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Structure–function relationship of β -lactoglobulin in the presence of sodium dodecylbenzenesulfonate

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Abstract

Bovine β -lactoglobulin (β -lg) present in milks has been found “*in vivo*” in complexes with lipids such as butyric and oleic acids. To elucidate the still unknown structure–function relationship in this protein, the structural changes of β -lactoglobulin variant A (β -lg A) were investigated in the presence of sodium dodecylbenzenesulfonate (SDBS) as an anionic surfactant using spectrofluorimetry. Subsequently, the retinol binding was investigated by β -lg in the presence of various amounts of this surfactant as its extrinsic functional binding fluorophore. The comparison of the results allowed for determining the binding of retinol by β -lg in the presence of SDBS.

The results of fluorescence studies showed a higher denaturing effect of SDBS at acidic pH that can be due to the positive charge density of β -lg at this pH which was calculated using the Henderson–Hasselbalch equation and pK_a values of its ionizable groups. For each transition curve, the conventional method of analysis which assumed a linear concentration dependence of the pre- and post-transition base lines gave the most realistic values for $\Delta\Delta G_o(H_2O)$. The value of about $21.6 \text{ kJ} \cdot \text{mol}^{-1}$ was obtained for $\Delta\Delta G_o(H_2O)$ at various pH from transition curves. The results of retinol binding studies represented the substantial enhancement of retinol binding affinity of β -lg in the presence of this surfactant at various pH levels. Moreover, the obtained results confirmed that the β -lg/retinol binding was pH-dependent.

Highlights

► Stability parameters and retinol binding property of β -lg in the presence of SDBS have been determined at various pH. ► Higher denaturing effect of SDBS at acidic pH can be due to

positive charge density of β -lg at this pH. ► SDBS enhances the retinol binding affinity of β -lg in all of its concentration range. ► The β -lg/retinol binding is pH-dependent.

Keywords

β -Lactoglobulin

Sodium dodecylbenzenesulfonate

Denaturation

Spectrofluorimeter

Protein stability

Binding affinity

1. Introduction

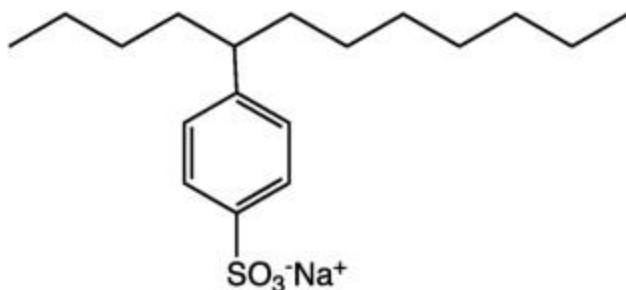
Surfactant is a group of amphipathic substance composed of both hydrophilic and hydrophobic groups. Surfactants can induce the unfolding of proteins and, in some special cases, stabilize proteins at very low concentration [1]. They are also solubilizing agents for membrane proteins [1]. Studies on protein–surfactant interactions have been carried out for many decades [2]. The great number of recent studies, however, indicates that this issue continues to interest the researchers in academics [3], [4], [5], [6], [7], [8]. That is because, first, the heterogeneity of surfactant binding sites in protein imparts significant changes in the physicochemical properties of protein molecule, which reflects that solute, *i.e.* the protein–surfactant complex, and solvent structures are mutually modified. Second, more importantly, because (protein + surfactant) systems are qualitatively consistent with the biological membrane, the information with respect to the intermolecular protein–surfactant interactions may have important implications for the behavior of biological membrane. Recent studies on such systems have used NMR [9], SANS [10], fluorescence [4], [8], [11], [12], ESR [13], microcalorimetry [2], [14], [15], light scattering [16], and other techniques for advancing the current understanding of protein–surfactant interactions. However, the classical techniques of physical chemistry still remain relevant [14], [16], [17]. It is generally accepted that binding of ionic surfactant molecules to proteins disrupts the native structure of most globular proteins. This initially involves the ionic binding of surfactants to the

ionic sites of the protein. Further binding occurs by hydrophobic cooperative interactions. Hence, ionic surfactants interact with proteins through a combination of electrostatic and hydrophobic forces [18]. It is well known that anionic surfactants can interact strongly with proteins, modify protein conformation and denature proteins [19], [20].

β -Lactoglobulin (β -lg) is a globular whey protein with the molar mass of $1.84 \cdot 10^4 \text{ g} \cdot \text{mol}^{-1}$ and isoelectric point, pI , of around 5.2 [21], [22]. β -lg may play important functions in the binding and the transportation of hydrophobic ligands, such as retinoids, alkenes, and fatty acids, attracts more and more attention [6], [23], [24], [25], [26], [27], [28], [29]; also, its interactions with amphiphilic and hydrophobic ligands such as retinoids, long chain fatty acids and surfactants have been investigated by different methods. This small globular protein has a three-dimensional structure consisting of one α -helix and nine anti-parallel β -strands with eight β -sheets folded into a cone-shaped barrel forming a hydrophobic pocket [30]. β -lg is composed of 162 amino acid residues and has two disulfide bonds at the positions of Cys66–Cys160 and Cys106–Cys119 and one sulfhydryl residue at Cys121 in each molecule [31].

Interactions between amphiphiles (surfactants/emulsifiers) and β -lg are highly diverse and have received much attention because of their relevance to *e.g.* two-component food systems [32], [33]. At the pH 7, the amphiphilic palmitic acid binds as a natural ligand in the central calyx according to heteronuclear NMR spectroscopy and fluorescence spectroscopy [34]. Affinity is mediated by hydrophobic interactions within the calyx with the head group protruding toward the solvent. Therefore, ionic surfactants are thought to bind in a similar manner at low concentrations. At higher concentrations, ionic surfactants such as the cationic *n*-decyltrimethyl ammonium bromides (C_n TABs) unfold the protein [4]. In our previous investigations, a series of systematic studies were done on the interaction of various surfactants on β -lg [3], [4], [5], [6]. The results represented the induction of cooperative unfolding in β -lg by some cationic surfactants. β -lg had conformational changes during its interaction with sodium *n*-dodecyl sulfate (SDS) and Triton X-100 while retinol binding properties of β -lg did not show significant changes in the presence of these surfactants. However, the variation of structural index parameters of β -lg *versus* these surfactants concentration did not show sigmoidal feature so that no cooperative unfolding transitions were detected. The values of $\Delta \Delta G_{\text{Do}}(\text{H}_2\text{O})$ as the quantitative measure of the thermodynamic stability of β -lg were extracted from the transition denaturation curves in the presence of cetylpyridinium chloride (CPC) and *n*-decyltrimethyl ammonium bromides (C_n TABs). The results represented the

increase in the denaturing power of surfactants with an increase in hydrocarbon chain length (n). Moreover, the retinol binding affinity was measured in the presence of C_n TABs. The relevant results represented the enhancement of retinol binding affinity in the presence of these surfactants. To continue previous studies, the interaction of sodium dodecylbenzenesulfonate (SDBS) (scheme 1), as an anionic surfactant, with β -lg was investigated at various pH levels using the spectrofluorimetry technique. The results of this study not only determine the nature of forces in the interaction process but also provide the sufficient results for evaluating the accuracy and validity of the applied data analysis method. Moreover, the effect of electrostatic forces on the structure and stability of β -lg was investigated by running the measurement at various pH levels.



SCHEME 1. Chemical structure of SDBS.

2. Material and methods

2.1. Materials

β -lg genetic variant A, retinol, and SDBS were purchased from Sigma Chemical Company and were used without further purification. The purities of these materials were $\geq 90\%$, $\geq 95\%$, and 98% for BLG, retinol, and surfactant, 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.

2.2. Methods

Fluorescence spectroscopy is used for studying the binding and conformational changes of proteins exploiting the intrinsic fluorescence of tryptophan (Trp) residues, which are particularly sensitive to the changes of their microenvironments [35]. During fluorescence measurements, the samples were put in the quartz cuvettes of 1 cm optical path length. In typical experiments, 2.0 mL of β -lg

solution was placed into the cuvette. Emission spectra were recorded after each addition of SDBS stock solution (1 mM) at 298 K. The excitation was performed at 295 nm and the emitted light was recorded between 300 and 400 nm for SDBS binding studies. The observed fluorescence intensities were corrected for dilution. The band slits for excitation and emission were set at 5 nm. To avoid the inner filtering effect, absorbance of the β -lg solutions in 279 nm should not exceed 0.1.

The binding of retinol was measured by following the differences of retinol fluorescence at λ_{\max} . The following procedure was used during the titration of retinol solutions or various [surfactant]/[retinol] solutions with β -lg: 1 mL of retinol solutions or various [surfactant]/[retinol] solutions was placed in a cuvette and small increments (0 μ L to 100 μ L) of the β -lg solution were injected in the cuvette. These spectra were recorded from 350 nm to 550 nm with an excitation wavelength of 325 nm. Spectral resolution was 5 nm for both excitation and emission wavelengths.

3. Results and discussion

3.1. Influence of various anionic surfactant concentrations on the structure of β -lg

When ionic surfactants are added to the β -lg solutions, the ionic surfactant monomers first bind electrostatically to the oppositely charged residues on the β -lg surface (site-specific binding) and this binding induces the expansion of the β -lg structure. This expansion allows for more hydrophobic interactions of the surfactant hydrophobic tails with the exposed β -lg non-polar interior (nonspecific, cooperative binding) sites. To investigate the influence of protein net charge on the denaturing power of SDBS, the net charge of β -lg was calculated using the net charge of its ionizable groups. Their net charge as a function of pH was calculated using the Henderson–Hasselbalch equation and their pK_a values [36]. Figure 1 shows the β -lg net charge at different pH levels. The isoelectric point (the pH associated with zero net charge) was about 4.6 which was in good agreement with the experimental reported data [37]. The net charges of β -lg were about 17.8, -11.1 , and -15.5 in the pH 2.0, 6.7, and 8.0, respectively. At the pH 2.0, below the pK_a of aspartates and glutamates, β -lg was positively charged and could constitute the binding sites for anionic surfactant; so, it could form precipitates with oppositely charged surfactants because of the creation of neutral complexes between β -lg and anionic surfactant. During the further increase of concentrations of anionic surfactant, more surfactants were bound to the electrostatically neutral

complexes, so the secondary complexes of β -lg/anionic surfactant gradually became negatively charged and the precipitate was dissolved. At the pH 6.7 and 8.0, β -lg was negatively charged and when anionic surfactant was bounded to β -lg, the β -lg/anionic surfactant complex was always negatively charged and the precipitation was not observed.

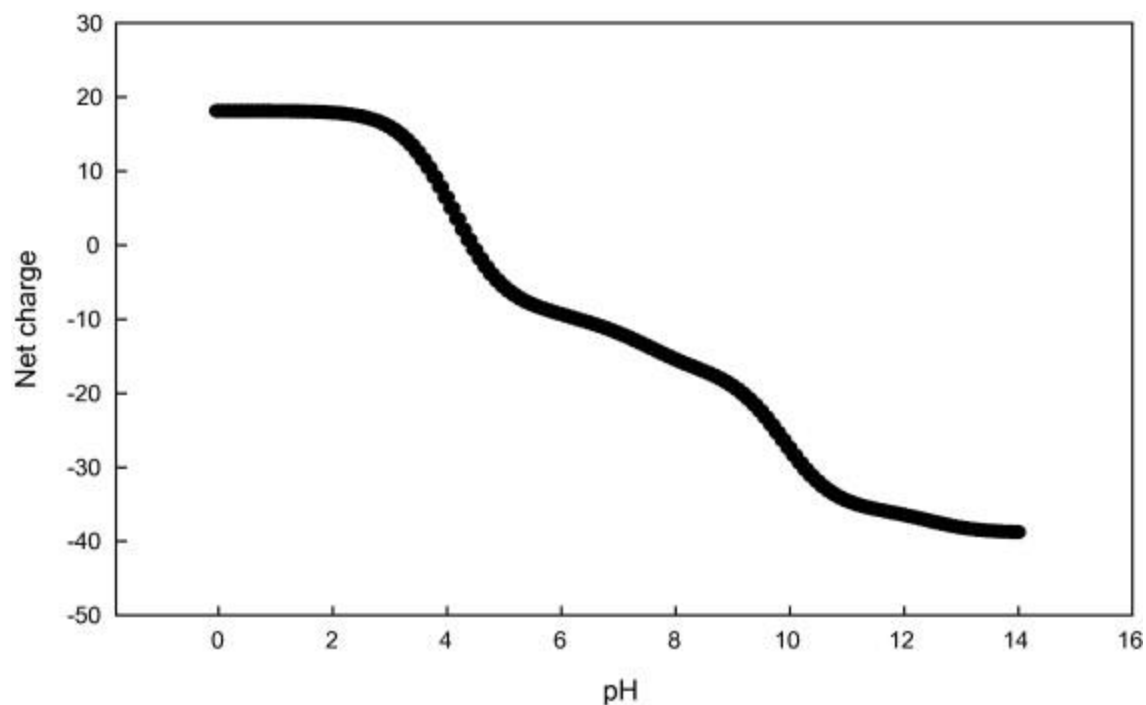


FIGURE 1. Net charge *versus* pH curve of β -lg.

3.2. Denaturation of β -lg

As mentioned above at the pH 2.0, β -lg was positively charged, so it can form precipitates with oppositely charged surfactant SDBS because of the formation of neutral complex β -lg/SDBS. With the further increase of SDBS concentration, more anionic surfactant was bound to the already electrostatically neutral complexes, so the complex of β -lg/SDBS became negatively charged and the precipitate was dissolved. Therefore, upon the increase of SDBS concentration, the mixtures of β -lg/SDBS underwent a transition from homogeneous solution to precipitates, and then to another homogeneous solution. It should be noted that the transition curve was recorded after dissolving the precipitate at this pH. No precipitate was observed at the pH 6.7 and 8.0.

The relative emission intensity of β -lg solution (F/F_0) at 330 nm and various concentrations of surfactants ($[\text{surfactant}]$) is shown in figure 2, where F and F_0 are the emission intensities in the presence and absence of surfactant, respectively.

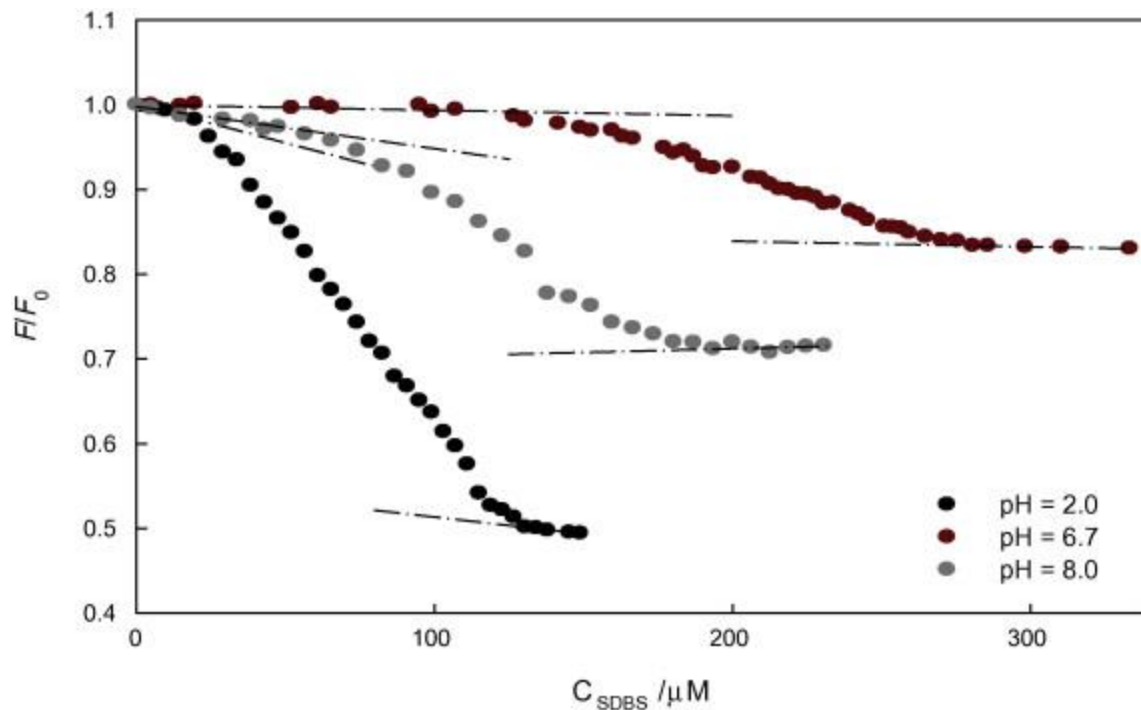


FIGURE 2. SDBS denaturation curves for β -lg in various pH. The excitation wavelength was 295 nm and band slits were 5 nm for both excitation and emission wavelengths. The relative fluorescence intensities were measured at 330 nm. F and F_0 are the emission intensities in the presence and absence of surfactant, respectively.

The sigmoidal feature of the curves in figure 2 can be related to the characteristic of a cooperative transition. Similar sigmoidal transitions that have been also reported for thermal and chemical denaturation of β -lg [3], [38] can be taken as another reason for the cooperative denaturation of β -lg by this anionic surfactant. It seems that this surfactant can induce an unfolding transition in the tertiary structure of β -lg. With respect to the position of the transition curves in figure 2, it can be judged that the denaturing power of surfactant in the pH 2.0 is greater than that of other pHs. As mentioned before, β -lg is positively charged at this pH and can constitute the binding sites for

SDBS. It represents the significant role of electrostatic interaction in the process of surfactant binding.

The standard free energy of denaturation, ΔG_D^0 , was calculated as a function of surfactant concentration by assuming a two-state mechanism [39], [40] and using equations (1), (2) [4], [38]

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

$$\Delta G_D^0 = -RT \ln K = -RT \ln(F_D)/(1 - F_D), = -RT \ln[(y_N - y_{\text{obs}})/(y_{\text{obs}} - y_D)], \quad (2)$$

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 and y_N and y_D are the optical properties of the native and denatured protein molecules, respectively. The two latter ones were 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:

$$y_N = a_N + b_N[\text{surfactant}], \quad (3)$$

$$y_D = a_D + b_D[\text{surfactant}]. \quad (4)$$

Figure 3 shows the standard free energy of denaturation, ΔG_D^0 (which was calculated from equation (2) based on the data in figure 2) varies linearly with surfactant concentration. ΔG_D^0 was plotted against [surfactant] and a linear least-squares analysis was used to fit the ΔG_D^0 and [surfactant] data to the following relation [41],

$$\Delta G_D^0 = \Delta G_D^0(\text{H}_2\text{O}) - m[\text{surfactant}], \quad (5)$$

where $\Delta G_D^0(\text{H}_2\text{O})$ is the value of ΔG_D^0 at 0 M denaturant and m is the slope of the curves which gives the linear dependence of ΔG_D^0 on surfactants concentration.

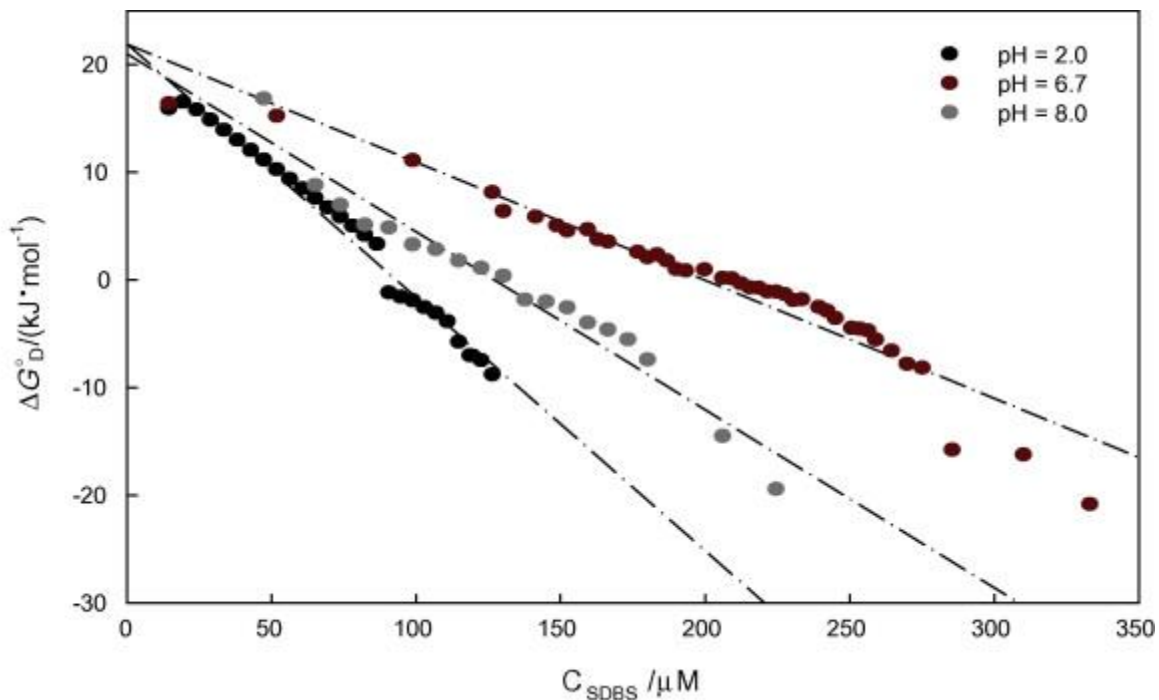
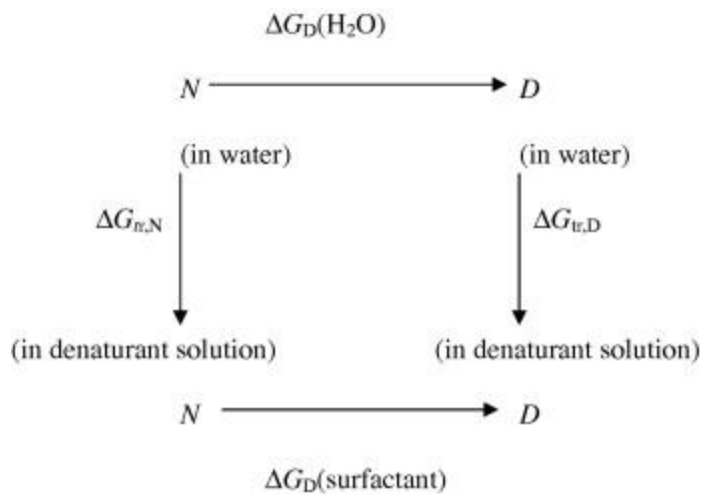


FIGURE 3. The variation of ΔG_D^0 versus SDBS concentration. ΔG_D^0 is the standard free energy of denaturation.

Unfolding of β -lg in an isothermal chemical denaturation process can be divided to thermodynamically defined stages according to scheme 2[38].



SCHEME 2. Unfolding of BLG in an isothermal chemical denaturation process.

It follows that ΔG_{Do} , for example, for the process of β -lg (in water) \rightarrow β -lg (in surfactant) is given by the following relation:

$$\Delta G_{\text{tr,N}}^{\circ} + \Delta G_{\text{D}}^{\circ}(\text{surfactant}) = \Delta G_{\text{D}}^{\circ}(\text{H}_2\text{O}) + \Delta G_{\text{tr,D}}^{\circ} \quad (6)$$

or

$$\Delta G_{\text{tr}}^{\circ} = \Delta G_{\text{D}}^{\circ}(\text{surfactant}) - \Delta G_{\text{D}}^{\circ}(\text{H}_2\text{O}) = \Delta G_{\text{tr,D}}^{\circ} - \Delta G_{\text{tr,N}}^{\circ},$$

where $\Delta G_{\text{tr,N}}^{\circ}$ and $\Delta G_{\text{tr,D}}^{\circ}$ represent the transfer standard free energies of native and denatured states of β -lg from water to surfactant solution at a given concentration of surfactant. $\Delta G_{\text{Do}}(\text{surfactant})$ is the standard free energy change for this process: native β -lg (in surfactant solution) \rightarrow denatured β -lg (in surfactant solution).

Figure 4 shows the linearity of the plot of ΔG_{Do} versus $\ln[\text{surfactant}]$. The standard free energy in the surfactant solution, $\Delta G_{\text{Do}}(\text{surfactant})$, was obtained at $\ln[\text{surfactant}] = 0$ from the least-squares analysis of these plots. The 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 $\Delta G_{\text{Do}}(\text{surfactant})$, $\Delta G_{\text{Do}}(\text{H}_2\text{O})$, and ΔG_{tro} are shown in table 1. $\Delta G_{\text{Do}}(\text{H}_2\text{O})$ has positive values but $\Delta G_{\text{Do}}(\text{surfactant})$ and ΔG_{tro} have negative values. The values of $\Delta G_{\text{Do}}(\text{H}_2\text{O})$ (about $21.6 \text{ kJ} \cdot \text{mol}^{-1}$) are in good agreement with the previously reported data for the denaturation of β -lg in the presence of *n*-decyltrimethyl ammonium bromides [4]. This similarity confirms the validity and accuracy of the applied data analysis method. The values of $\Delta G_{\text{Do}}(\text{surfactant})$ show that the minimum stability occurs at the pH 2.0 (the protein is completely surrounded by denaturant). ΔG_{tro} is also less negative at this pH, which is the best state for the denatured protein. The comparison of $\Delta G_{\text{Do}}(\text{surfactant})$ and ΔG_{tro} values with the results of the our previous study on the interaction of β -lg with C_n TABs represents that β -lg has less stability in the presence of SDBS.

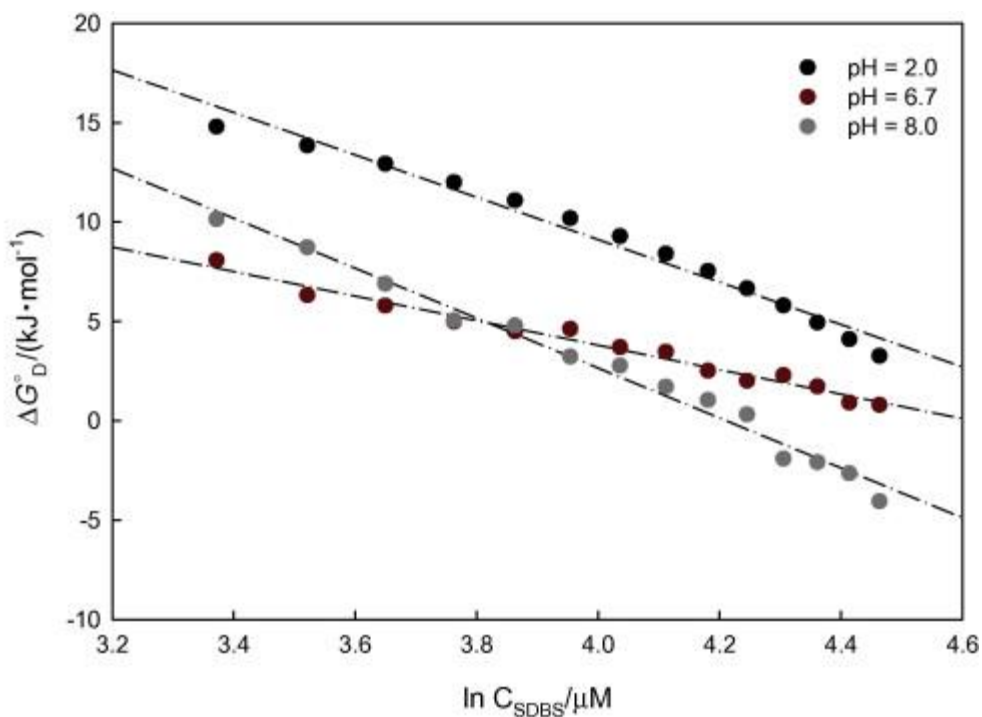


FIGURE 4. The variation of ΔG_D° versus $\ln C_{SDBS}$. ΔG_D° is the standard free energy of denaturation.

TABLE 1. Parameters characterizing the SDBS denaturation for β -lg at various pH. $\Delta G_D^\circ(\text{H}_2\text{O})$ 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, $\Delta G_D^\circ(\text{surfactant})$ is the free energy change at 1 M denaturant, and $\Delta G_D^\circ(\text{tro})$ is difference between $\Delta G_D^\circ(\text{surfactant})$ and $\Delta G_D^\circ(\text{H}_2\text{O})$.

pH	$m/(\text{kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1})$	$\Delta G_D^\circ(\text{H}_2\text{O})/(\text{kJ} \cdot \text{mol}^{-1})$	$\Delta G_D^\circ(\text{surfactant at 1 M})/(\text{kJ} \cdot \text{mol}^{-1})$	$\Delta G_D^\circ(\text{tro})/(\text{kJ} \cdot \text{mol}^{-1})$
2.0	235,256	21.9	-155.2	-177.2
6.7	109,643	21.9	-153.5	-175.4
8.0	156,221	21.0	-152.3	-173.3

3.3. Retinol binding of β -lg

The gradual increase in retinol fluorescence intensity was observed at various pH levels as β -lg concentration increased, which suggested that retinol transferred 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 [42]. Likewise, there may be equilibrium between free and β -lg-bound retinol. For 1:1 complexes, the retinol fluorescence data can be analyzed by the following equation [43]:

$$1/\Delta F = 1/\Delta F_{\max} + 1/(K_a \cdot \Delta F_{\max} \cdot [\beta\text{-lg}]). \quad (7)$$

ΔF is the change of retinol fluorescence intensity in the presence and absence of β -lg ΔF_{\max} is the maximal change of fluorescence intensity, K_a is the binding constant and $[\beta\text{-lg}]$ is the concentration of β -lg. The linear plots of $1/\Delta F$ as a function of $1/[\beta\text{-lg}]$ in the presence of SDBS at the pH 2.0, 6.7, and 8.0 are given in FIGURE 5, FIGURE 6, FIGURE 7, respectively. From the slope of the straight line, K_a can be calculated according to equation (7). The values of $12.8 \cdot 10^4 \text{ M}^{-1}$, $9.8 \cdot 10^4 \text{ M}^{-1}$, and $5.0 \cdot 10^4 \text{ M}^{-1}$ were obtained for K_a in the absence of any surfactant at the pH 2.0, 6.7, and 8.0, respectively. The apparent β -lg/retinol binding constants decrease between the pH levels 2.0 and 8.0. This observation does not support the suggestions of Fugate and Song [23] who claimed that the binding of retinol to β -lg is pH-independent in the pH range of 2.0 to 7.5 but agrees with the fluorescence spectroscopy results reported by Dufour *et al.* [25]. In the case of this study, the obtained results show that the β -lg/retinol binding is pH-dependent. All the obtained data suggest the change of conformation and retinol binding properties of β -lg with pH. The obtained values for K_a in the presence of SDBS at various pH levels are given in table 2. These values represent a large enhancement of retinol binding affinity of β -lg in the concentrations range of this surfactant located in the pre-transition state of denaturation curves (figure 2). Although, at all concentrations of surfactant, the tertiary structure of protein changes in such a manner that the retinol binding site of β -lg is more accessible and causes an increase in the retinol binding affinity of β -lg. Moreover, the K_a values in the presence of SDBS are lower than C_n TABs [4]. Thus, it can judge conclude that, in comparison with SDBS, the increase of hydrophobic patches in the protein surface is more in the presence of C_n TABs.

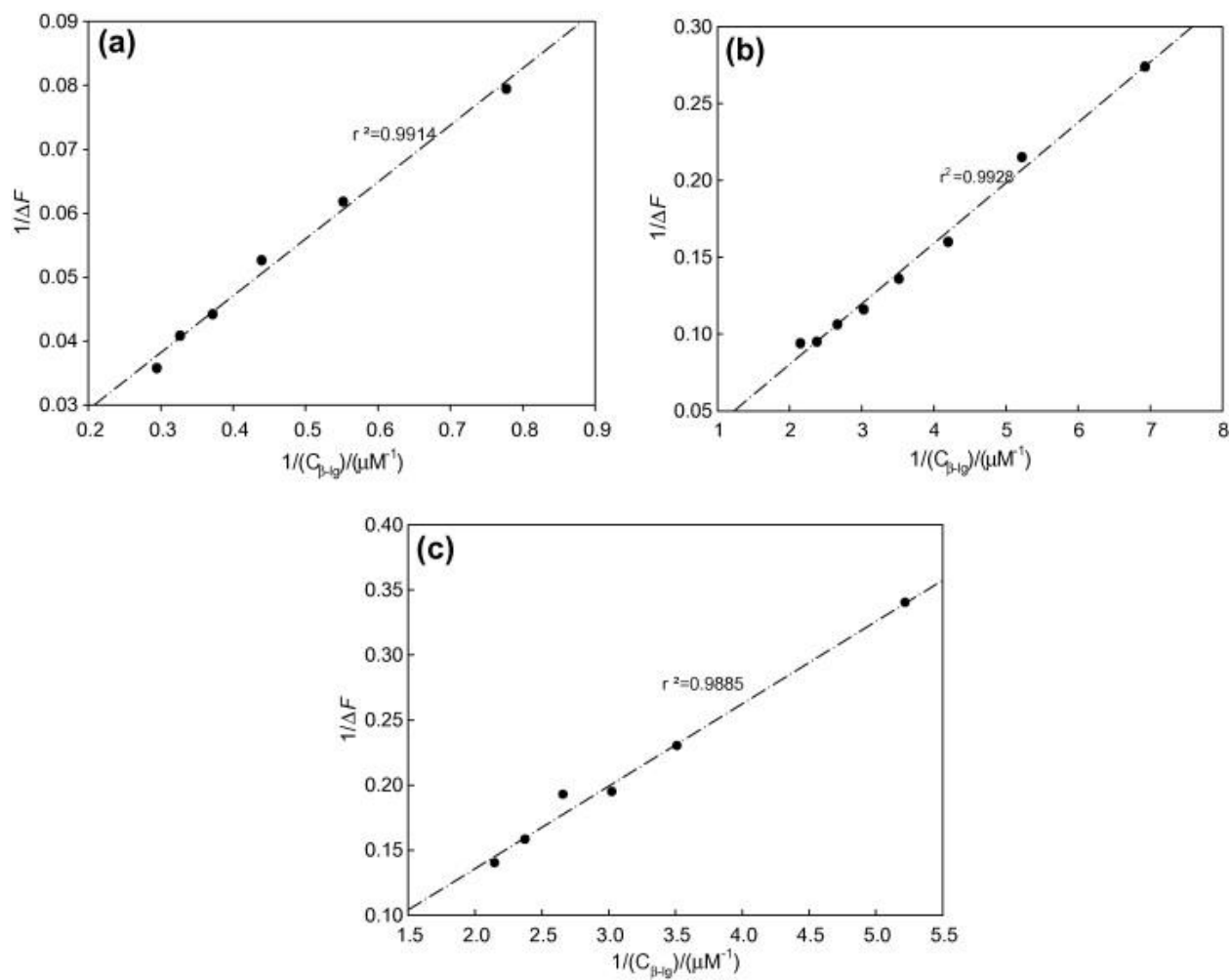


FIGURE 5. The linear plots of $1/\Delta F$ as a function of $1/C_{\beta-Ig}$ based on equation (7) and in the presence of various SDBS concentrations at pH 2.0; (a) 0 μM , (b) 50 μM and (c) 150 μM . ΔF is the change of retinol fluorescence intensity in the presence and absence of β -Ig.

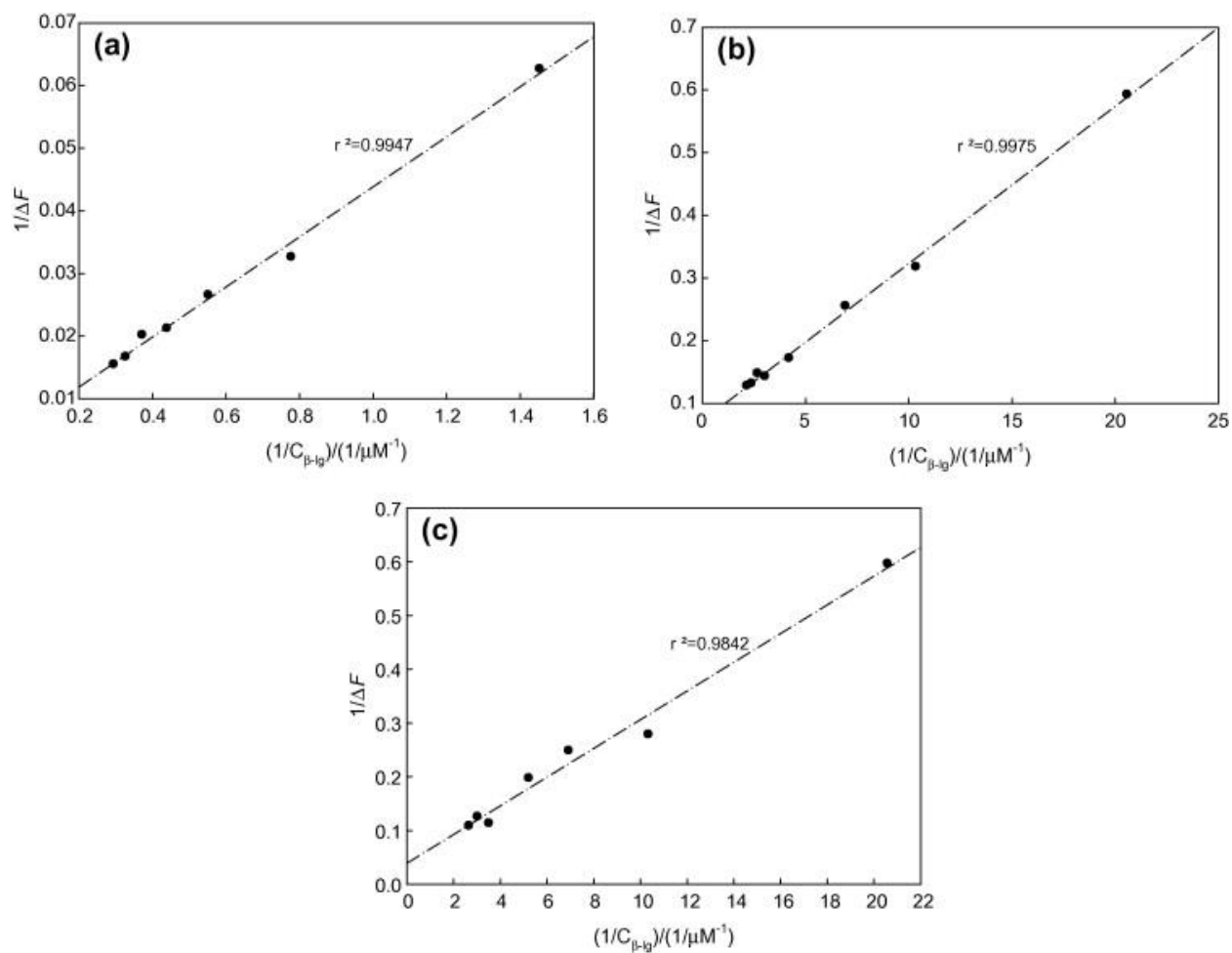


FIGURE 6. The linear plots of $1/\Delta F$ as a function of $1/C_{\beta\text{-Ig}}$ based on equation (7) and in the presence of various SDBS concentrations at pH 6.7; (a) 0 μM , (b) 200 μM , and (c) 330 μM . ΔF is the change of retinol fluorescence intensity in the presence and absence of β -Ig.

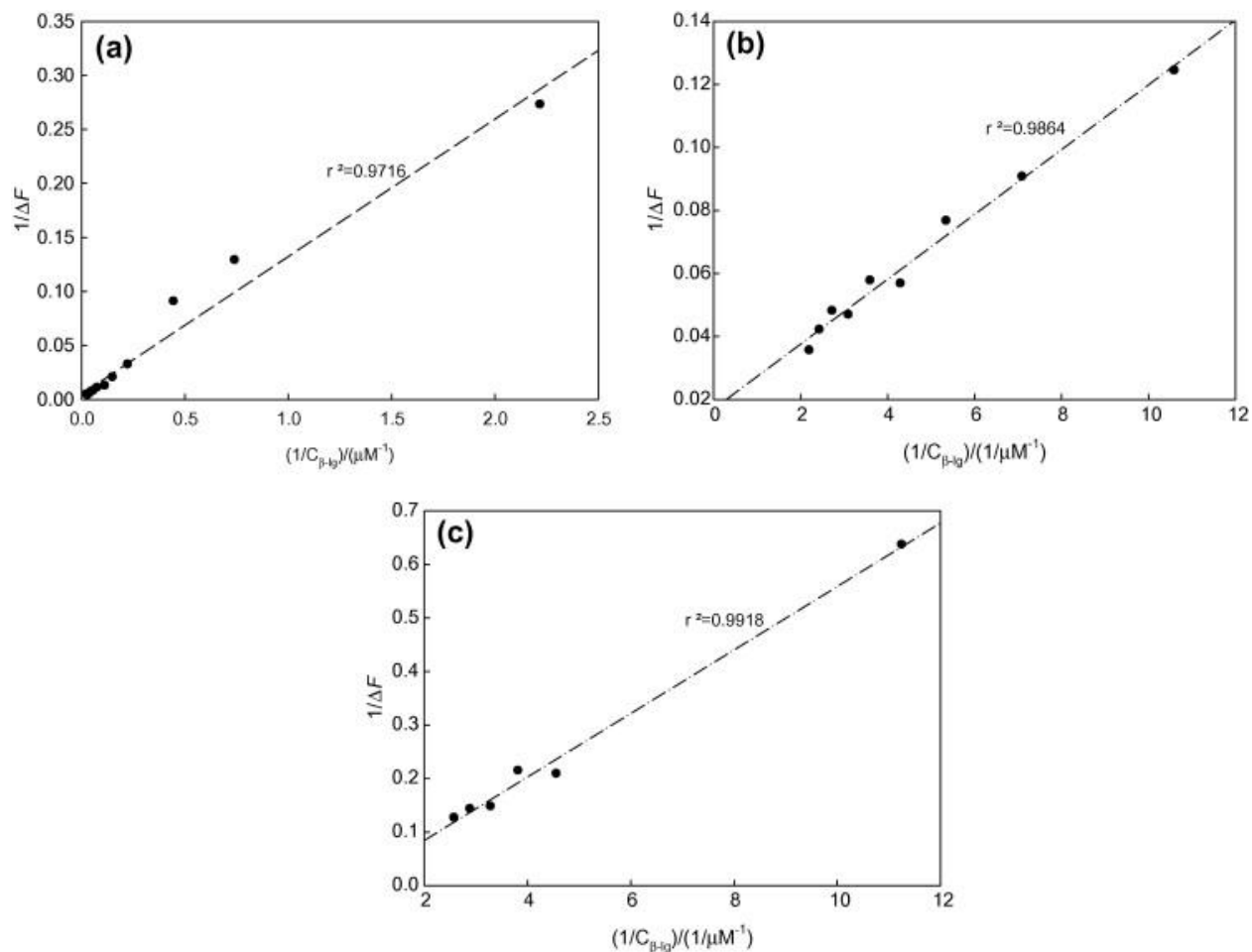


FIGURE 7. The linear plots of $1/\Delta F$ as a function of $1/C_{\beta\text{-Ig}}$ based on equation (7) and in the presence of various SDBS concentrations at pH 8.0; (a) $0 \mu\text{M}$, (b) $30 \mu\text{M}$, and (c) $120 \mu\text{M}$. ΔF is the change of retinol fluorescence intensity in the presence and absence of β -Ig.

TABLE 2. The obtained values for K_a in the presence of SDBS. K_a is the binding constant.

pH	[Surfactant]/(μM)	$K_a \cdot 10^{-4}/(\text{M}^{-1})$	pH	[Surfactant]/(μM)	$K_a \cdot 10^{-4}/(\text{M}^{-1})$
2.0	0	12.8	6.7	280	174.3
	10	32.5		330	130.0

pH	[Surfactant]/(μM)	$K_a \cdot 10^{-4}/(\text{M}^{-1})$	pH	[Surfactant]/(μM)	$K_a \cdot 10^{-4}/(\text{M}^{-1})$
	20	229.0	8.0	0	5.0
	50	15.8		10	25.0
	90	19.4		30	170.0
	130	12.3		90	44.0
	150	15.5		120	14.8
6.7	0	9.8		200	3.2
	30	414.0			
	90	1360.0			
	200	242.0			
	230	162.3			

4. Conclusions

The studied surfactant denatured β -lg in a cooperative manner at various pH levels. The denaturation effect of this surfactant in the pH 2.0 was greater than that in other pHs because of the positive net charge of BLG at this pH and represented the predominate role of electrostatic interaction.

The values of about $21.92 \text{ kJ} \cdot \text{mol}^{-1}$, $21.89 \text{ kJ} \cdot \text{mol}^{-1}$, and $21.00 \text{ kJ} \cdot \text{mol}^{-1}$ were obtained at the pH 2.0, 6.7, and 8.0, respectively, for $\Delta G_{\text{Do}}(\text{H}_2\text{O})$, as thermodynamic stability of β -lg in the absence of surfactant. These values were consistent with the previously reported data for the interaction of β -lg and C_n TABs. This similarity approved the validity of the applied data analysis method. The values of $\Delta G_{\text{Do}}(\text{surfactant})$ and ΔG_{tro} represented the less stability of β -lg in the

presence of SDBS in comparison with C_n TABs. The apparent β -lg/retinol binding constants decreased between the pH levels of 2.0 and 8.0. The obtained data suggested that β -lg conformation changed in the studied pH range and binding of retinol by β -lg was pH-dependent. The used surfactant enhanced the retinol binding affinity of β -lg in all of their concentration range at various pH levels. In the presence of surfactant, the tertiary structure of protein was changed in such a manner that its retinol binding site became more accessible and obtained higher affinity. However, this enhancement was much more in the region of pre-transition. It seems that binding of initial surfactant ions increases the hydrophobic patches in the protein surface and, subsequently, increases the hydrophobic interaction of retinol molecule with β -lg. This enhancement for the pH 6.7 was more than others. However, the authors' previous study showed more enhancement of K_a in the presence of C_n TABs.

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