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Thermodynamic stability and retinol binding property of β-lactoglobulin in the presence of cationic surfactants

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ABSTRACT

In this work the stability parameters of bovine β-lactoglobulin, variant A (BLG-A), with regard to their transition curves induced by dodecyltrimethylammonium bromide (C_{12}TAB), tetradecyltrimethylammonium bromide (C_{14}TAB) and hexadecyltrimethylammonium bromide (C_{16}TAB) as cationic surfactants, were determined at 298 K. For each transition curve, the conventional method of analysis which assumes a linear concentration dependence of the pre- and post-transition base lines, gave the most realistic values for $\Delta G_D(H_2O)$. The results represent the increase in the denaturating power of surfactants with an increase in hydrocarbon chain length. The value of about 22.27 kJ · mol^{-1} was obtained for $\Delta G_D(H_2O)$ from transition curves. Subsequently, the retinol binding property of BLG as its functional indicator was investigated in the presence of these surfactants using the spectrofluorimeter titration method. The results represent the substantial enhancement of retinol binding affinity of BLG in the presence of these surfactants.

1. Introduction

The small globular protein β-lactoglobulin (BLG), as a member of the family called lipocalins, is the major protein in the whey of ruminant milk [1]. It exists at neutral pH as a dimer. Each monomer is made of 162 amino acids including two disulfide bonds (Cys66–Cys160 and Cys109–Cys119) and one free cysteine (Cys121) [2], with a molecular weight of about 18,400 Da. Like retinol binding protein (RBP), BLG is able to bind a wide variety of hydrophobic molecules such as retinoids, alkenes and fatty acids [2,3]. Binding to BLG protects retinol and β-carotene from degradation due to heat, oxidation, and irradiation [4]. It has therefore been proposed that BLG could be used as a versatile carrier of hydrophobic molecules in controlled delivery applications [5]. BLG is known to bind tightly, *in vitro,* one retinol molecule per monomer [6].

An understanding of protein–surfactant interactions, particularly at a molecular level, is an important research area that attracts the interest of researchers [7–11]. The interactions between biopolymers and surfactants depend strongly on the type of biopolymer and surfactant as well as on medium and its physicochemical properties such as pH, ionic strength, and temperature [12–20]. Moreover, these interactions are also the driving force for the structural transition of proteins [21]. In some cases these induced transitions can be monitored and analyzed in order to obtain the thermodynamic parameters of protein stability [22,23]. The interaction of ionic surfactants with BLG has been investigated by various researchers [16,22,24–26]. It has been shown that sodium n-dodecyl sulfate (SDS) is a stronger denaturant than Triton X-100; however, the analysis of binding data represents the absence of considerable changes in retinol binding properties of BLG in the presence of various amounts of these surfactants [16]. The interaction of dodecyltrimethylammonium bromide (C_{12}TAB) with BLG has also been investigated at various pH representing an increase in the binding strength of BLG/C_{12}TAB complex with an increase in the pH although C_{12}TAB binding does not have the significant effect on retinol binding affinity of BLG [26]. The stability parameters of BLG in relation to its transition curves induced by cetylpyridinium chloride (CPC) at various temperatures have already been determined [22]. This has been done by analysis of induced transition curves by conventional method, assuming a linear concentration dependence of the pre- and post-transition base lines. However, the binding properties of BLG have not been investigated in the presence of CPC.

In the present study, the structural changes of BLG have been investigated initially in the presence of a homologous series of cationic surfactants (dodecyltrimethylammonium bromide (C_{12}TAB), tetradecyltrimethylammonium bromide (C_{14}TAB), and hexadecyltrimethylammonium bromide (C_{16}TAB)) using fluorescence spectroscopy. A comprehensive thermodynamic analysis has been carried out on induced sigmoidal transition curves in order to determine the stability parameters of BLG at various

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concentrations of these surfactants. The nature of the interactions has been discussed by considering the surfactant and BLG structure. Moreover, the retinol binding properties of BLG, as its functional indicator, were investigated in the presence of the various amounts of these surfactants using the spectrofluorimeter titration method. Comparison of the results paves the way for the determination of some aspects of the structure–function relationship of BLG in the presence of these surfactants. Finally, the obtained information can be useful in the application of these cationic surfactants in dairy food industry. This may also give some guidance as to what could be expected during interactions of charged and neutral lipids with BLG.

2. Material and methods

2.1. Materials

β-Lactoglobulin genetic variant A, retinol, C_{12}TAB, C_{14}TAB and C_{16}TAB were purchased from Sigma Chemical Co. and used without further purification. The purities of these materials were ⩾90%, ⩾95% and 98% for BLG, retinol and surfactants, respectively. All salts used for buffer preparation were of analytical grade and were dissolved in double distilled water. All solutions were used freshly after preparation.

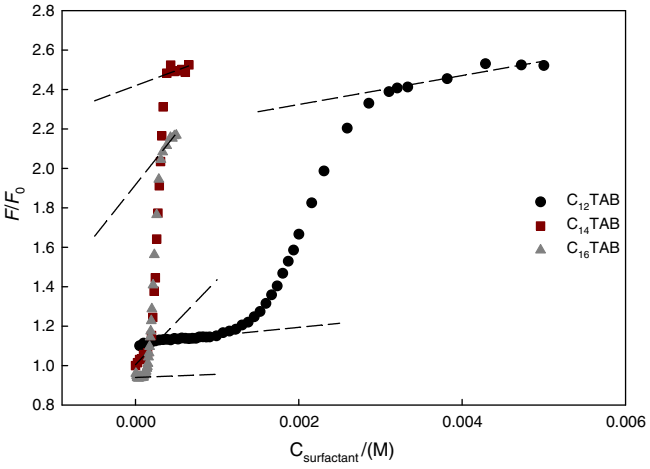


FIGURE 1. Surfactant denaturation curves for β-lactoglobulin in 50 mM phosphate buffer, pH 8.0. The excitation wavelength was 295 nm and band slits were 5 nm for both excitation and emission wavelengths. The relative fluorescence intensities were measured at 330 nm.

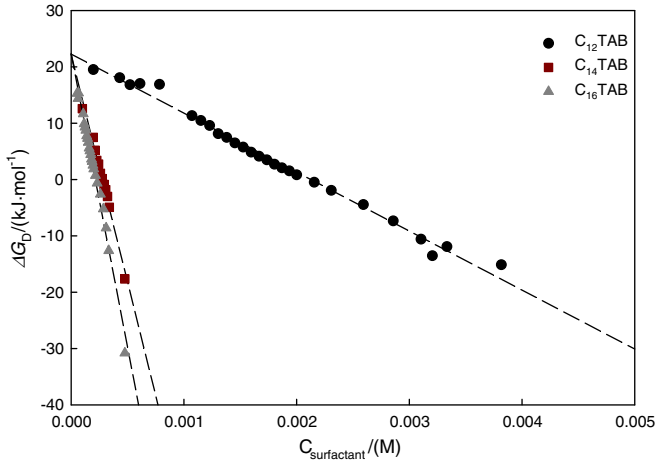
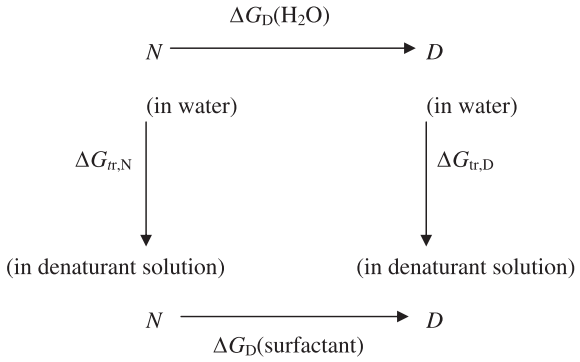


FIGURE 2. The variation of ΔG_D *versus* surfactants concentration.

2.2. Methods

The concentrations of BLG and retinol were determined spectrophotochemically using the value of 17,600 $M^{-1} \cdot cm^{-1}$ for the molar absorption coefficient (ε) of BLG at 279 nm [27], and 52,480 $M^{-1} \cdot cm^{-1}$ for the molar absorption coefficient (ε) of retinol at 325 nm [28]. For optical measurements all solutions were prepared in 50 mM phosphate buffer at pH 8.0. The experiments were conducted at 298 K.

Fluorescence spectroscopy is used to study the conformational changes of proteins containing tryptophan residues during the denaturation because the intrinsic fluorescence of indol chromophores in tryptophan (Trp) residues is particularly sensitive to



SCHEME 1. Unfolding BLG in an isothermal chemical denaturation process.

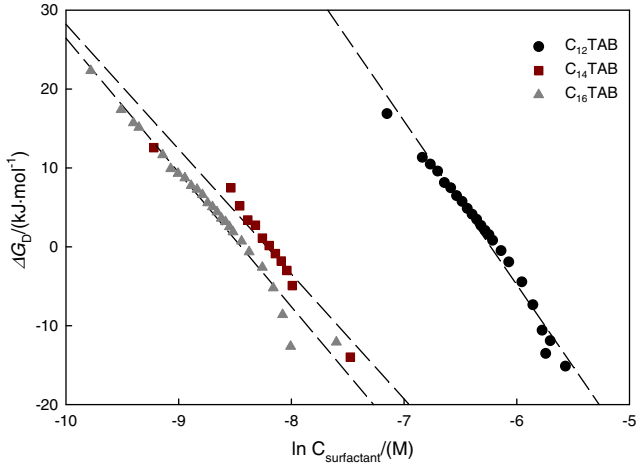


FIGURE 3. The variation of ΔG_D *versus* ln $C_{surfactant}$.

TABLE 1
Parameters characterizing the surfactants denaturation for β-lactoglobulin at 298 K. $\Delta G_D(H_2O)$ is the value of ΔG_D at 0 M denaturant, *m* is the slope of the curves which gives the linear dependence of ΔG_D on surfactants concentration, ΔG_D(surfactant) is the free energy change at 1 M denaturant and ΔG_{tr} is difference between ΔG_D(surfactant) and $\Delta G_D(H_2O)$.

Surfactant	*m*/ (kJ · mol^{-1} · M^{-1})	$\Delta G_D(H_2O)$/ (kJ · mol^{-1})	ΔG_D(surfactant at 1 M)/(kJ · mol^{-1})	ΔG_{tr}/ (kJ · mol^{-1})
C_{12}TAB	10,742	22.2	−129.3	−151.6
C_{14}TAB	80,609	22.2	−130.0	−152.2
C_{16}TAB	104,111	22.4	−143.6	−166.0

their microenvironments. The intrinsic fluorescence of aromatic amino acids in proteins has long been used as a means of monitoring unfolding/refolding transitions induced by chemical denaturants, pH changes, temperature, and pressure [29]. Surfactants denaturation curves were determined by measuring the relative emission intensity of the solutions containing BLG at 330 nm using a Jasco model FP-750 spectrofluorimeter. During fluorescence measurements, the samples were put in quartz cuvette of 1 cm optical path and thermostated to maintain the temperature at ±0.l K. In typical experiments 2.0 mL of BLG solution was placed in the cuv-

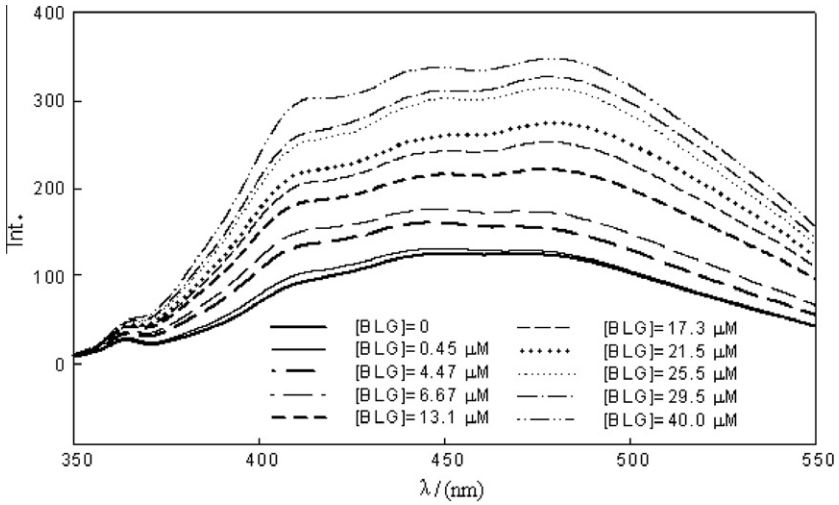


FIGURE 4. Fluorescence emission spectra of retinol in the presence of various BLG concentrations in 50 mM phosphate buffer, pH 8.0. The excitation wavelength was 325 nm and band slits were 5 nm for both excitation and emission wavelengths.

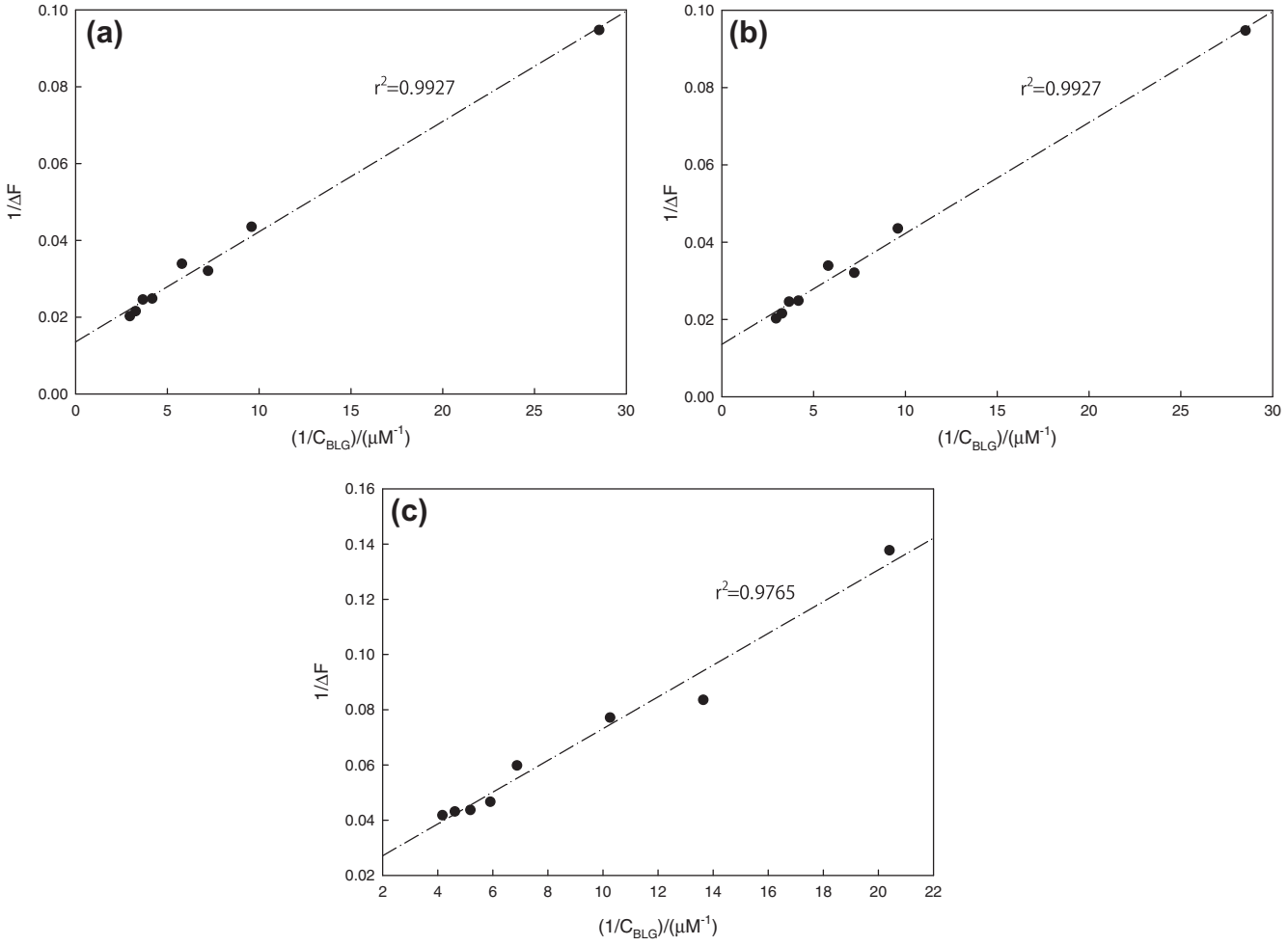


FIGURE 5. The linear plots of $1/\Delta F$ as a function of $1/C_{BLG}$ based on equation (7) and in the presence of various C_{12}TAB concentrations; (a) 500 μM, (b) 2.0 mM, and (c) 2.8 mM.

ette and emission spectra were recorded after each addition of surfactant stock solution. The excitation was performed at 295 nm and the emitted fluorescence was recorded between (300 and 400) nm. The band slits for both excitation and emission were set at 5 nm. Absorbances of the BLG solutions in 279 nm should not exceed 0.1 to avoid the inner filtering effect. The final surfactant concentrations were about (5.0, 0.6, and 0.5) mM for C_{12}TAB, C_{14}TAB, and C_{16}TAB, respectively and there was no inner filter effect due to the presence of these surfactants. The critical micelle concentrations (cmc) of surfactants are 14 mM, (4 to 5) mM and (0.92 to 1) mM for C_{12}TAB, C_{14}TAB, and C_{16}TAB, respectively.

The binding of retinol was measured by following the differences of retinol fluorescence at λ_{max}. The following procedure was used during titration of retinol solutions or various [surfactant]/[retinol] solutions with BLG: 1 mL of retinol solutions or various [surfactant]/[retinol] solutions was placed in a cuvette and small increments (0–100 μL) of the BLG solution were injected in the cuvette. These spectra were recorded from (350 to 550) nm with an excitation wavelength of 325 nm. Spectral resolution was 1 nm for both excitation and emission wavelengths.

3. Results and discussion

3.1. Denaturation of BLG

Relative emission intensity of BLG solution (F/F_0) at 330 nm and various concentrations of surfactants ([surfactant]) is shown in figure 1, where F and F_0 are the emission intensities in the presence and absence of surfactant, respectively.

The fluorescence properties of tryptophan residues apparently are sensitive to perturbations of protein structure. Interaction of surfactants with BLG changes the environment of tryptophan residue and causes differences in fluorescence intensity. The sigmoidal feature of the curves in figure 1 can be related to the characteristic of a cooperative transition. Similar sigmoidal transitions that have also been reported for thermal and chemical denaturation of BLG [22,23] can be taken as other reason for cooperative denaturation of BLG by these cationic surfactants. It seems that these surfactants can induce an unfolding transition in tertiary structure of BLG. With respect to the position of the transition curves in figure 1, it can be judged that the denaturating power of surfactant has been increased by increasing its hydrocarbon tail. It represents the significant role of hydrophobic interaction in the process of surfactant binding. However, it appears that there is not a linear relation between these phenomena. The standard free energy of denaturation, ΔG_D, was calculated as a function of surfactants concentration by assuming a two-state mechanism [30,31], and by using equations (1) and (2) [22,23,32]

$$F_D = (y_N - y_{obs})/(y_N - y_D) \quad (1)$$

$$\Delta G_D = -RT \ln K = -RT \ln(F_D/(1 - F_D)) = -RT \ln[(y_N - y_{obs})/(y_{obs} - y_D)] \quad (2)$$

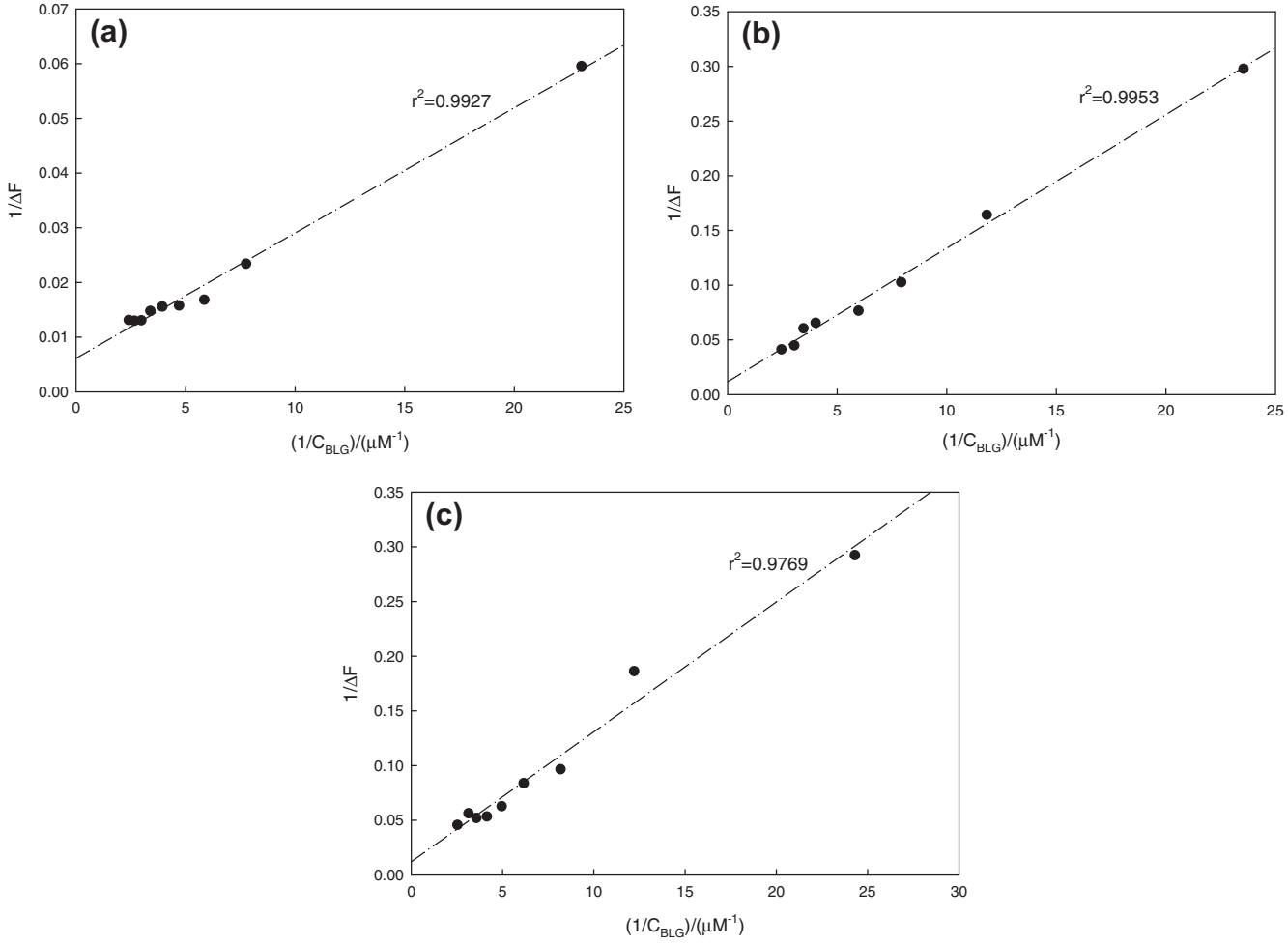


FIGURE 6. The linear plots of $1/\Delta F$ as a function of $1/C_{BLG}$ based on equation (7) and in the presence of various C_{14}TAB concentrations; (a) 100 μM, (b) 300 μM, and (c) 600 μM.

where K is the equilibrium constant, F_D is the fraction of denatured protein, R is the gas constant, y_{obs} is the observed optical property, y_N and y_D are the optical properties of the native and denatured protein molecules, respectively. The two latter ones have been extrapolated from the pre- and post-transition baselines to the considered denaturant concentration. These pre- and post-transition baselines were determined by a linear fit of the corresponding data before and after the transition according to the following equations:

$$Y_N = a_N + b_N \quad [\text{surfactant}] \tag{3}$$

$$Y_D = a_D + b_D \quad [\text{surfactant}] \tag{4}$$

Figure 2 shows the standard free energy of denaturation, ΔG_D (which is calculated from equation (2) based on the data in figure 1), which varies linearly with surfactants concentration. ΔG_D was plotted against [surfactant] and a linear least-squares analysis was used to fit the ΔG_D and [surfactant] data to the following relation [33],

$$\Delta G_D = \Delta G_D(H_2O) - m \quad [\text{surfactant}], \tag{5}$$

where $\Delta G_D(H_2O)$ is the value of ΔG_D at 0 M denaturant and m is the slope of the curves which gives the linear dependence of ΔG_D on surfactants concentration.

Unfolding BLG in an isothermal chemical denaturation process can be divided into thermodynamically defined stages according to the scheme 1 [23].

It follows that ΔG_D, for example, for the process of BLG (in water) → BLG (in surfactant) is given by the relationship:

$$\Delta G_{tr,N} + \Delta G_D(\text{surfactant}) = \Delta G_D(H_2O) + \Delta G_{tr,D}$$

or

$$\Delta G_{tr} = \Delta G_D(\text{surfactant}) - \Delta G_D(H_2O) = \Delta G_{tr,D} - \Delta G_{tr,N}, \tag{6}$$

where $\Delta G_{tr,N}$ and $\Delta G_{tr,D}$ represent the transfer standard free energies of native and denatured states of BLG from water to surfactant solution at a given concentration of surfactant. ΔG_D(surfactant) is the standard free energy change for this process: native BLG (in surfactant solution) → denatured BLG (in surfactant solution).

Figure 3 shows the linearity of the plot of ΔG_D *versus* ln[surfactant]. From the least-squares analysis of these plots, the standard free energy in the surfactant solution, ΔG_D(surfactant), was obtained at ln[surfactant] = 0. A concentration of surfactant of 1 M is in good agreement with the transition of the native state of protein (from water) to the denatured state of protein (to surfactant solution). The estimated value of ΔG_D(surfactant), $\Delta G_D(H_2O)$, and ΔG_{tr} are shown in table 1. $\Delta G_D(H_2O)$ has positive values but ΔG_D(surfactant) and ΔG_{tr} have negative values. The values of ΔG_D(surfactant) show that the minimum stability occurs in the presence of C_{16}TAB at 298 K (the protein is completely surrounded by denaturant). ΔG_{tr} is also less negative in the presence of this surfactant, which is the best state for denatured protein.

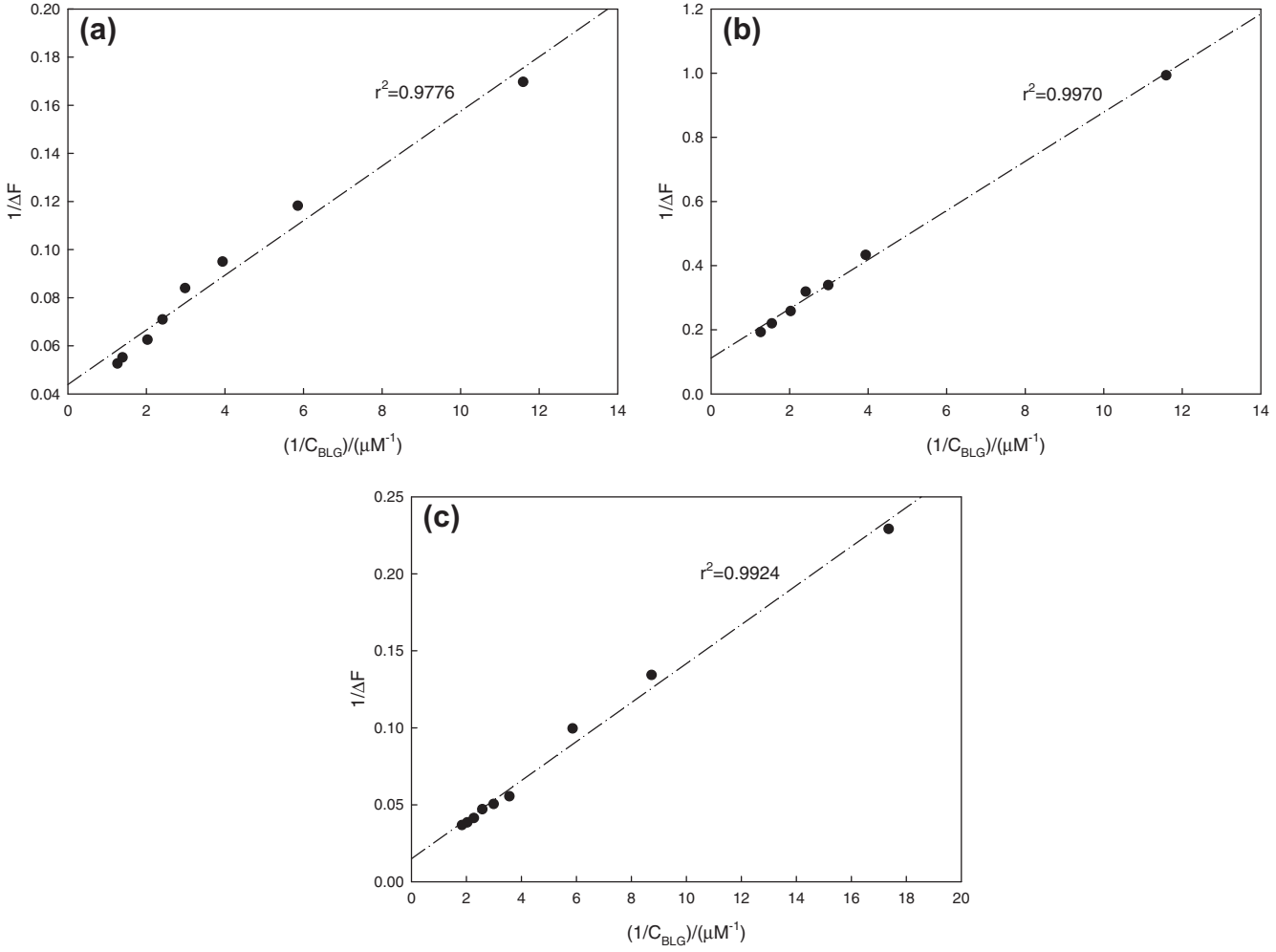


FIGURE 7. The linear plots of $1/\Delta F$ as a function of $1/C_{BLG}$ based on equation (7) and in the presence of various C_{16}TAB concentrations; (a) 50 μM, (b) 200 μM, and (c) 500 μM.

TABLE 2
The obtained values for K_a in the presence of C_{12}TAB, C_{14}TAB, and C_{16}TAB. K_a is the binding constant.

Surfactant	[Surfactant]/(mM)	$K_a \cdot 10^{-4}/(M^{-1})$	Surfactant	[Surfactant]/(mM)	$K_a \cdot 10^{-4}/(M^{-1})$
C_{12}TAB	0	5.0 ± 0.9	C_{14}TAB	0.35	135.0 ± 28.3
	0.3	1933.3 ± 113.1		0.45	168.0 ± 39.5
	0.5	1487.5 ± 320.2		0.6	120.0 ± 26.1
	1.0	520.0 ± 98.8	C_{16}TAB	0	5.0 ± 0.9
	2.0	453.3 ± 93.4		0.02	126.3 ± 30.6
	2.8	260.0 ± 52.0		0.05	1439.0 ± 289.2
	5.0	106.0 ± 22.4		0.1	124.0 ± 27.9
C_{14}TAB	0	5.0 ± 0.9		0.2	139.7 ± 32.9
	0.05	1285.7 ± 255.8		0.25	134.8 ± 30.5
	0.1	305.0 ± 62.8		0.4	145.9 ± 29.0
	0.15	127.5 ± 25.5		0.5	149.0 ± 30.2
	0.3	117.0 ± 25.8			

3.2. Retinol binding of BLG

Fluorescence is a useful approach to investigating intermolecular interactions because the photophysical character of the fluorophore is sensitive to the polarity of its surrounding environment. Generally, fluorescence intensity increases as this polarity decreases. As shown in figure 4, the gradual increase in retinol fluorescence intensity is observed as BLG concentration increases 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 equilibrium between retinol and retinol–protein complexes [34]. Likewise, there may be an equilibrium between free and BLG-bound retinol. For 1:1 complexes, the retinol fluorescence data in figure 4 can be analyzed by the following equation [35]:

$$1/\Delta F = 1/\Delta F_{max} + 1/(K_a \cdot \Delta F_{max} \cdot [BLG]) \quad (7)$$

ΔF is the change of retinol fluorescence intensity in the presence and absence of BLG; ΔF_{max} is the maximal change of fluorescence intensity; K_a is the binding constant; and [BLG] is the concentration of BLG. The linear plots of $1/\Delta F$ as a function of 1/[BLG] in the presence of C_{12}TAB, C_{14}TAB, and C_{16}TAB are given in figures 5 to 7, respectively. From the slope of the straight line, K_a can be calculated according to equation (7). The value of $(5.0 \pm 0.9) \cdot 10^4\ M^{-1}$ was obtained for K_a in the absence of any surfactant. This value is in consistent with previously reported data [36]. The obtained values for K_a in the presence of C_{12}TAB, C_{14}TAB, and C_{16}TAB are given in table 2. These values represent a large enhancement of retinol binding affinity of BLG in the concentrations range of these surfactants that located in the pre-transition state of denaturation curves (figure 2); although, at all concentrations of surfactants, the tertiary structure of protein will change in such a manner that the retinol binding site of BLG will be more accessible and that it will cause an increase in the retinol binding affinity of BLG.

4. Conclusions

All of the studied surfactants denature BLG in a cooperative manner. The denaturation effect of these surfactants increases with an increase in their hydrocarbon tail length, representing the predominate role of hydrophobic interaction. However, this does not obey in a linear trend. This nonlinearity can be deduced by considering the m, ΔG_D, and ΔG_{tr} values in table 1 (there is not any linear relation between these parameters and number of carbon atoms in hydrocarbon tail of surfactants).

The value of about $22.27\ kJ \cdot mol^{-1}$ has been obtained for $\Delta G_D(H_2O)$, as thermodynamic stability of BLG in the absence of surfactant. This value is obtained from the identical extrapolated values of all lines in figure 1. This can represent the accuracy of applied analyzing method. With respect to the negative values of ΔG_D(surfactant) and ΔG_{tr} and hydrophilic nature of surfactants, it looks that hydrophobic interactions have substantial role in denaturation process.

All of the used surfactants enhance the retinol binding affinity of BLG in all of their concentration range. In the presence of surfactants, the tertiary structure of protein has been changed such a manner that its retinol binding site became more accessible and obtained higher affinity. However, this enhancement is much more in the region of pre-transition. It seems that binding of initial surfactant ions increases the hydrophobic patches in the protein surface that subsequently increases the hydrophobic interaction of retinol molecule with BLG. This enhancement for C_{12}TAB is more than others. However, higher concentration of C_{12}TAB is required in order to reach this enhancement.

Acknowledgment

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