Thermodynamic Study of Intermediate State of Papain Induced by n-Alkyl Sulfates at Two Different pH Values: A Spectroscopic Approach

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Abstract: The formation of the intermediate state of papain was induced by n-alkyl sulfates including sodium octyl sulfate, SOS; sodium decyl sulfate, SDeS; and sodium dodecyl sulfate, SDS at different concentrations. A systematic investigation of n-alkyl sulfates induced conformational alteration in molten globule state under an acidic condition and the native state of papain was examined by tryptophan fluorescence, 1-anilino 8-naphtalene sulfonic acid (ANS) binding and UV absorbance. The addition of n-alkyl sulfates to molten globule state at pH 2 shows a decrease in tryptophan fluorescence intensity and quenched ANS fluorescence relative to the native state that leads to enhancement in tryptophan fluorescence and an increase in ANS fluorescence as well. In the presence of n-alkyl sulfates in various conditions, two intermediate (I_A and I_B) with different conformations were obtained at acidic and native states, respectively. Thus, we can assume that the intermediate states in folding and unfolding pathways in various conditions have different structures. The results show that SDS is much more effective than SDeS and SOS for the formation of the intermediate states for papain in the two different pathways due to the presence of its hydrophobic tail. Therefore, hydrophobic interactions play an important role in inducing the two different intermediates along the two various thermodynamic pathways.

Keywords: Papain, n-Alkyl sulfates, intermediate state, thermodynamic, refolding, unfolding.

1. INTRODUCTION

The globular protein, papain (EC.3.4.22.2) is a thiol enzyme from the latex and unripe fruit of carica papaya (tropical melon or pawpaw). The cystein protease, papain is unusually resistant to high temperatures and to high concentrations of denaturing agents, such as, 8 M urea or organic solvent like 70% EtOH [1, 2]. Papain is a carbohydrate free, basic, single chain protein. Papain has a molecular weight of 23,000 Da and consists of 212 amino acid residues (metionin absents; IP 8.7) with four disulfide bridges and catalytically important cystein (position 25) and histidin residues (position 158) [3, 4].

The study of the folding intermediates and denatured states provides an insight to understanding how and when various forces come to play their roles in the folding process of the protein [5, 6]. The development of a broad range of techniques has led to the identification and characterization of stable folding intermediates, termed "molten globule", (MG) which have been shown to be compact structures with a pronounced secondary structure which lack rigid tertiary structures [7-10]. Recent evidence, however, supports the idea that the molten globules may also possess well-defined tertiary contacts [11-14]. At pH 2, a papain exhibit substantial secondary structure as β-sheet and is relatively less denatured compared to 6 M guanidium hydrochloride (GnHCl) but loses the persistent tertiary structure of the native state [15]. The addition of HFIP and TFE caused an induction of α-helical structure as evident from the increase in the mean residue ellipticity value at 208 and 222 nm. In addition tryptophan fluorescence studies indicate a change in the environment of the tryptophan residues on the addition of HFIP and TFE to MG state of papain. Maximum ANS binding occurs at 13% (V/V) HFIP and 30% (V/V) TFE, suggesting a compact "molten globule" - like conformation with enhanced exposure of hydrophobic surface area [16]. In the molten globule state (pH 2), papain shows a great tendency to aggregate at lower concentrations of GnHCl or a high concentration of salt [17, 18]. Also, the conformational behavior of papain in aqueous solution has been investigated in the presence of SDS. The results show the high α-helical content and unfolded structure of papain in the presence of SDS is due to strong electrostatic repulsion [19]. In this paper we have reported for the first time that there are different structures of intermediate states with various stability under acidic and native conditions in the presence of n-alkyl sulfates along papain denaturation pathway.

2. MATERIALS AND METHODS

2.1. Materials

Fresh latex from green fruits of Carica papaya was collected and immediately transported in ice path to the lab, papain was isolated by anionic exchange chromatography on a column of Diethylaminoethyl sepharos 6B Purity, and homogeneity of the preparation was checked by SDS-polyacrilamide gel electrophoresis [20-22] (Fig. **1**). To avoid any hindrance due to the autolytic nature of papain, the cystein was blocked by carboxymethylation [23]. Such a blocked protein is similar to the active form in all its physical properties. Therefore, for all the studies presented here, such inactive protein (1RCM-papain) was used. An extinction coefficient of ε1% = 25 was used for the determination of concentration of papain solutions [24]. Sodium octyl sulfate (SOS) and sodium dodecyl sulfate (SDS) were purchased from Sigma. Sodium decyl sulfate

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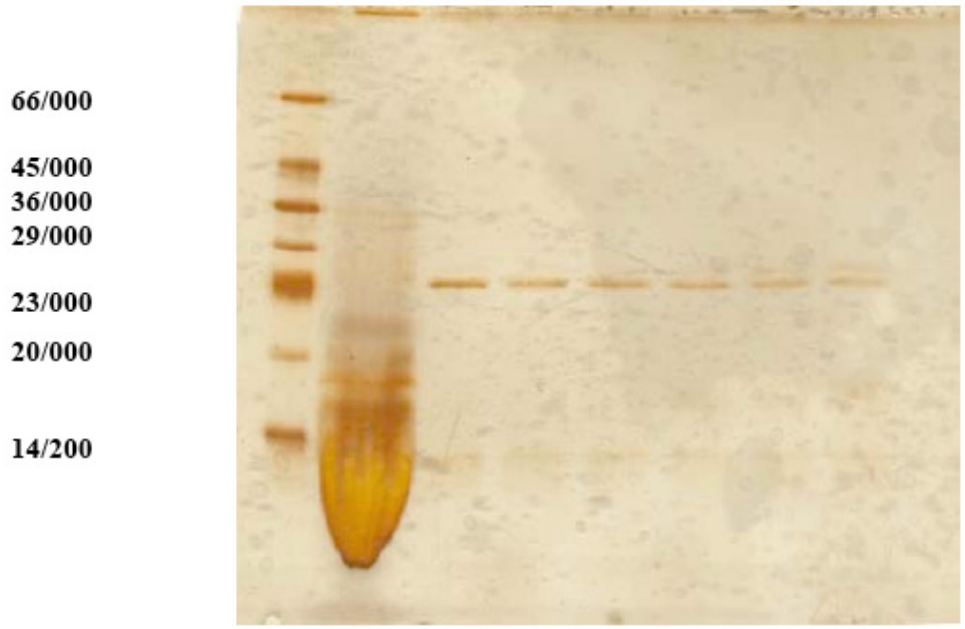

Fig. (1). Polyacrilamyde gel electrophoresis of papain. The samples in the different lanes represent: Standard (lane 1), crude latex extract (lane 2), different fractions of papain after purification by anionic exchange chromatography (lane 3-8).

(SDeS) was obtained from Merck. The concentration of the n-alkyl sulfates, used in all experiments was under the critical micelle concentration (CMC) and the critical aggregation concentration (CAC) [25, 26]. 8-Anilino-1-naphthalen sulfonic acid (ANS) was purchased from Aldrich-Sigma, USA. A stock solution of ANS was prepared in minimum amount of methanol and the concentration was determined with an extinction coefficient of 5000 $M^{-1}cm^{-1}$ at 380 nm [27]. All other used reagents were of analytical grade, the highest quality commercially available.

2.2. Solution Preparation

The protein solution was dialyzed against buffer (20 mM HCl, pH 2 and 50 mM phosphate buffer, pH 8.7). The extinction coefficients were used to calculate the concentration of the native protein at different pH values. If the initial concentration and volume of protein solution are $[P]_0$ and V_0 respectively, and the stock ligand concentration is $[L]_0$, then the total concentration of protein ($[P]_t$) and ligand ($[L]_t$)can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment [28]:

$$[P]_t = [P]_0 V_0/(V_0 + V),\ [L]_t = [L]_0 V_0/(V_0 + V_c). \quad (1)$$

The aliquots of the n-alkyl sulfate were injected in to the papain solution at 5 min intervals to allow for equilibration. Each experiment was repeated three times [24].

2.3. Methods

2.3.1. Absorption Measurements

The absorption spectra of papain were obtained with a spectrophotometer, Model Shimadzu pc 1650, with cells of one cm diameter. The protein concentration was determined at 0.04%. Samples containing different concentrations of n-alkyl sulfates were equilibrated at room temperature for 5 min before recording for absorbance measurements.

2.3.2. Fluorescence Measurements

Fluorescence spectra were recorded with a JASCO FP-2600 spectrofluorometer. Samples containing different concentrations of n-alkyl sulfates were equilibrated at room temperature for 5 min before recording for tryptophan fluorescence measurements. The excitation wavelength was at 278 nm and the emission was recorded from 300 to 400 nm. ANS spectra were reported between 400 and 700 nm with an excitation at 350 nm. Protein concentration was 0.04 % for all experiments.

3. RESULTS

The 15 ml fresh milky latex extracted from samples of C. papaya contained 121.6 mg/ml papain with 3648000 units of protease activity/ml. As papain is a protease of broad specificity and no specific synthetic substrate is available, casein was used as a substrate to determine the total protease activity present in the latex while the purity of papain was determined by electrophoretic and chromatographic techniques. On anionic gel electrophoresis, the protein in the latex separated as seven bands (Fig. **1**). One of these proteases was identified as papain according to its mobility that was equal to that of the standard papain and its in situ proteolytic activity on polyacrylamide gel [29, 30].

3.1. Absorbance Measurements

Absorbance spectroscopic technique in UV region can be used to study the binding and folding-unfolding of protein initiated by surfactant. The addition of low concentrations of n-alkyl sulfates to the papain at pH 2 and 8.7 decreases and enhances the absorption intensities respectively. The marked change in the absorbance value of the mixture at low concentrations of n-alkyl sulfates corresponds to the change of the conformation of the protein, i.e. the protein is folding [31]. When the hydrophobic interactions between protein and high concentrations of anionic surfactants predominate, protein will be unfolded [32]. In fact, the binding of DS of n-alkyl sulfates to cationic sites of papain increases the absorption [33].

UV-visible spectra show the effect of chain length of n-alkyl sulfates on cited interactions, indicating that higher chain lengths of n-alkyl sulfates (higher hydrophobicity) increase the probability for the presence of intermediate

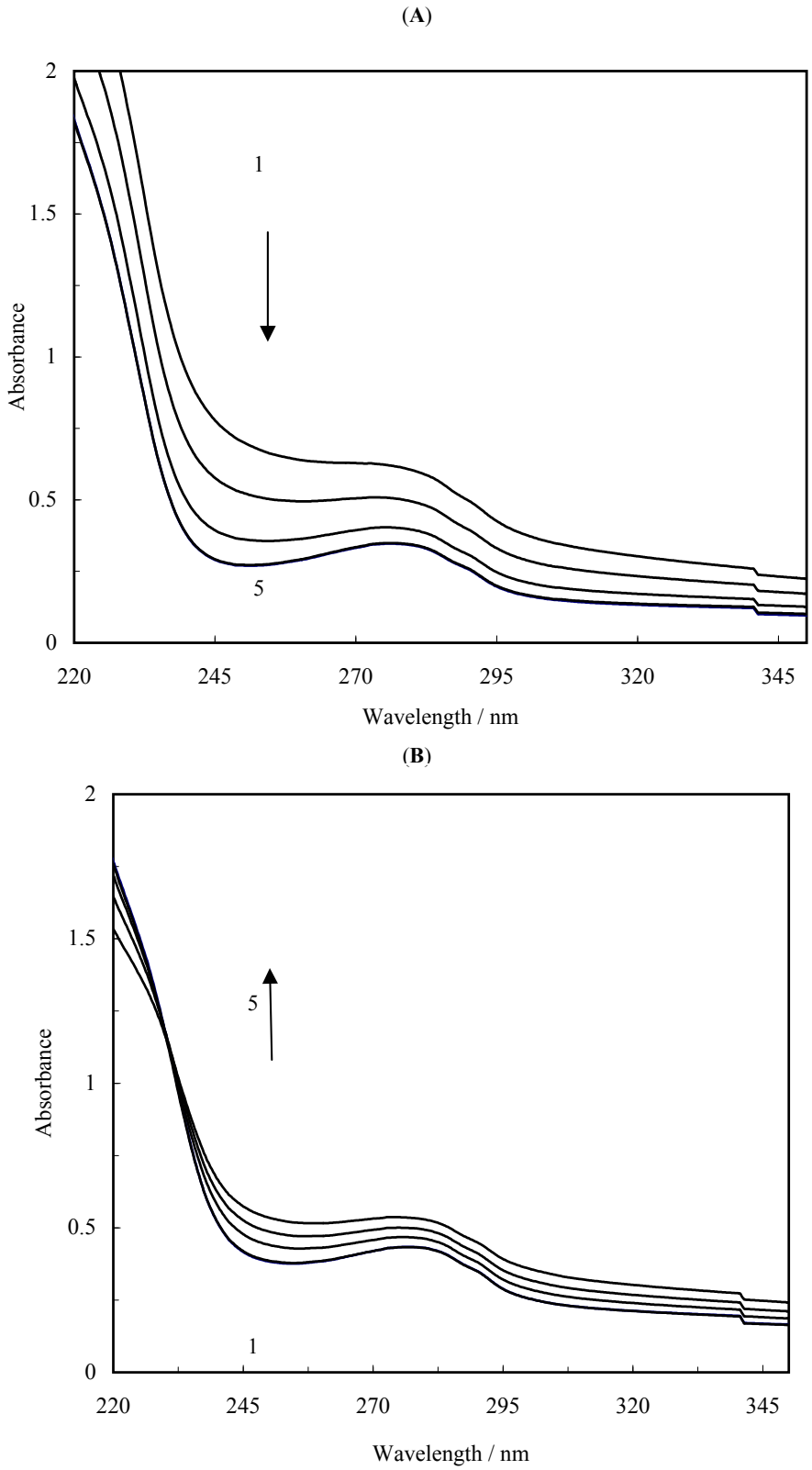

Fig. (2). (**A**) Absorption spectra of papain in the presence of different concentration of SDeS at pH 2. Curves 1-5 are papain upon the addition of 0, 0.037, 0.111, 0.185, 0.257 mM SDeS, respectively. Inset: Plot of absorption spectra versus concentrations of n-alkyl sulfates at pH 2. (**B**) Absorption spectra of papain in the presence of different concentrations of SDeS at pH 8.7. Curves 1-5 are papain upon the addition of 0, 0.611, 0.68, 0.781, 0.882 mM SDeS, respectively.

state. The results show that SDS is much more effective than SDeS and SOS for the formation of the intermediate state (Fig. **2**). Fig. (**2A**, **B**) shows the spectra of the papain upon the addition of SDeS, at various concentrations, in the region of UV, at pH 8.7.

3.2. Fluorescence Measurement

Fluorescence is an excellent probe to investigate conformational changes of proteins. Fig. (**3A**, **B**) show the effect of SDeS on the fluorescence spectra of the acid-unfolded state of papain at pH 2 and 8.7 respectively. The

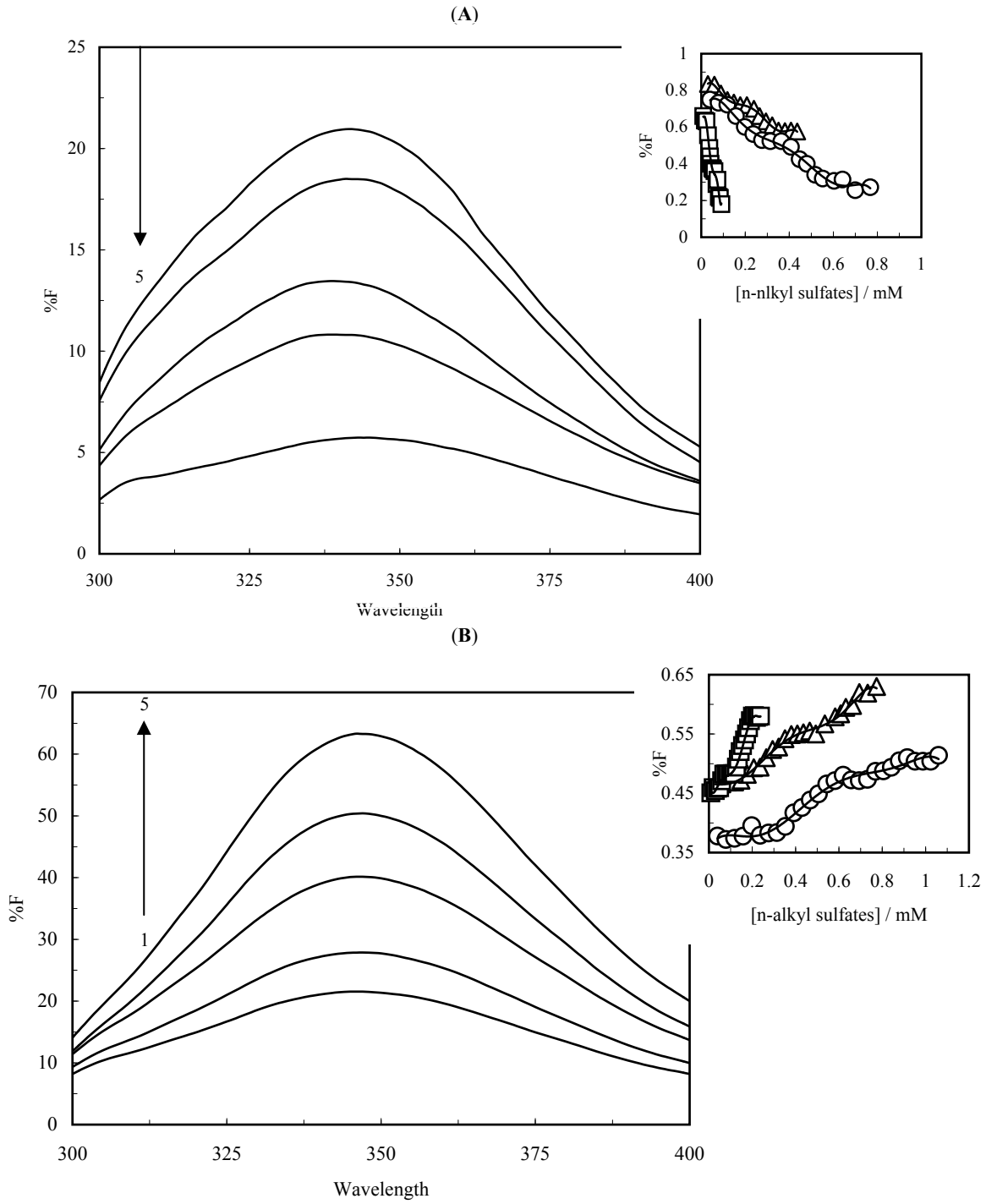

Fig. (3). (**A**) Fluorescence spectra of papain at different concentrations of SDeS at pH 2. Curves 1-5 are papain upon the addition of 0, 0.037, 0.576, 0.915, 0.981 mM SDeS at pH 2. Inset: Plot of intensity fluorescence versus concentration of n-alkyl sulfates at pH 2. (**B**) Fluorescence spectra of papain at different concentrations of SDeS at pH 8.7. Curves 1-5 are papain upon the addition of 0, 1.425, 1.813, 2.012, 2.234 mM SDeS at pH 8.7. Inset: Plot of intensity fluorescence versus concentration of n-alkyl sulfates at pH 8.7. SOS (*o*), 20 mM; SDeS (Δ), 15 mM; SDS (□) 5 mM.

inset of Fig. (**3A**, **B**) show the profile transitions of the unfolded to the molten globule state of papain induced by various concentrations of *n*-alkyl sulfates. According to Fig. (**3A**, **B**), the addition of low concentrations of SDeS to the acid-unfolded state and native state of papain causes a decrease and enhances fluorescence intensity. The Protein showed some tendency to unfold at the presence of n-alkyl sulfates with an increasing number of buried tryptophan residues being exposed at the surface of the molecule. However, the addition of n-alkyl sulfates to MG state at pH 2 shows quenched ANS fluorescence the native state as opposed to in which a similar addition leads to an increase in ANS fluorescence. Decreasing the binding affinity of the hydrophobic dye (ANS) in the presence of n-alkyl sulfates indicates that the exposure of hydrophobic region has been decreased due to the presence of n-alkyl sulfates, and, hence, the burial of the hydrophobic groups further into the interior of the molecule (data not shown).

Sigmoidal curves – Fig. (**3A**, **B**) show the profile transitions of the MG state to the native state pathway (MG $\rightarrow$ N') and the Native to the unfolded state process (N $\rightarrow$ U) of papain, at various concentrations of n-alkyl sulfates in the presence of ANS. These figures also show the effects of the number of hydrophobic chains on the inflection points of the cited profiles. When the hydrophobic tail of n-alkyl sulfate becomes longer, the inflection points of the profiles decrease. These results show that the refolding and unfolding ability of papain increase according to the length of hydrophobic chains.

3.3. Thermodynamic Analysis of Intermediate State Formation

Fig. (**3A**, **B**) show the sigmoidal curves (drawn by a numerical analysis method, called qubic-spline in the MATLAB program, version 6.1) for the transition MG state to the native state (MG $\rightarrow$ N') at pH 2 and transition of the native state turns to the unfolded state of papain N $\rightarrow$ U) at pH 8.7 upon the addition of n-alkyl sulfates. Papain is a small protein with a single subunit. Therefore, a two state analysis based on the pace theory was performed [31]. It is now possible to obtain the equilibrium constant (K) for the MG $\rightarrow$ I and I $\rightarrow$ N' pathways and to calculate the corresponding Gibb's free energy changes, ΔG^0, as follows:

$$\Delta G^0 = -RT \ln (A_{obs} - A_{MG})/(A_I - A_{obs}) \quad (2)$$

where R is the gas constant, T is the absolute temperature, A_{MG}, A_I and A_{obs} are the physical parameters of extinction coefficient, the percentage of fluorescence of MG, I, and any observed states, respectively. Fig. (**4A**, **B**) show the plot of ΔG^0 against total n-alkyl sulfates concentrations ([n-alkyl sulfate]$_{total}$=[n-alkyl sulfate]$_{free}$ + [n-alkyl sulfate]$_{bound}$). The free energies of Intermediate formation in the absence of n-alkyl sulfates, ΔG^0 (H_2O), were calculated by the least-square method from the following equation [34].

$$\Delta G^0 = \Delta G^0 (H_2O) - m[\text{n-alkyl sulfate}] \quad (3)$$

when m is the slope of linear curve reflecting the cooprativity and also hydrophobicity of the transition state. The m-value correlates very strongly with the extend to which the protein surface is exposed to the solvent upon unfolding [35]. Thus, for proteins that undergo a simple two-state unfolding mechanism, the extra of the surface exposed to the solvent upon unfolding is a main structure to determine the m-values and hydrophobicity. The ΔG^0 (H_2O) and m-values at pH 2 and pH 8.7 are tabulated in Tables **1** and **2**. The free energy values are shown as the dotted linear line in Fig. (**4A**, **B**) at pH 2 and 8.7, respectively.

It is apparent from Tables **1** and **2** that the values of ΔG^0 (H_2O) and m-values of the conformational transitions induced by SOS, SDeS and SDS increase with the chain lengths of n-alkyl sulfates increase. $\Delta G^0{}_1$ (H_2O) and $\Delta G^0{}_2$ (H_2O) are for inducing of I_1 and I_2 at both different pH respectively. The inset of Fig. (**4A**, **B**) show the $\Delta G^0{}_2$ (H_2O) against n-alkyl sulfates concentrations that related to the second sigmoid curve in Fig. (**3A**, **B**).

3.4. Binding of n-Alkyl Sulfates

In this paper the possible number of binding sites "b" was also determined by using the Scatchard plot:

$$1/1-\theta = K[Q] / \theta - Kbp \quad (4)$$

where θ equals (θ: $\Delta F/\Delta F_{max}$); ΔF, ($F_0 - F$); ΔF_{max}, ($F_0 - F_{saturation}$); [Q], quencher (n-alkyl sulfates concentration); K, affinity constant; p, protein concentration and b, number of binding sites on each protein molecule [36]. The binding set analysis would be considered as a parameter to help us study and distinguish the two different sets of binding sites which are at work during protein folding and unfolding, induced by n-alkyl sulfates. The binding of many oppositely charged surfactants to the protein molecule is supposed to cause a charge reversal of the protein - surfactant complex, compared with that of the native protein. It is also supposed that, at a specific binding ratio, the protein-surfactant complex will be uncharged, and, therefore the aggregation and precipitation of the complex are expected. Furthermore, when specific binding sites are occupied, the bound surfactant molecules may impose significant surface hydrophobicity on the complex, which may enhance hydrophobic interactions between individual protein-surfactant complexes. The effects of various n-alkyl sulfates on the papain at pH 2 and 8.7 show that the longer chain length increases the ability of n-alkyl sulfates to bind the protein molecule [37].

The anionic surfactant plays a variety of roles with regard to protein conformation, depending on its concentration. Surfactants have found wide applications in biology [38, 39]. The wide spread application of surfactants in the field of biochemistry has given impetus to fundamental studies of the nature of the interactions between

Table 1. ΔG^0 (H_2O), m-Value for the Intermediate States of Papain at pH 2 Upon Interaction with n-Alkyl Sulfates

m_2 kJ.mol.$^{-1}$M^{-1}	m_1 kJ.mol.$^{-1}$M^{-1}	$\Delta G^0{}_{1+2}$(H_2O) M kJ.mol^{-1}	$\Delta G^0{}_2$ (H_2O) kJ.mol^{-1}	$\Delta G^0{}_1$(H_2O) kJ.mol^{-1}	
12.175	10.052	10.6925	7.4954	3.1971	SOS
35.509	11.651	11.887	11.885	2.1701	SDeS
162.7	109.16	19.7699	14.108	5.6619	SDS

Table 2. ΔG^0 (H_2O), m-Values for Intermediate States of Papain at pH 8.7 Upon Interaction with n-Alkyl Sulfates

m_2 kJ.mol.$^{-1}$M^{-1}	m_1 kJ.mol.$^{-1}$M^{-1}	$\Delta G^0{}_{1+2}$ (H_2O) kJ.mol^{-1}	$\Delta G^0{}_2$ (H_2O) kJ.mol^{-1}	$\Delta G^0{}_1$ (H_2O) kJ.mol^{-1}	
10.949	15.682	20.1816	11.243	8.9386	SOS
19.763	10.064	23.1989	19.004	4.1949	SDeS
76.025	26.155	18.5615	15.065	3.4965	SDS

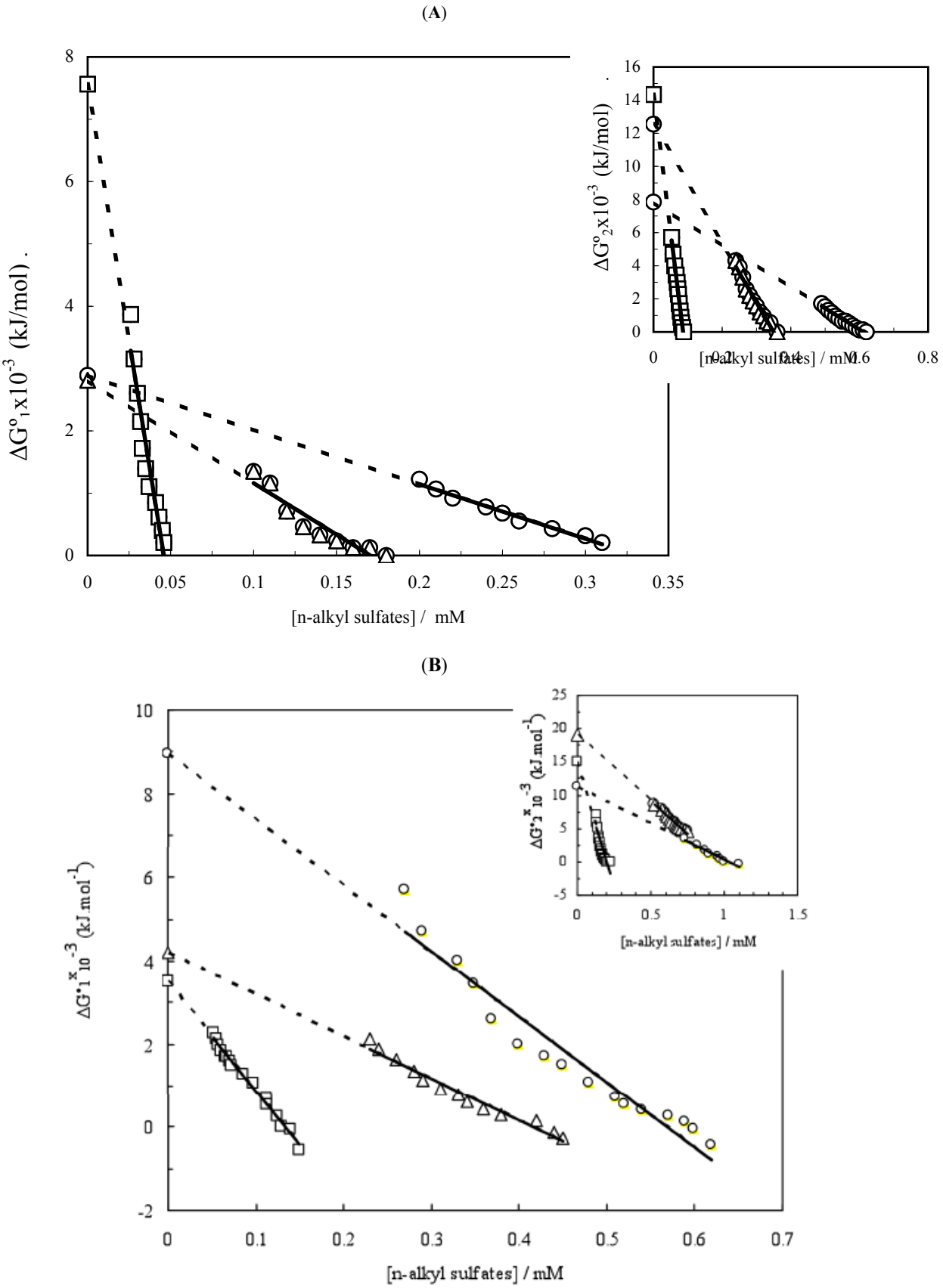

Fig. (4). (**A**) Free energy values ΔG^0_1 (H_2O), versus concentrations of n-alkyl sulfates at pH 2. Inset: Free energy values ΔG^0_2 (H_2O), versus concentrations of n-alkyl sulfates at pH 2. (**B**) Free energy values ΔG^0_1 (H_2O), versus concentrations of n-alkyl sulfates at pH 8.7. Inset: Free energy values ΔG^0_2 (H_2O), versus concentrations of n-alkyl sulfates at pH 8.7.

proteins and surface active agents in Biological phenomena such as Biological membranes and protein solubilization [40-42]. Fig. (**5A**, **B**) show the Schatchard plot of various concentrations of SDeS upon interaction with papain to determine the number of binding sites at pH 2 and 8.7 respectively.

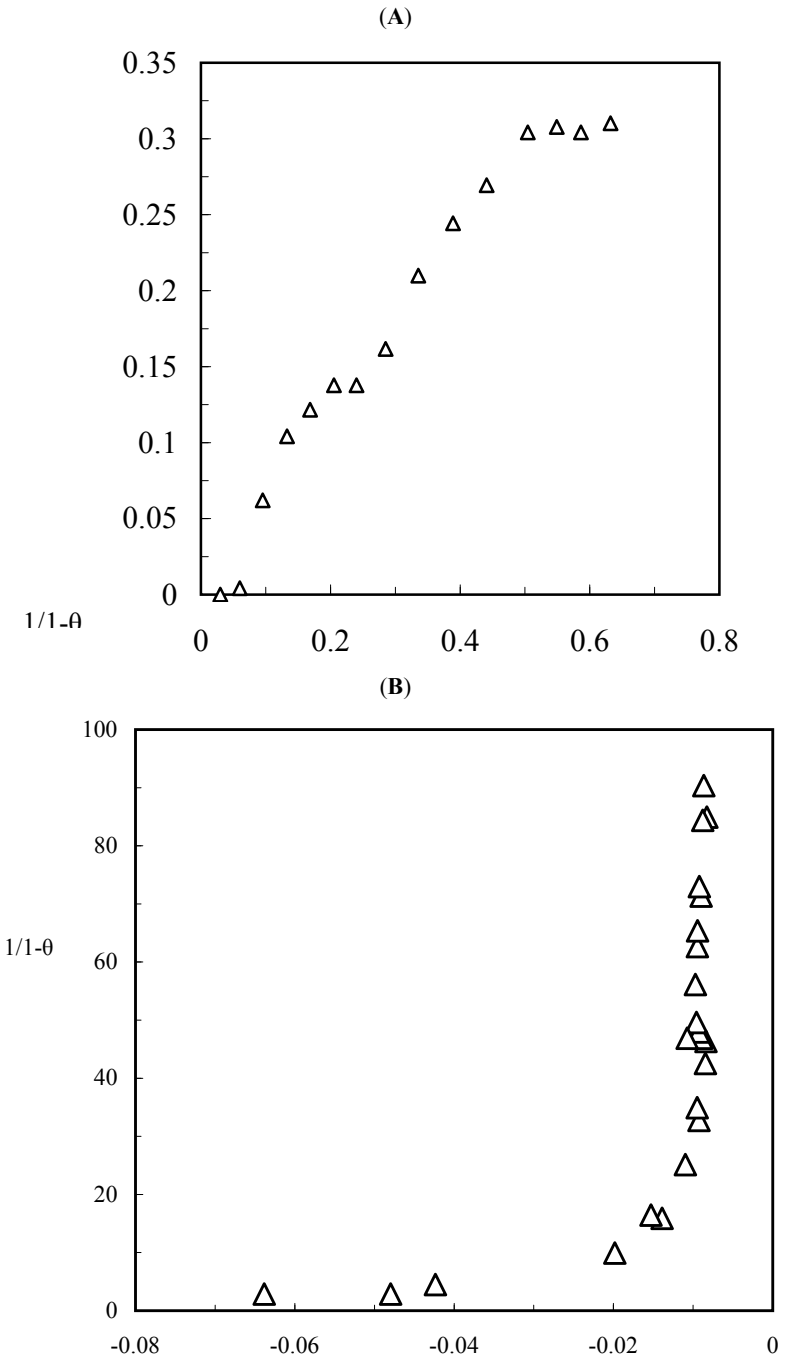

Fig. (5). Scatchard plot of papain upon interaction with SDeS at pH 2 and pH 8.7 for determination of the number of binding sites.

DISSCUSION

It has been suggested that surfactant- protein systems can be used as a model for biological membranes. Since phospholipids membranes help to stabilize the three-dimensional structure of membrane proteins, the use of detergents for the reconstitution of proteins seems plausible. Further more, there are similarities in the structure of some detergents and some of the phospholipids of cell membranes. It has been shown that both the head groups and the hydrophobic tails of surfactants are important for the stabilizing of membrane proteins [31, 37, 43]. The effect of surfactants, such as n-alkyl sulfates, on protein folding and unfolding depends on the concentration of n-alkyl sulfates and protein. Anionic surfactants such as SDS, SDeS and SOS, bind to proteins in the mono-meric state and in the micellar condition. In this paper, n-alkyl sulfates were selected because of their dual electrostatic and hydrophobic interactions. These are amphiphatic compounds with a polar head and a non-polar tail group. Studies of n-alkyl sulfates with identical polar heads but different non polar tails will allow us to determine the contribution of electrostatic and hydrophobic forces to the induction of intermediate state conformation in protein denaturation pathway. Here we report the presence of intermediate state in papain denaturation pathway under acidic and native conditions in the presence of n-alkyl sulfates including SOS, SDeS and SDS. The conformation of the intermediate state is determined by the balance of charge repulsion among positive groups. When n-alkyl sulfates are added to the MG state of papain, in acidic condition, the shielding of intra-molecular electrostatic repulsive forces in the MG state by the negative polar heads of n-alkyl sulfate binding reflects the intrinsic forces that favor the formation of the

intermediate state. It was previously reported that two binding sets exist in protein-surfactant complexes, the first involving electrostatic interactions, and the second having a hydrophobic nature [44-48]. Once all the ionic sites are saturated, the hydrophobic contribution predominates. n-Alkyl sulfates play an important role in electrostatic interactions at low concentrations, and they have a hydrophobic nature at high concentrations. Due to the nature of the MG state of papain with a positively charged surface, it was also suggested that the first interaction of a mono-valent anionic surfactant is electrostatic. It is important to note that the presence of a hydrophobic moiety together with the electrostatic contribution of n-alkyl sulfates to the interaction with papain produces the intermediate state. In this manner, short range interactions between non polar groups on a protein and the non-polar tail of n-alkyl sulfates at low concentrations induce the compaction state [49-51]. Papain assumes a native conformation at pH 8.7. Although the anionic head of n-alkyl sulfate is an important factor determining the n-alkyl sulfate effects, we consider that the direct interaction between hydrophobic tails of n-alkyl sulfates and hydrophobic groups of protein is responsible for the n-alkyl sulfates effects. When n-alkyl sulfates interact with native state of papain at pH 8.7, the interior hydrophobic groups of the protein are exposed to solvent, and then the polarity around the papain will decrease. This leads to stabilization of the intermolecular hydrogen binds, and, consequently, the formation of intermediate state. On the other hand, the presence of free hydrophobic tails of n-alkyl sulfates (unbounded) causes the low polarity of the solvent which decreases the hydrophobic interactions stabilizing the papain, and then local hydrogen bonds are strengthened and the intermediate state is induced. SDS has different tail than SDeS and SOS. The interaction between SDS with papain at acidic condition causes the electrostatic interactions neutralized the positive charge repulsion and then short range interactions between hydrophobic tail of SDS and non-polar group of papain causes the folding of protein. It was previously reported that two binding sets exist in protein-surfactant complexes, the first involving electrostatic interactions and the second having a hydrophobic nature. Once all the ionic sites are saturated, the hydrophobic contribution predominates. Alkyl sulfates play an important role in electrostatic interactions at low concentrations and they have a hydrophobic nature at high concentrations. Due to the nature of the acid-unfolded state of papain with a positively charged surface, it was also suggested that the first interaction of a mono-valent anionic surfactant is electrostatic. It is important to note that the presence of a hydrophobic moiety together with the electrostatic contribution of *n*-alkyl sulfates upon interaction with papain produces the MG state, whereas the interaction of protein with *n*-alkyl sulfates at high concentration produces the predominant hydrophobic force that induces protein denaturation. In this manner, short-range interactions between non-polar groups on a protein and the non-polar tail of *n*-alkyl sulfates at low concentrations induce the compaction state, but at high concentrations of *n*-alkyl sulfates, the hydrophobic interactions predominate relative to the electrostatic contribution and protein unfolds.

One of the best criteria for determining protein stability is free energy in the absence of ligand, ΔG^0 (H_2O) or m-value. m-value for a given transition is generally interpreted as a measure of change in the solvent exposure for that transition [52] and this makes them to be useful estimates of the gross compactness of different states on the folding pathway relative to the two end-stations, the denatured state D and the native state N. Ligand–induced refolding is useful for understanding the mechanism of conformational stability. In particular, the m-value is important since it is a measure of the cooprativity of the folding and unfolding transitions and is proposed to be approximately proportional to the difference in solvent – accessible surface area between the folded and unfolded states [53, 54]. Tables **1** and **2** show the m-values and ΔG^0 (H2O) for the intermediate state of papain upon the addition of n-alkyl sulfates such as SOS, SDeS, SDS (below the CMC) at pH 2 and 8.7, respectively. Tables **1** and **2** show the increase in ΔG^0 (H_2O) and m-values that corresponds to the length of the hydrophobic chains. It will be noted that the m-value for the intermediate states induced by a low concentration of n-alkyl sulfates at pH 2 is significantly higher than that at pH 8.7. What is more, m-value is a sign of cooprativity; therefore, the intermediate states induced by n-alkyl sulfates at pH 2 are more cooperative than the intermediate state at pH 8.7. However, the effects of various n-alkyl sulfates on papain at pH 2 and 8.7 indicate that the longer the chain, the higher the ability of binding n-alkyl sulfates to the protein molecule. Therefore, the quality of a solvent with various ionic strengths can determine different levels of stability and conformation of the intermediate state. The binding constant values obtained from Schatchard equation show that the binding affinity of n-alkyl sulfates to papain at pH 2 is more than pH 8.7. Thus the intermediate structures induced by n-alkyl sulfates at different pH values are dependent on the ion quality in papain solution. On the other hand, the base of hydrophobic forces is electrostatic interactions between various kinds of ions in solution and there is a direct relationship between hydrophobicity and the stability of the intermediate states. In addition, our results show that there are two different intermediate states (I_A and I_B) induced by n-alkyl sulfates at acidic and native conditions, respectively.

CONCLUSION

The results described in this paper strongly indicate that there are different structures of intermediate states of papain induced by various concentrations of n-alkyl sulfates along refolding and unfolding pathways in two different conditions. In addition, evidence for the stabilization of the intermediate states of papain in the two different ionic solutions of n-alkyl sulfates with a hydrophobic chain has been presented. Here, n-alkyl sulfates in various conditions induced two intermediate states (I_A and I_B) with different conformations at acidic and native states, respectively. Thus, we can assume that the intermediate states in folding and unfolding pathways in various conditions have different structures. The results also indicate the appearance of higher stabilized intermediate states, which corresponds to the interaction of papain with *n*-alkyl sulphates. Therefore, our results show a direct role of hydrophobicity to the stability of the molten globule state.

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