 298 K. The excitation was performed at 280 nm and the emitted light was recorded between 300 to 400 nm. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were 3 and 5 nm. The experiments were performed in 50 mM glycine buffer at pH 2.0 and 50 mM phosphate buffer at pHs 6.7 and 8.0.

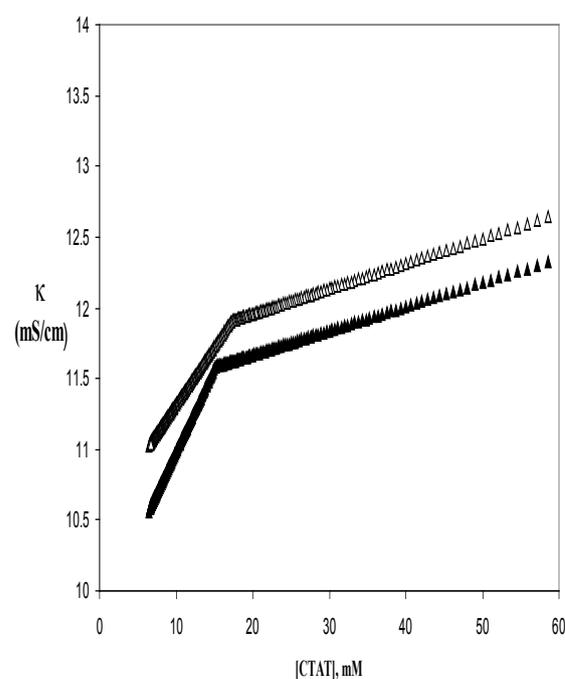
### 3. Results and discussion

#### 3.1. Conductivity



**Fig 1:** Variation of the specific conductivity of CTAB solutions at 298K in water (◆) and in BLG solution (1.0g/L, ◇).

Figs. 1 and 2 showed the conductivity of CTAB and CTAT aqueous solutions with or without BLG. The break point in the curves corresponded to the critical micelle concentration of cationic surfactants (listed in Table 1). Table 1 showed that the cmc of cationic surfactants decreased with the increase of counterion size. It indicated that surfactant with larger counterion has stronger tendency to form aggregates. In the presence of BLG, the cmc of surfactants increased. It implied that surfactants bound to BLG and higher concentration of surfactants was needed to form surfactant micelles. The variation of cmc in the presence and absence of BLG,  $\Delta$ cmc, decreased with counterion size (Table 1). It corresponded to the decrease of the binding



**Fig. 2:** Variation of the specific conductivity of CTAT solutions at 298K in water (▲) and in BLG solution (1.0g/L, Δ).

**Table 1.** Values of the cmc, the micelle binding degree at the cmc of surfactants ( $\beta$ ), the variation of cmc with and without BLG ( $\Delta$ cmc), and the binding ratio of surfactants to BLG (R) at 298K

Surfactants	In Water		In BLG		
	cmc(mM)	$\beta$	cmc(mM)	$\Delta$ cmc(mM)	R
CTAT	15.48	0.85	17.51	2.03	37.15
CTAB	22.64	0.58	25.80	3.16	57.83

ratio of surfactants to BLG (R) with increasing the surfactant counterion size (Table 1).

As shown in Table 1, the cmc of surfactants (CTAB and CTAT) both in single surfactants and in surfactant–BLG mixtures decreases with the increase of counterion size, that is, the aggregation of surfactants is affected by counterion size. On one hand, the CTAT with the more hydrophobic counterion has a greater tendency to form aggregates on BLG, which causes CTAT to form aggregates on BLG at lower concentration. On the other hand, the increase of hydrophobicity of surfactant counterion decreases the binding ratio of surfactants to BLG (R, shown in Table 1). The reason might be that, with the increase of the hydrophobicity of surfactant counterion, the tendency of surfactant counterion binding to surfactant aggregates ( $\beta$  values in Table 1) increases, which might screen the BLG–CTAT interaction. Lu and et.al [16] showed the cmc of perfluorooctanoate surfactants both in single perfluorooctanoate surfactants and in perfluorooctanoate surfactants–BLG mixtures decreases with the increase of counterion size, that is, the aggregation of perfluorooctanoate surfactants is affected by counterion size. Consequently, in the cooperative binding process, the counterions affect the aggregation of perfluorooctanoate ion on BLG.

### 3.2. UV–Vis Absorption Spectra

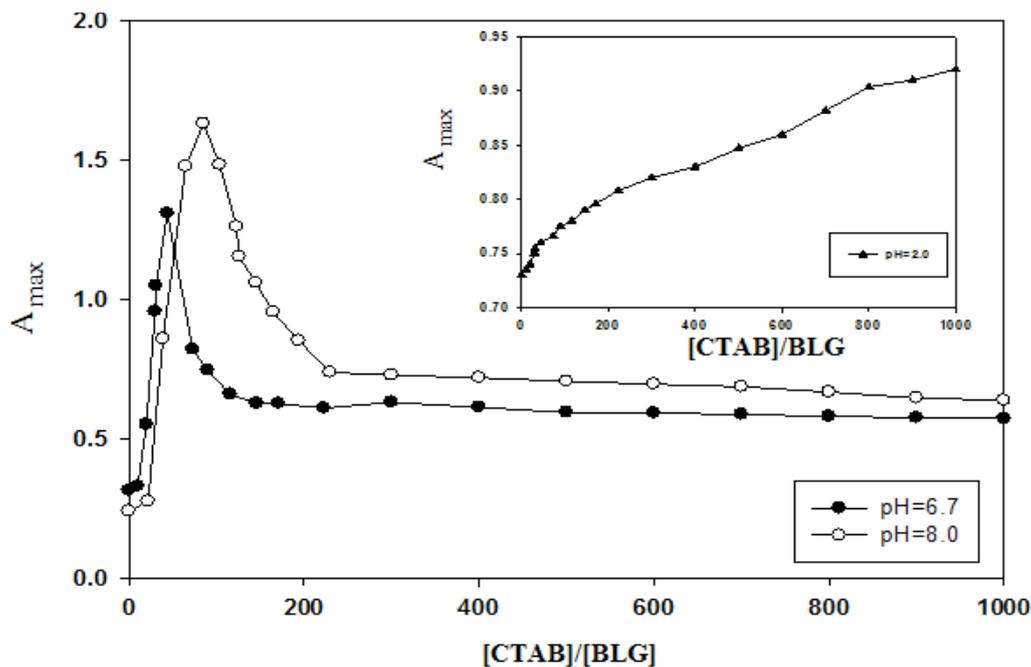
UV-visible spectroscopy was used to analyze the binding of CTAB and CTAT to BLG. The absorption maximum of BLG depends upon the microenvironment in which the probe is located although this dependence is significantly smaller than in case of fluorescence measurements. The study of the absorbance of BLG during its interactions with surfactants allows the determination of the micro polarity of the local environment surrounding the probe.

BLG molecule contains 14 Glu and 10 Asp residues, negatively residues which could be the binding sites of cationic surfactant [5]. At pHs 6.7 and 8.0, BLG is negatively charged

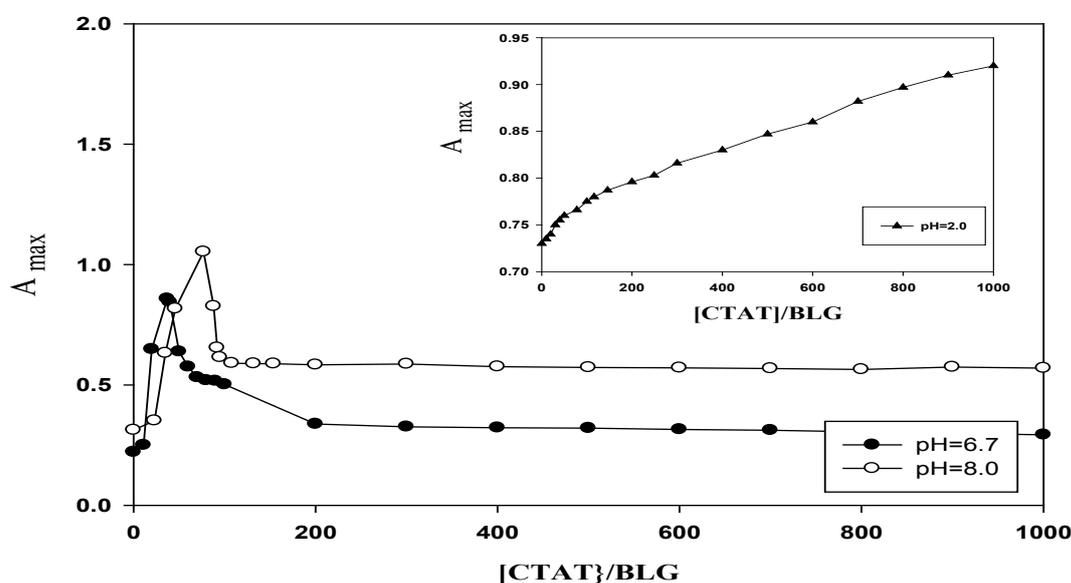
(the isoelectric point of  $\beta$ -LG is 5.2) [5], so it can form precipitates with oppositely charged surfactants CTAB and CTAT outstanding to the formation of neutral complexes of BLG / cationic surfactant. With the further increase of CTAB and CTAT concentrations, more cationic surfactants bind to the electrical neutral complexes, so the complexes of BLG /CTAB and BLG /CTAT are positively charged, and the precipitates are dissolved. Therefore, upon the increase of CTAB and CTAT concentrations, the mixtures of BLG / cationic surfactant underwent a transition from homogeneous solution to precipitates, and then to another homogeneous solution again. At pH 2.0, below the pKa of aspartates and glutamates BLG is positively charged and when cationic surfactants bind to BLG, the BLG /cationic surfactant complex is always positively charged and the precipitation is not observed. These results are similar to those of other oppositely charged protein-surfactant systems [17, 18].

Figs. 3 and 4 show the plot of the maximum of absorbance of BLG vs. molar ratio of [CTAB]/[BLG] and [CTAT]/[BLG] at pHs 2.0, 6.7 and 8.0 at 298 K. As shown in these figures, we have the same procedure at pHs 6.7 and 8.0 but different procedure at pH 2.0. Two distinct conformational changes can be distinguished at pHs 6.7 and 8.0. First, precipitation is observed at 10 and 22 molar ratio of [CTAB]/[BLG] at pHs 6.7 and 8.0, respectively and at 11 and 23 molar ratio of [CTAT]/[BLG] at pHs 6.7 and 8.0, respectively. The observed sharp increasing of absorbance is due to the formation of precipitate. These points are corresponding to the first break in this figure. Second precipitates dissolve at 120 and 230 molar ratio of [CTAB]/[BLG] at pHs 6.7 and 8.0, respectively and at 70 and 110 molar ratio of [CTAT]/[BLG] at pHs 6.7 and 8.0, respectively.

The cooperative character of binding is obvious at pH 2.0 and two notable point that has observed in case of pHs 6.7 and 8.0 was absent at this pH. These results are in good agreement with the data reported by Taheri-



**Fig.3:** Variation of absorbance of  $\beta$ -LG at 280 nm vs molar ratio of [CTAB]/[BLG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 and 8.0 in temperature of 298 K.



**Fig. 4:** Variation of absorbance of  $\beta$ -LG at 280 nm vs molar ratio of [CTAT]/[BLG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 and 8.0 in temperature of 298 K.

al[11] that BLG has conformational changes during its interaction with dodecyltrimethyl ammonium bromide (DTAB).

### 3.3. Fluorescence spectra

The BLG fluorescence mostly related with the microenvironment of the Trp residues of BLG

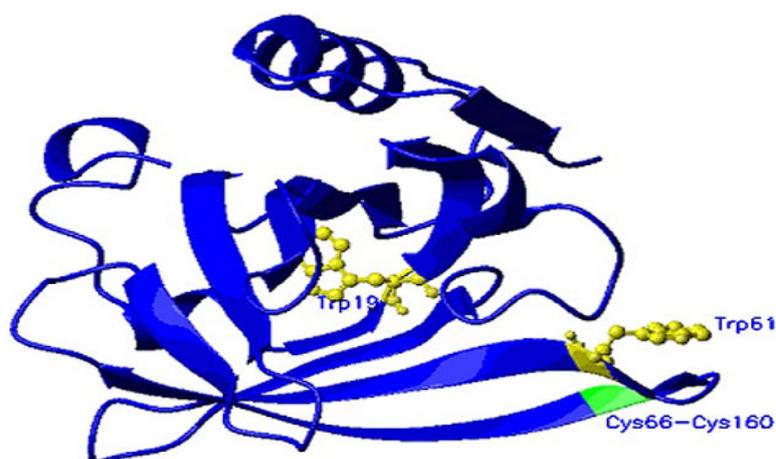
[19-20]. BLG contains two Trp residues, Trp 19 and Trp 61 [5]. Fig. 5 the localization of the Trp residues in the native BLG structure was illustrated using MOLMOL, a molecular graphics program for displaying, analyzing, and manipulating the three-dimensional structure of biological macromolecules [21,22]. As it shown in this Figure, Trp 19 is

Kafrani et al. in an apolar environment in the main cavity of BLG, whereas Trp 61 protrudes beyond the surface of the molecule and is quite close to the Cys 66-Cys 160 disulfide bridge [23-24]. Since the disulfide bridges are effective Trp fluorescence quenchers, the intrinsic fluorescence of BLG is almost exclusively attributed to Trp 19 [25, 26].

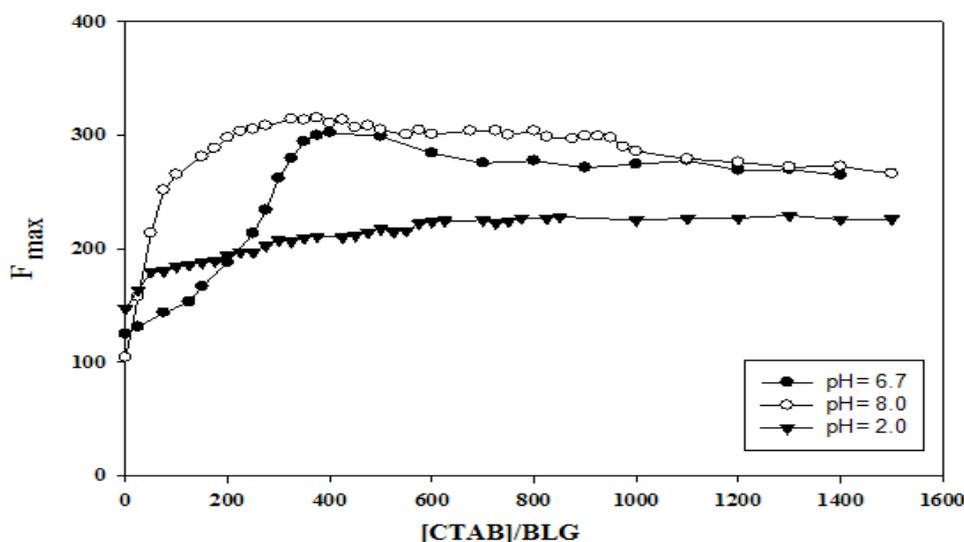
Figs. 6 and 7 show the changes of maximum of fluorescence intensity vs. [CTAB]/[BLG] and [CTAT]/[BLG] molar ratio at various pH and 298 K. Despite UV-vis results, no precipitation of  $\beta$ -LG was observed at any of studied pHs that is due to low concentration of protein at fluorescence experiments compared to UV-

vis experiments. As shown in this figure, the cooperative character of binding is obvious at all studied pH. Analysis of the location of these transition curves, shows that the binding strength decreases with the increase of the pH. This fact can be related to larger hydrophobic surface area of BLG /cationic surfactant complex at lower pH due to predominant nature of hydrophobic interactions.

It is known that the transfer of Trp to a hydrophilic environment and increase in hydrophobic interactions leads to a red shift in wavelength and to an increase in intensity of the emission maximum. As seen in 3D structure of native BLG, Trp 19, situated at the bottom



**Fig.5:** Localization of the Glu and Asp residues in the native BLG structure. The Glu and Asp residues are labeled as yellow and green, respectively.



**Fig. 6:** Changes of fluorescence emission maximum intensity of  $\beta$ -LG vs molar ratio of [CTAB]/[BLG] in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 and 8.0 in temperature of 298 K.

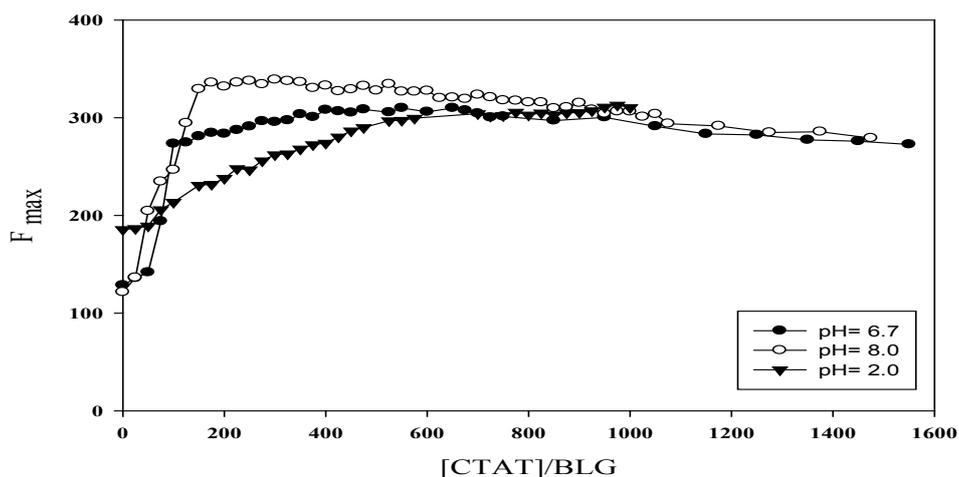


Fig. 7: Changes of fluorescence emission maximum intensity of  $\beta$ -LG vs molar ratio of  $[\text{CTAT}]/[\text{BLG}]$  in 50 mM glycine pH 2.0 and 50 mM phosphate buffer pH 6.7 and 8.0 in temperature of 298 K.

of the calyx formed by 8 antiparallel  $\beta$ -strands, contributes about 80 % to the total fluorescence [27] and a disulfide bond quenches Trp 61 emission [28]. Therefore, smaller quenching of Trp 61 by disulfide bond, when BLG binds to cationic surfactant, probably leads to an increase of the fluorescence intensity of BLG. It indicates also that the protein unfolds partially during its interaction with cationic surfactant.

The maximum of emission wavelength shifts from 223 to 229, 295 to 310 and 300 to 310 nm in the presence of CTAB at pH 2.0, 6.7 and 8.0, respectively. Also 301 to 312, 306 to 314 and 329 to 332 nm in the presence of CTAT at pH 2.0, 6.7 and 8.0, respectively. A red shift of the emission maxima indicates that at least part of the Trp residues is transferred /moved into more hydrophilic environment during the interaction of BLG with the cationic surfactant. Lu and et.al [29] showed addition of surfactants C10SO<sub>3</sub> and C10NE resulted in the enhancement of BLG fluorescence and the red shift of the emission maximum. This result is different from those in lysozyme–surfactant systems or BSA–surfactant systems, in which the addition of surfactants quenches the fluorescence of protein [30]. Similar results were also observed in BLG–SDS systems [31].

## Conclusions

In conclusion, the results clearly confirm the cmc of cationic surfactants decreases with increasing of counterion size. On the other hand, the increase of hydrophobicity of surfactant counterion decreases the binding ratio of surfactants to BLG. The cooperative character of binding is obvious at pH 2.0 and two distinct conformational changes can be distinguished at pHs 6.7 and 8.0. The binding strength of  $\beta$ -lactoglobulin / surfactant complex decreases with increasing of the pH.

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