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# Fluorescence spectroscopic study on interaction of retinol with $\beta$ -lactoglobulin in the presence of cetylpyridinium chloride

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**Abstract.** In our previous study thermodynamic denaturation of bovine  $\beta$ -lactoglobulin variant A (BLG-A), has been investigated in the presence of cetylpyridinium chloride (CPC) as a cationic surfactant. Here, the retinol binding property of BLG was determined at 298 K and pH 8.0 by spectrofluorimeter titration method, in the presence of CPC to elucidate the still unknown structure–function relationship in this protein. Comparison of the results allowed determining the binding of retinol by BLG in the presence of CPC. The two-way chemometrics method was used, to estimate the equilibrium concentration of components by analysis of fluorescence emission spectrum, in order to obtain its equilibrium concentration. The results indicate that the retinol binding properties of BLG do not show significant changes in the presence of this surfactant.

Keywords:  $\beta$ -Lactoglobulin, retinol, binding constant, cetylpyridinium chloride, spectrofluorimeter titration

## 1. Introduction

Fluorescence spectroscopy is a rapid and sensitive method for characterizing molecular environments and events. It can be used as a non-destructive analytical technique to provide information on the presence of fluorescent molecules and their environment in all sorts of biological samples. Because of its high sensitivity to the conformational state of macromolecule, fluorescence techniques are very useful tools to obtain thermodynamic and kinetic information about transitions of macromolecules, such as protein folding reactions [10]. The intrinsic fluorescence of tryptophanyl residues (Trp) are particularly sensitive to their micro-environments and provide appropriate methods to perform this kind of studies. Creamer and Busti et al. reported a red shift in  $\beta$ -lactoglobulin intrinsic fluorescence emission in accordance with the loss of the protein native structure promoted by urea denaturation [4,7]. This red shift indicates that the Trp residues in  $\beta$ -lactoglobulin moved from an apolar environment to a more polar region. The degree of exposure of Trp residues in  $\beta$ -lactoglobulin molecules can also be evaluated by following the external quenching of the intrinsic protein fluorescence by added solutes [3,7,11,20]. In a recent work, the surface hydrophobicity of whey protein concentrates was evaluated by application of this method [17]. Steady state polarization measurements can also be useful in determining the unfolding profile of proteins. Besides this, polarization of intrinsic Trp fluorescence can be useful in discriminating between dissociation and denaturation in multimeric proteins [22]. The objective of this work was to

propose a novel method to monitor the  $\beta$ -lactoglobulin equilibrium denaturation profiles by urea based on protein fluorescence quenching by acrylamide and to analyze the results using the new DCU model proposed by Galani and Apenten [13].

$\beta$ -lactoglobulin (BLG), a member of the so-called lipocalins, is the major protein of whey ruminant milk [28]. An enormous number of papers exist in the literature on this topic due to its ease of purification and importance for the food industry. BLG may play important roles in the binding and transporting hydrophobic ligands such as retinoids, alkenes and fatty acids. Different methods have been used for investigating its interactions with amphiphilic and hydrophobic ligands such as retinoids, long chain fatty acids and surfactants [26,27]. 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 [25]. The core of the BLG molecule includes a structural motif similar to that found in retinol binding protein (RBP) [21]. Like RBP, BLG is able to bind a wide variety of hydrophobic molecules [5,14]. Binding to BLG provides protection for retinol and  $\beta$ -carotene and they are not degraded due to heat, oxidation and irradiation [15]. BLG is known to bind tightly, *in vitro*, one retinol molecule per monomer [12].

Several studies on the retinol binding of BLG in the presence of various surfactants have been done, recently [23,24,26,27]. BLG has conformational changes during its interaction with sodium *n*-dodecyl sulfate (SDS) and Triton X-100 while retinol binding properties of BLG do not show significant changes in the presence of these surfactants [27]. Moreover, the retinol binding affinity has been measured in the presence of cationic *n*-decyltrimethyl ammonium bromides ( $C_n$ TABs). The relevant results represent the enhancement of retinol binding affinity in the presence of these surfactants [24].

In the previous study, the thermodynamic parameters of BLG denaturation were determined in the presence of cetylpyridinium chloride (CPC) [23]. In this work, the two-way chemometrics method was used for analyzing the fluorescence spectrum in order to precise estimate of binding affinity of retinol to BLG in the presence of CPC. These results may be valuable by considering this fact that CPC is a mild antibacterial used often in the antibacterial protection in buccal treatments and also in food conservation [2,19].

## 2. Experiment section

### 2.1. Materials and methods

$\beta$ -Lactoglobulin genetic variant A, retinol and CPC were purchased from Sigma Chemical Co. and used without further purification. All the salts that used for buffer preparation were analytical grade and were dissolved in doubly distilled water. All the solutions were freshly used after preparation.

The samples were measured on a Jasco FP-750 fluorescence spectrometer with a 10 mm quartz cuvette at 298 K. Chemometrics evaluations were conducted using the N-way toolbox for Matlab version 2.1 [9], available at <http://www.models.kvl.dk/source>.

### 2.2. Procedures

The concentrations of BLG and retinol were determined experimentally using the values of 17,600 and 52,480  $M^{-1} \cdot cm^{-1}$  for the molar absorption coefficient ( $\epsilon$ ) at 279 nm and at 325 nm, respectively [6, 8]. For optical measurements, all the solutions were prepared in 50 mM phosphate buffer at pH 8.0. The binding of retinol was measured by following the changes in retinol fluorescence at  $\lambda_{max}$ . The following

procedure was used during the titration of retinol solutions or various [CPC]/[retinol] solutions, with BLG: 1 ml, retinol solutions or various [CPC]/[retinol] solutions were placed in a cuvette and small increments (0–100  $\mu\text{l}$ ) of the BLG solution were injected into the cuvette. These spectra were recorded from 350 to 550 nm with an excitation wavelength of 325 nm. Spectral resolution was 5 nm for both excitation and emission. The final CPC concentration was about 150  $\mu\text{M}$  when there was no inner filter effect due to the presence of CPC.

### 3. Theory section

A two-way array or data matrix usually has rows representing objects and columns representing variables. This array has  $I \times J$  entries, e.g.,  $I$  objects and  $J$  variables measured on each object. A two-way model of matrix  $\mathbf{X}(I \times J)$ , with typical elements  $x_{ij}$ , based on a singular value decomposition truncated to  $R$  components reads in summation notation as

$$x_{ij} = \sum_{r=1}^R a_{ir} g_{rr} b_{jr} + e_{ij}, \quad i = 1, \dots, I, j = 1, \dots, J, \quad (1)$$

where,  $\mathbf{A}(I \times R)$ , with the elements  $a_{ir}$ , and  $\mathbf{B}(J \times R)$ , with the elements  $b_{jr}$ , are both orthogonal matrices, and  $\mathbf{G} = \text{diag}(g_{11}, \dots, g_{rr})$  is a diagonal matrix called the singular value or core-matrix containing the  $R$  largest singular values of  $\mathbf{X}$  in decreasing order and  $e_{ij}$  are the residuals. Written in matrix notation, Eq. (1) becomes

$$X = AGB' + E, \quad (2)$$

where  $E$  contains the  $e_{ij}$  and  $\mathbf{B}\mathbf{B}' = \mathbf{I}$  (identity matrix). The values of  $g_{rr}$  can be absorbed in  $a_{ir}$ ,  $b_{jr}$  or in both, which is only a matter of choice. This leads to the following model:

$$X = AB' + E. \quad (3)$$

The model may alternatively be written as:

$$x_{ij} = \sum_{r=1}^R a_{ir} b_{jr} + e_{ij}. \quad (4)$$

### 4. Results and discussion

Fluorescence is a useful approach for investigating intermolecular interactions because the photophysical character of the fluorophore is sensitive to the polarity of its surrounding environment. Generally, fluorescence intensity increases by decreasing polarity. As shown in Fig. 1, a gradual increase in retinol fluorescence intensity is observed by increasing BLG concentration, suggesting that retinol transfers from the hydrophilic environment of the aqueous solution to a more hydrophobic environment.

Generally, complexes involving noncovalent bonds are reversible; for example, the binding of retinol to retinol binding protein is involved in the equilibrium between retinol and retinol–protein complexes

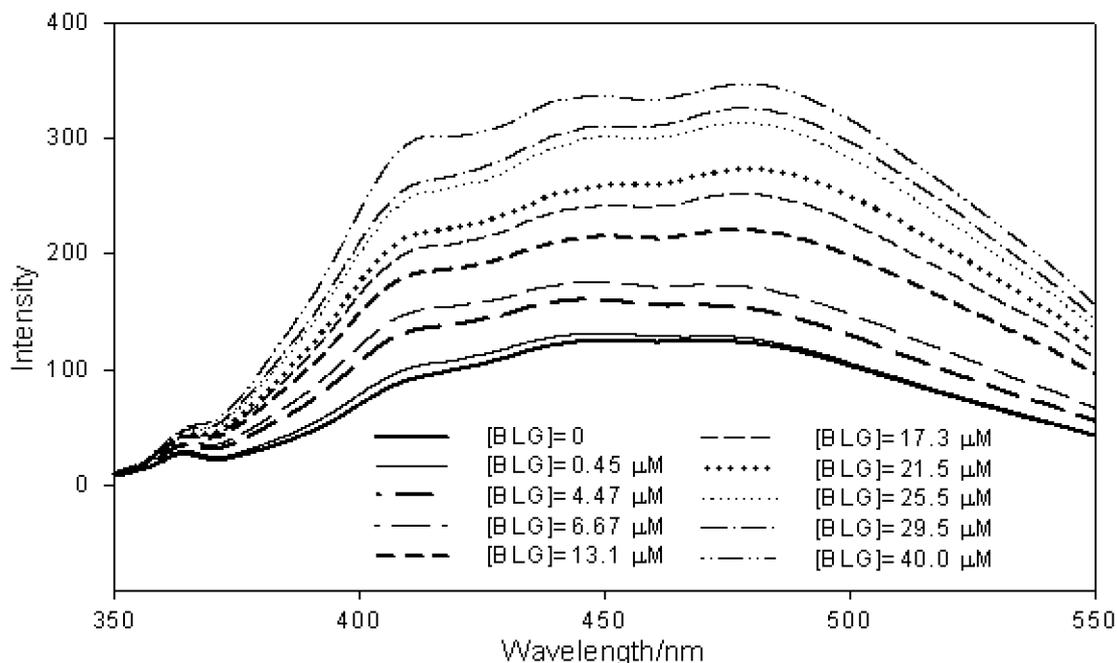


Fig. 1. Fluorescence emission spectra of retinol in the presence of various BLG concentrations ( $\lambda_{\text{ex}} = 325 \text{ nm}$ ,  $[\text{retinol}] = 10 \text{ } \mu\text{M}$ , spectral band-width was 5 nm for both excitation and emission wavelength). The measurements were done in 50 mM phosphate buffer at pH 8.0.

[16]. Likewise, free and BLG-bound retinol may be in equilibrium. For 1:1 complexes, the retinol fluorescence data in Fig. 1 can be analyzed using Eq. (5) [18]:

$$1/\Delta F_i = 1/\Delta F_{\text{max}} + 1/(K_a \Delta F_{\text{max}} [\text{BLG}]_i), \quad (5)$$

where  $\Delta F_i$  is the change of retinol fluorescence intensity for  $i$ th titration step;  $\Delta F_{\text{max}}$  is the maximal change of fluorescence intensity;  $K_a$  is the binding constant and  $[\text{BLG}]_i$  is the total concentration of BLG at  $i$ th titration step.  $K_a$  can be calculated from the slope of the straight line of  $1/\Delta F_i$  as a function of  $1/[\text{BLG}]_i$  using Eq. (5). It has been found out that using total concentration of BLG in Eq. (5) is an approximation; thus, this equation cannot predict the actual binding constant. BLG concentration is changed during the spectrofluorimeter titration. Therefore, the equilibrium concentration of BLG in each titration step is needed for calculating the actual binding constant. In order to overcome to this shortcoming, the two-way chemometrics method was used. The main advantage of this is that it allows concentration information of an individual component to be extracted in the presence of other constituents. Thus, it is highly useful for solving analytical problems involving a complex matrix. Figure 2 shows the separated fluorescence emission spectrum of BLG-retinol solution using this method. The equilibrium concentration of BLG can be calculated from emission intensity of separated fluorescence spectra.

Equation (5) can thus be rewritten as follows:

$$1/\Delta F_i = 1/\Delta F_{\text{max}} + 1/(K_a \Delta F_{\text{max}} [\text{BLG}]_{\text{eq},i}), \quad (6)$$

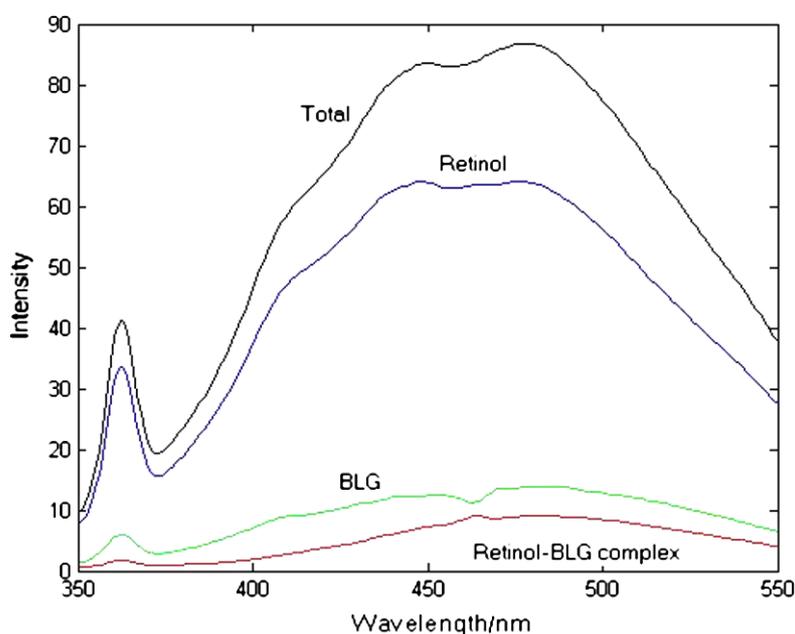


Fig. 2. Separated fluorescence emission spectrum of BLG–retinol solution using the two-way chemometrics method. (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/SPE-2012-0565>.)

Table 1

Binding constants for retinol with BLG in the presence of various CPC concentrations

[CPC] ( $\mu\text{M}$ )	$K_a$ ( $\text{M}^{-1}$ )
0	$5.20 \times 10^7$
10	$0.10 \times 10^7$
20	$0.95 \times 10^7$
40	$0.57 \times 10^7$
100	$0.15 \times 10^7$
150	$0.40 \times 10^7$

where  $[\text{BLG}]_{\text{eq},i}$  is the equilibrium concentration of BLG in  $i$ th titration step.  $K_a$  can now be calculated from the slope of the straight line of  $1/\Delta F_i$  as a function of  $1/[\text{BLG}]_{\text{eq},i}$  according to Eq. (6). A value of  $5.2 \times 10^7 \text{ M}^{-1}$  was obtained for  $K_a$  which was consistent with the previously reported data [16].

According to Eq. (6), the linear plots of  $1/\Delta F_i$  as a function of  $1/[\text{BLG}]_{\text{eq},i}$  and in the presence of various CPC concentrations are given in Fig. 3. The values obtained for  $K_a$  in the presence of CPC are given in Table 1. These values indicate that the retinol binding affinity of BLG decreases in the presence of CPC, at whole. But these changes do not show a distinct trend. Our previous study represents the cooperative denaturation of BLG by CPC [23]. The decreasing of  $K_a$  in pre-transition region (10 and 20  $\mu\text{M}$  of CPC) may be due to competition of CPC with retinol for occupation of the same binding site (central calyx). But the decreasing in the presence of higher concentration of CPC can be due to the conformational changes of BLG. While, the results of our study on interaction of BLG with  $C_n$ TABs represent a significant change in retinol binding affinity of BLG in all of the transition regions [24].

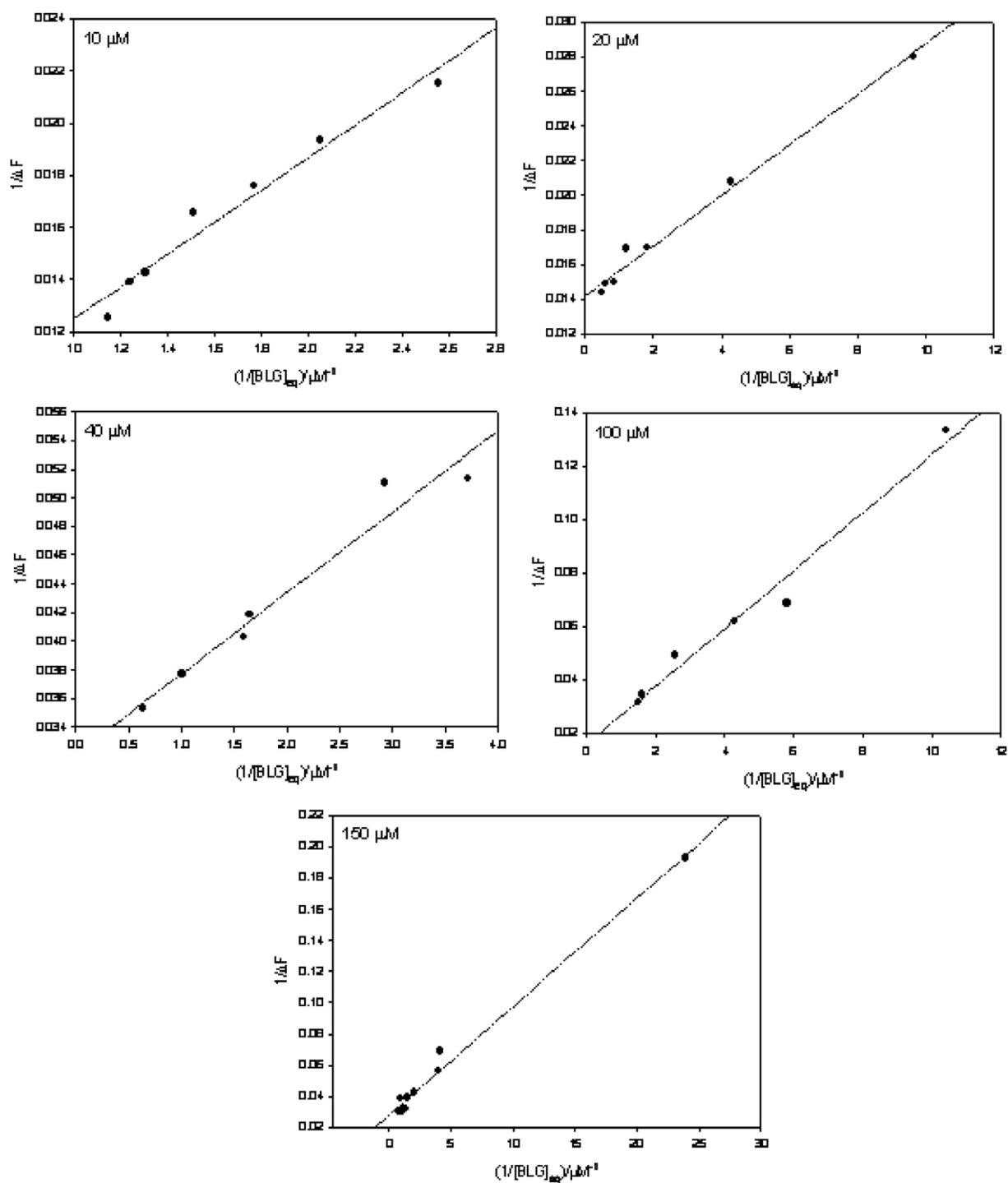


Fig. 3. The linear plots of  $1/\Delta F_i$  as a function of  $1/[BLG]_{eq,i}$  based on Eq. (6) and in the presence of various CPC concentrations.

## 5. Conclusions

Spectrofluorimeter titration method was used for determining the binding constant for retinol with BLG in the presence of CPC. The two-way chemometrics method was used for separating the contribution of BLG in the fluorescence emission spectrum in order to calculate its equilibrium concentration which was then used instead of its total concentration for estimating the actual binding constant. The results show that the two-way chemometrics method is a good approach for calculating a more accurate binding constant. The results also represent that the retinol binding affinity of BLG is decreased in the presence of CPC. This may be due to competition of CPC with retinol for binding in central calyx and structural changes of BLG. However, in comparison with cationic  $C_n$ TABs, which change the tertiary structure of protein such a manner that binding affinity increased, it looks that the nature of BLG–CPC complex is considerably different with BLG– $C_n$ TABs complexes.

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