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Evaluation of Insulin Stability in the Presence of Nonionic Surface Active Agents (Polysorbate Groups) by Circular Dichroism and Fluorescence Spectroscopy

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ABSTRACT

In this study, the possibility interactions of insulin with four non-ionic surfactants of polysorbates group using circular dichroism and fluorescence spectroscopy was investigated. The results showed that insulin structure is changed at pH 3 and 10 partially in comparison with pH 7. It was also was shown that polysorbates may cause a red shift of maximum emission fluorescence at all of the above pH values. Thermodynamic stability of insulin was determined in the presence of polysorbate group surfactants. The α-helix content of insulin exhibits a slight rising and thereby it causes an increasing in its free energy change (ΔG) and stability. The ΔG (H_2O) of insulin was about 65.6 kj mol^{-1} in the presence of polysorbate 80 at pH 7. It was shown that polysorbate 80 increased the free energy and the α-helix content of insulin structure more than the others, so they could be considered as stabilizer in the formulations of protein drugs.

Key words: Insulin, polysorbate, stability, interaction, spectroscopy

INTRODUCTION

Protein instability encompasses many complicated and interrelated chemical and physical process. Any of those processes could be occurred during the production, isolation, purification, analysis, delivery and storage of pharmaceutical proteins. Some of those reactions appear to be ubiquitous and of concern to the pharmaceutical sciences researchers. Physical instability refers to some conditions such as denaturation, aggregation, precipitation and adsorption to surface without any changes in the chemical structure of the protein (Suresh and Soll, 2005). Due to vast advances in biopharmaceutical technology the preparation of proteins as medicinal agents has become an integral part of the pharmaceutical industry. Formulation of proteins is greatly different from that of rigid small organic molecules. Proteins may undergo a variety of structural changes apart of chemical modification and this is because of their ability to adopt some structural forms (secondary, tertiary, quaternary) and also their polymeric nature. It has been demonstrated that proteins could be denatured while they still can keep some part of their secondary structure (Manning *et al*., 1989). It is more complicated to control the physical instability of proteins than their chemical instability and it also is as a greater concern. The surface active agents are one of the most

significant excipient in formulation of peptides and proteins. Surfactants, especially non-ionic types, are frequently added to prevent or reduce protein aggregation during fermentation, purification, freeze-drying, shipping and storage (Ruiz-Pena *et al*., 2010). Similarly to polymers, most proteins do not associate with non-ionic surfactants, with the general exception being those containing hydrophobic pockets or patches, such as serum albumins. The hydrophobic section of non-ionic surfactants can attach to hydrophobic patches on proteins which normally causes the surfactant to order itself so that more hydrophilic groups are solvent exposed. Consequently, the surfactant-protein complex becomes more hydrophilic than either the surfactant or the protein alone and the efficient increase of the complex solubility avoids formation of higher order aggregates (Randolph and Jones, 2002). Polysorbates are a group of non-ionic surfactants. They are oily liquids derived from PEGylated sorbitan (a derivative of sorbitol) esterified with fatty acids. Polysorbates are used as emulsifiers, defoamers, dispersants and stabilizers in food, cosmetics and pharmaceutical formulations (Perkins, 1998). Typical applications with CD analysis are included the estimation of the content of protein secondary structures and the detection of conformational changes in a protein due to changes in its solution environment (pH, excipients and presence of denaturants) and temperature. The study on protein surfactant stability has been done for some decades. Although there was a burst of protein-surfactant activity in the late 60s and early 70s and it caused to establish the general concept and principles of how charged surfactants bind to and denature the proteins but advances in techniques for more accurate and sophisticated analysis of the interaction between protein-surfactants such as analysis circular dichroism technique happened in 90s decade. To make fluorescence measurements the protein is excited at a wavelength corresponding to its excitation maximum and light is emitted during the protein's transition from the excited state to the ground state. Aromatic amino acids, including tryptophan, tyrosine, phenylalanine and histidine, provide intrinsic fluorescence signals that can be measured. The information obtained is related to the environment surrounding these residues, hence, the integrity of the tertiary structure of the molecule can be interpreted. For these reasons, fluorescence spectroscopy is a useful tool for studying the unfolding of a protein in different environments (pH, temperature, buffer excipients, etc.) (Uversky *et al*., 2003). Many researches have been done about protein surfactant interactions and they emphasize the amazing power of surfactants to both extend the protein conformational landscape and at the same time provide convenient and reversible shortcuts between the native and denatured state of proteins. Mollmann *et al*. (2005) showed that polysorbate 80 reduces the adsorption of insulin by competing for sites at the surface. Insulin is composed of an A chain of 21 amino acids and a B chain of 30 amino acids, the chains being held together by disulfide bonds. Third disulfide bond is present within the A chain. The A chain forms two anti-parallel alpha helices (A2-A8 and A12-A20) while the B chain forms a single alpha helix (B9-B19), followed by a turn and a p-strand (B21 and B30) (Chang *et al*., 2003; Gok and Ates, 2001; Yamada, 2009). It becomes evident that to efficiently design stable protein formulations we need a more comprehensive understanding of the interactions of proteins with the various components of a formulation and their effect on protein stability. In this study, interaction of human insulin with four non-ionic surfactants (Polysorbate 20, polysorbate 40, polysorbate 60 and polysorbate 80) in solution with different pH (3, 7 and 10) as a function of surfactant concentrations by using fluorescence and circular dichroism spectroscopy was investigated.

MATERIALS AND METHODS

The human insulin was purchased from Lilly. The surfactants (Polysorbate 20, 40, 60 and 80) were analytical grade from Fluka. Glycine, phosphate buffer with the highest purity, HCl and

NaOH were purchased from Merck. All solutions were prepared using double distilled water (ddH_2O). All measurements were performed in a 10 mM phosphate buffer at pH 7 and 50 mM glycine buffer at pH 3 and 10. The pH values for all solutions were measured separately using a TWT-Metrohm pH meter and an Accumet Ag/AgCl combination microelectrode. The absolute error of our pH-measurements was ±0.01 unit.

Circular dichroism data were obtained at 25°C using an AVIV model 215 spectropolarimeter (AVIV, Lakewood, NJ, USA). The CD spectroscopic measurements were carried out in a 1 mm path length cuvette at far UV region. For all CD measurements, protein concentration was about 0.25 g L^{-1}.

Fluorescence measurements were performed in a 10 mm path-length cuvette at 25°C using a Cary Eclipse spectrofluorometer (Cary Eclipse, USA). The protein samples were excited at 296 nm with the excitation polarizer oriented in the vertical position and horizontal components of the polarized emission light were recorded through a monochromator at 350 nm. The protein concentration for all fluorescence measurements was about 1 g L^{-1}. Titrations of human insulin solutions in phosphate buffer at pH 7 and glycine buffer at pH 3 and 10 with surfactants were performed while adding aliquots of polysorbate stock solution directly to the cuvette and fluorescence emission and CD spectra were registered.

RESULTS AND DISCUSSION

The structure of human insulin was studied with Circular Dichroism (CD) at acidic, neutral and basic pH (Fig. 1), at 25°C. The range of far-UV region CD spectra was 190-250 nm. At pH 7 the structure of human insulin is native and it was consider as physiologic pH. According to the CD spectrum of human insulin at pH 3 and 10, the value of α-helix is smaller than neutral pH which confirms that the structure of human insulin is different at these pH. In order to make further investigation for the effects of polysorbates on the insulin structure for all of the pH, a CD experiment were carried out. All far-UV CD spectra of insulin combination with polysorbates (Fig. 2-5) showed two minimum at 208 and 222 nm, in the range 200-250 nm, at 25°C which are typical of the predominant α-helix structure. The changes in the secondary structure of insulin upon increasing concentrations of the polysorbates are reflected as change in the intensity of the

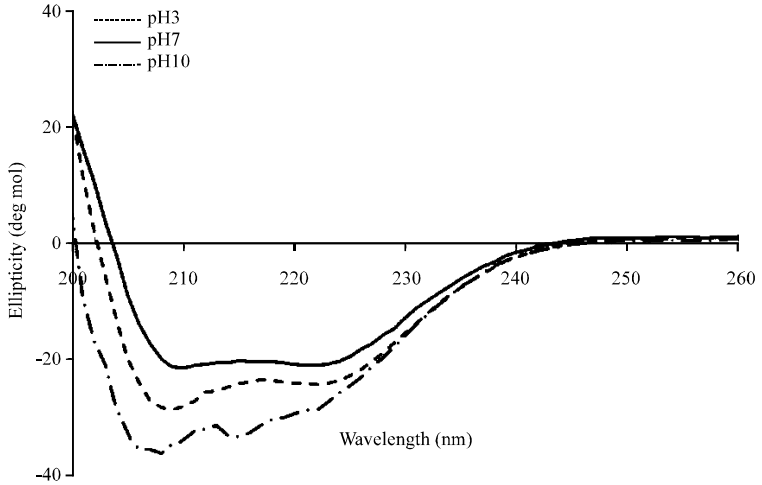

Fig. 1: Circular dichroism spectra of human insulin (Mean±SD) at three different pH values

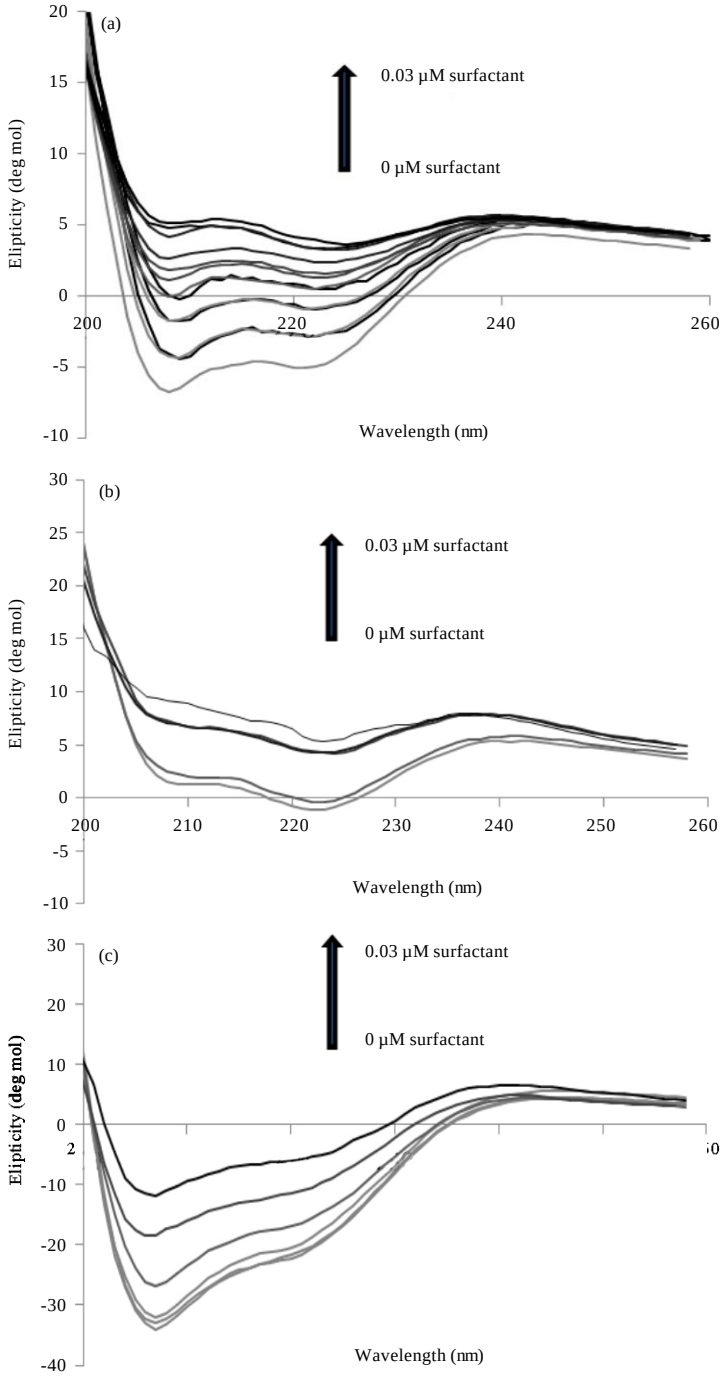

Fig. 2(a-c): Circular dichroism spectra of human insulin at different concentrations of polysorbate 20 in, (a) pH 3, (b) pH 7 and (c) pH 10

spectrum in the vicinity of two wavelengths. As it was seen from CD results, upon increasing the [polysorbate]/[insulin] molar ratio, the α-helical content of insulin exhibited a slight growing. Altogether, these results show that the polysorbates partially increase the α-helix content of human

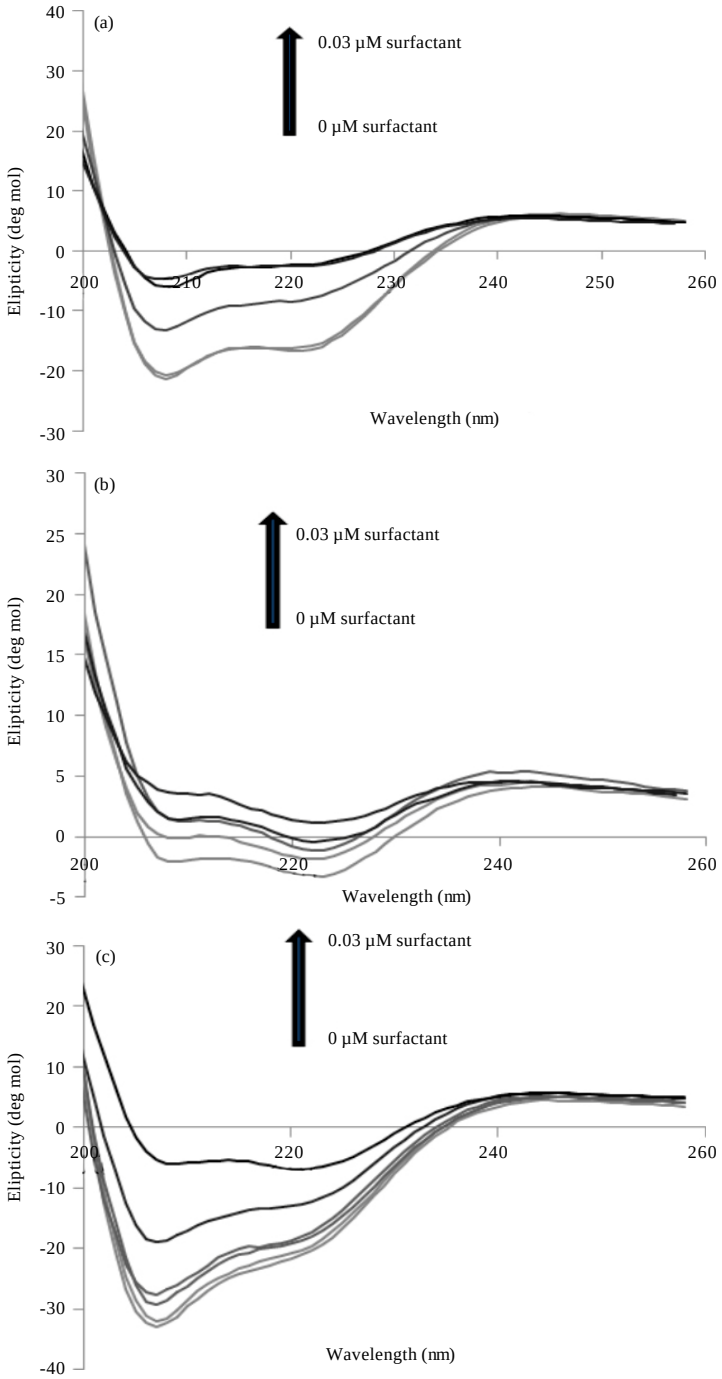

Fig. 3(a-c): Circular dichroism spectra of human insulin at different concentrations of polysorbate 40 in, (a) pH 3, (b) pH 7 and (c) pH 10

insulin. The effect of α-helix induction is also like to the other results and may be exerted by decrease of hydrophobic interactions; strengthening intra-protein hydrogen bonding and weaker shielding from electrostatic interactions (Pourhosseini *et al*., 2007; Yong *et al*., 2009).

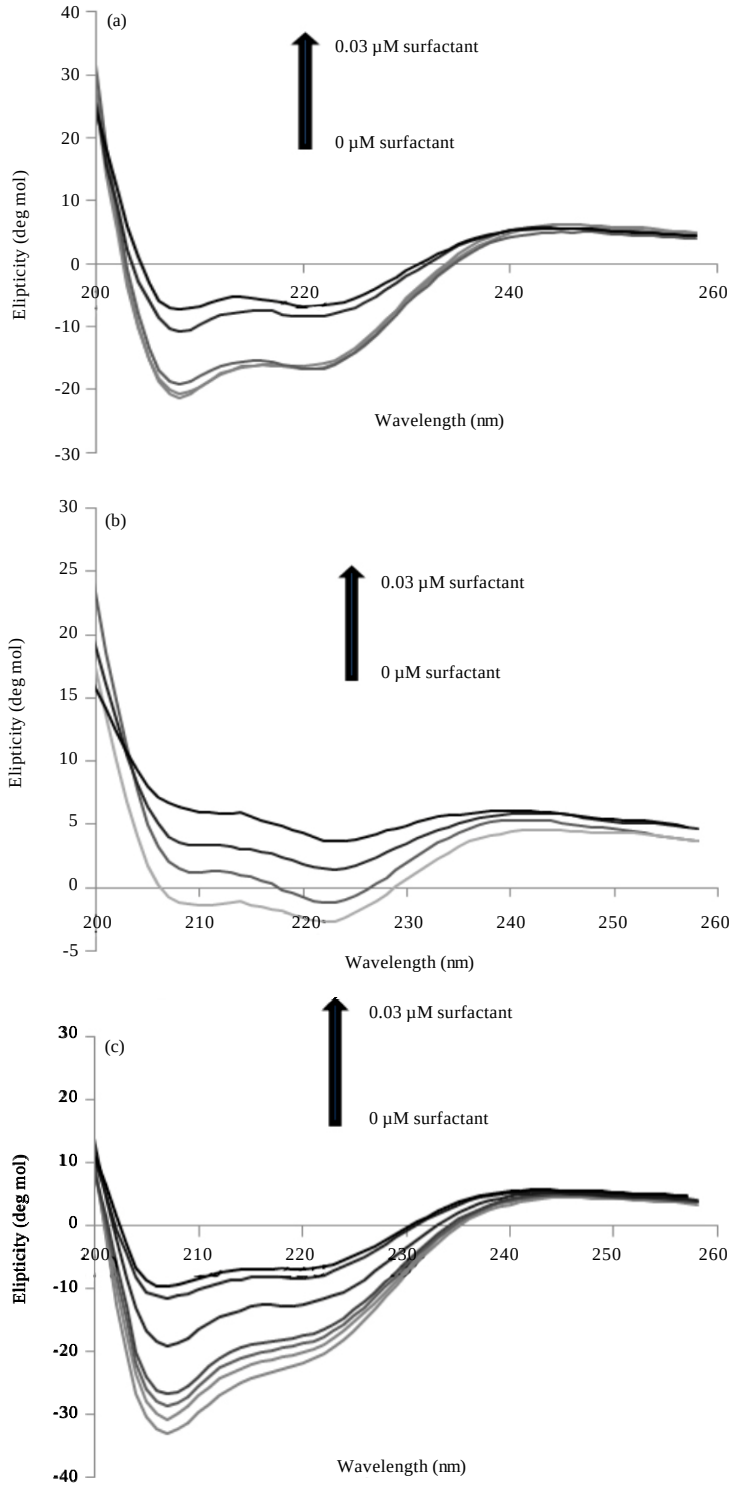

Fig. 4(a-c): Circular dichroismspectra of human insulin at different concentrations of polysorbate 60 in, (a) pH 3, (b) pH 7 and (c) pH 10

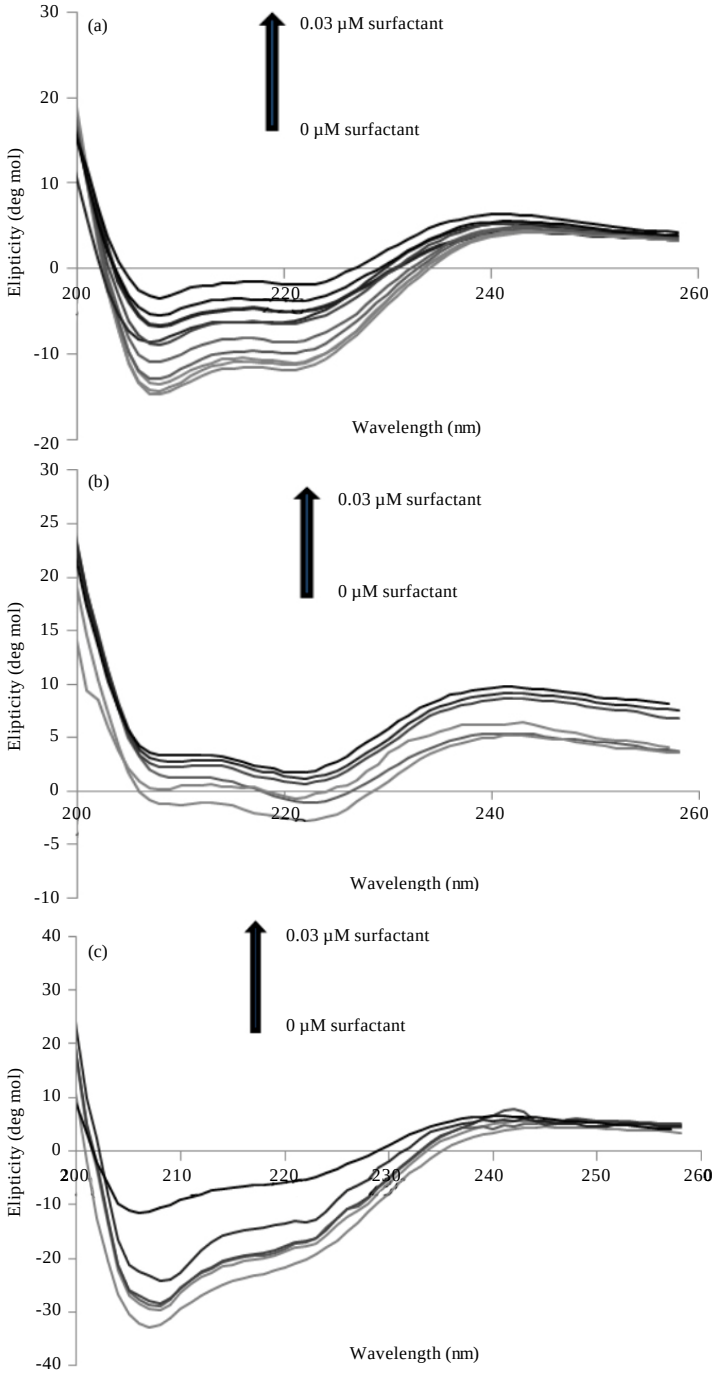

Fig. 5(a-c): Circular dichroism spectra of human insulin at different concentrations of polysorbate 80 in, (a) pH 3, (b) pH 7 and (c) pH 10

Figure 6 shows the fluorescence intensity of human insulin at different pH. The fluorescence spectra of human insulin at pH 3 and 10 showed the intensities were smaller than neutral pH. The same results were observed by CD measurements. This means that the α-helix content of human

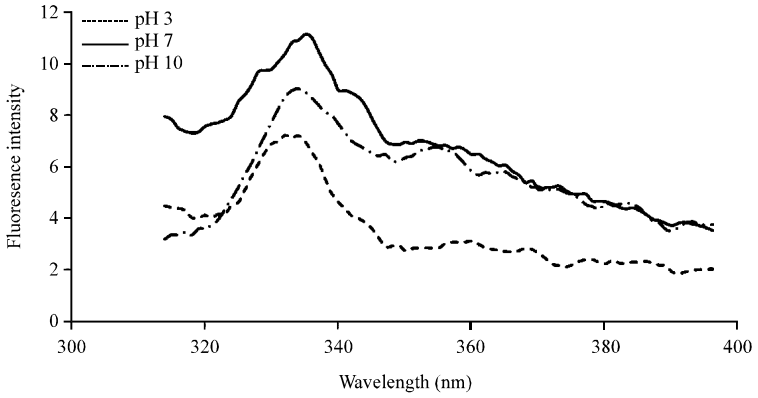

Fig. 6: Insulin fluorescence spectrum in different pH

insulin is more at this pH. The influences of concentration of polysorbates surfactants group on the fluorescence spectra of human insulin at different pH were also measured. Figure 7-10 show the fluorescence intensity of human insulin at different concentrations of the polysorbates and pH 3, 7 and 10. The presence of surfactants had an appreciable effect on both the height and wavelength of the fluorescence emission spectrum. Nevertheless, the influence of all investigated surfactants (with the same concentrations) on the emission fluorescence of human insulin was qualitatively different. As the results show, the fluorescence intensity of human insulin was increased regularly with increasing in the surfactants concentrations. The increase in the fluorescence spectra along with a red shift (as like the results of CD spectra) of human insulin with increasing surfactant concentrations suggests that the tyrosine residues of human insulin have been shown more tendency to less hydrophobic environment at higher concentrations. The observed red shift could therefore be accounted for by a change in the polarity of the environment of tyrosine groups after desorption (Madaeni and Rostami, 2008).

Since there are no tryptophan residues in insulin (Brange and Langkjoer, 1993), changes in the emission fluorescence spectra reflect the contributions due to tyrosine (Pourhosseini *et al.*, 2007). Spectral studies on insulin have led to the suggestion that tyrosyl-OH groups are involved as donors in hydrogen bonds to other protein side chains. In view of these and other data, it has been suggested that protein difference spectra may arise indirectly from general configurationally changes which lead to changes in polarity and polarizability in the vicinity of the tyrosyl groups (Leach and Scheraga, 1960).

There are two different aspects of protein stability. One is the chemical stability of the covalent structure which involves covalent changes and is usually irreversible. The other is the conformational stability of the folded state, in the absence of covalent changes. Measuring the conformational stability requires determining the equilibrium constant and the free energy change, ΔG, for the reactions:

$$\text{Folded (F)} \leftrightarrow \text{Unfolded (U)}$$

We refer to the value of ΔG at 25°C in the absence of a denaturant, ΔG (H_2O), as the conformational stability of a protein.

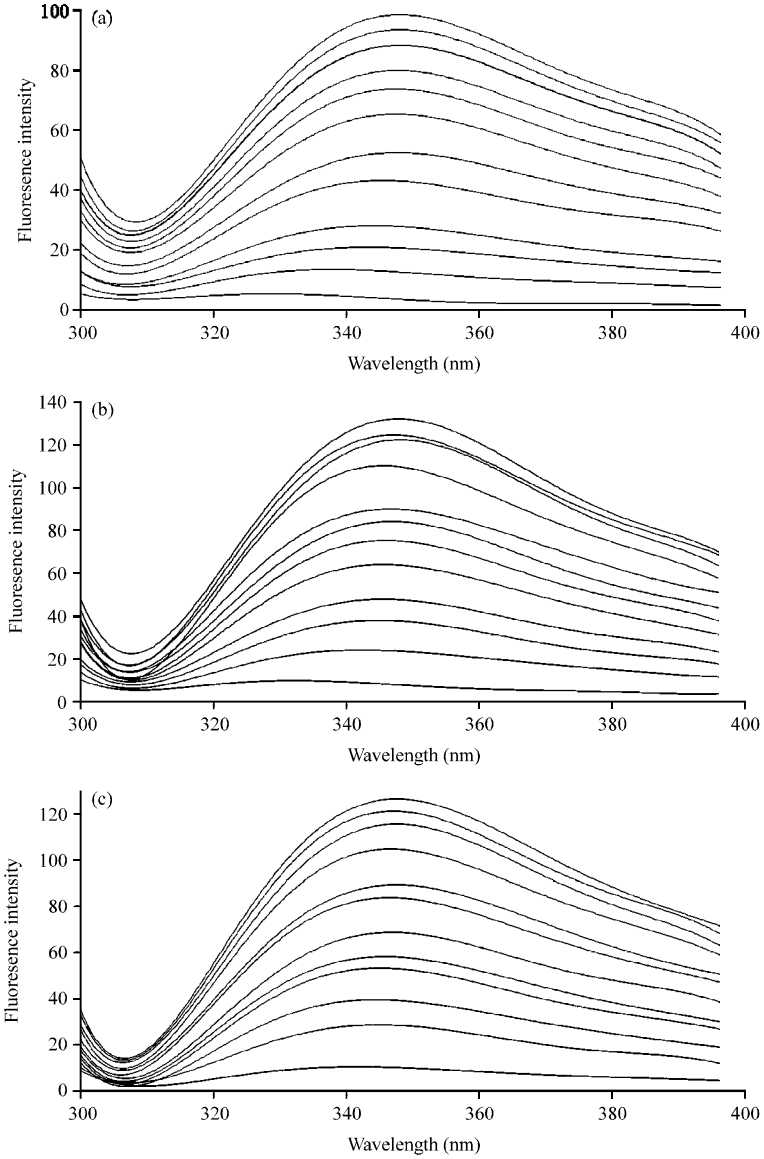

Fig. 7(a-c): Fluorescence emission spectra of human insulin at different concentrations of polysorbate 20 in, (a) pH 3, (b) pH 7 and (c) pH 10 (Down to up: 0, 0.0008, 0.0016, 0.0024, 0.004, 0.006, 0.007, 0.009, 0.0106, 0.012, 0.014 and 0.0155 μM)

Partially folded protein intermediates can be very difficult to detect and study even though they may be important for both kinetic and equilibrium properties. Here, we consider how these intermediates can affect the classical analysis of protein stability. Classical melting analysis provides the major method for measuring the stabilization free energy of protein molecules and changes in stability imposed by different factors. Denaturant, temperature, or pressure is used to drive proteins through their global unfolding transition where the population of Unfolded (U) and Native (N) forms can be measured. The U/N ratio measured through the melting transition region is then transformed into stabilization free energy (Eq. 1) and extrapolated to obtain the equilibrium stability at native conditions:

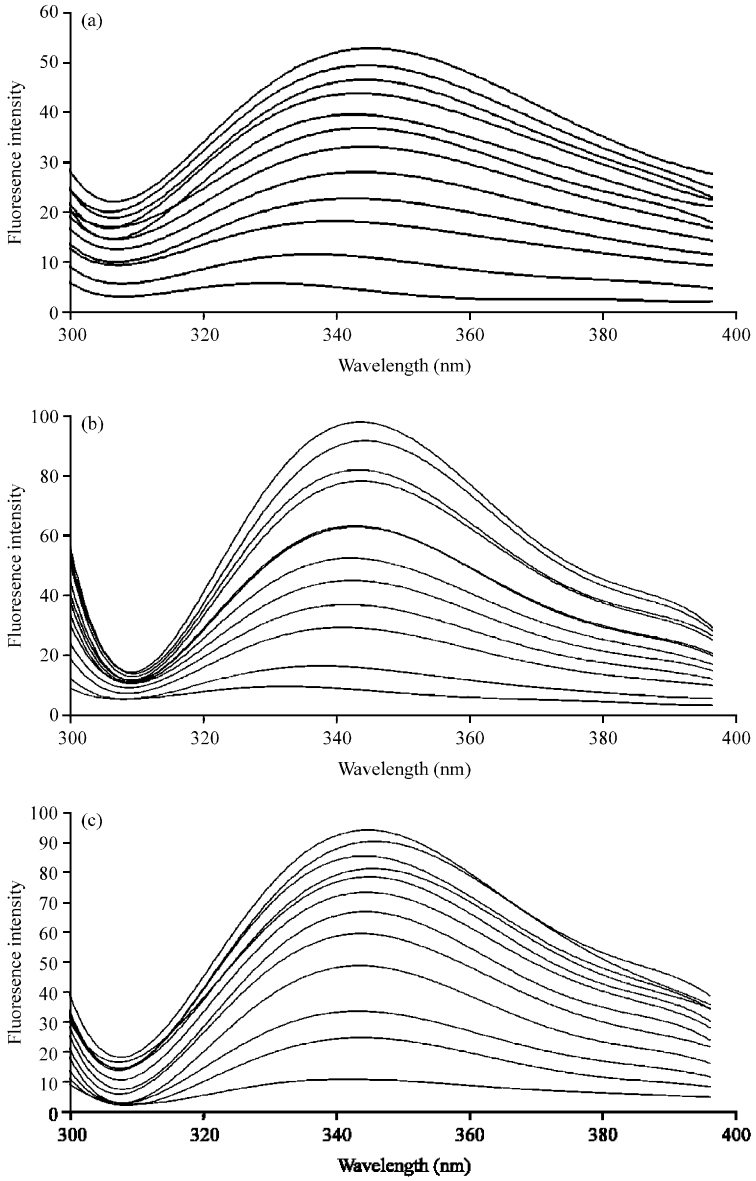

Fig. 8(a-c): Fluorescence emission spectra of human insulin at different concentrations of polysorbate 40 in, (a) pH 3, (b) pH 7 and (c) pH 10 (Down to up: 0, 0.00077, 0.0015, 0.0023, 0.0039, 0.0054, 0.007, 0.0086, 0.0101, 0.0117, 0.013 and 0.0148 μM)

$$\Delta G_U = RT \ln K = RT \ln U/N \quad (1)$$

If two-state unfolding be assumed, only the fully native and fully unfolded forms contribute to the measured data. This assumption is often justified by the fact that the measured melting behavior does not obviously reveal intermediate forms and fits well to the standard two-state melting equation. However, many observations (kinetic folding, molten globule structure, hydrogen exchange) now show that proteins are able to stabilize intermediates that lie between the N and U states (Mayne and Englander, 2000). The analysis of data was made assuming an approximation

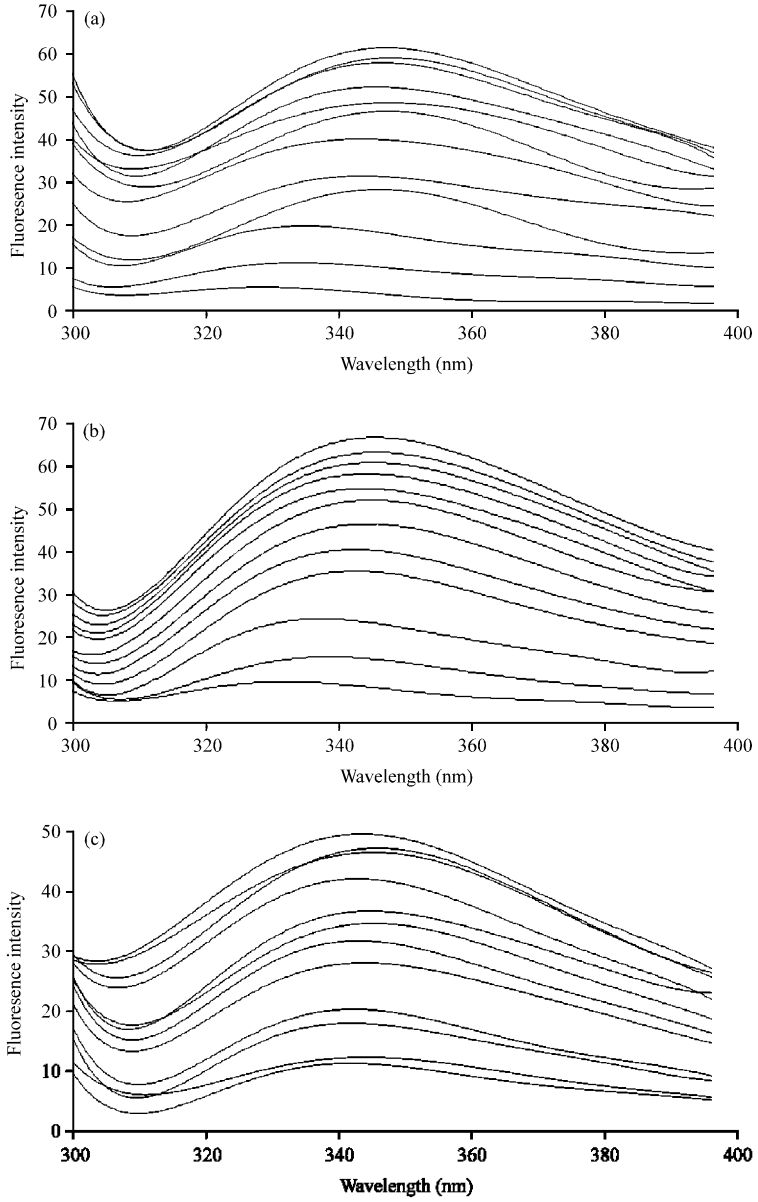

Fig. 9(a-c): Fluorescence emission spectra of human insulin at different concentrations of polysorbate 60 in, (a) pH 3, (b) pH 7 and (c) pH 10 (Down to up: 0, 0.00076, 0.0015, 0.0023, 0.0038, 0.0053, 0.0069, 0.0084, 0.099, 0.0114, 0.013 and 0.0144 μM)

of a two-state model for each step. We have used non-linear least squares to fit α versus polysorbate concentration curves. The dependence of ΔG° with polysorbates concentration, [D], fits very well with Pace Eq. 2:

$$\Delta G^\circ = \Delta G^\circ(H_2O) - m_D[D] \quad (2)$$

where, $\Delta G^\circ(H_2O)$ is the value of ΔG° in the absence of polysorbate and m_D is the slope of this strength line calculated around pre-and post-transition zone. Values of $\Delta G^\circ(H_2O)$ and

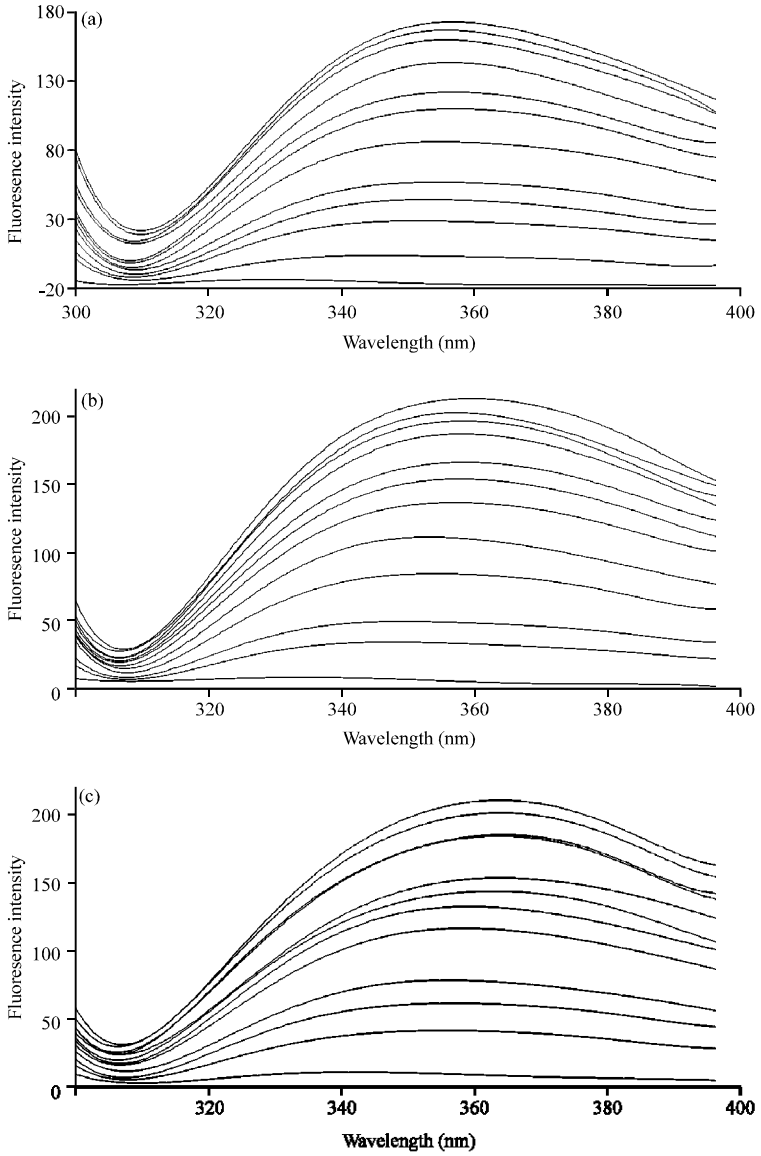

Fig. 10(a-c): Fluorescence emission spectra of human insulin at different concentrations of polysorbate 60 in, (a) pH 3, (b) pH 7 and (c) pH 10 (Down to up: 0, 0.00076, 0.0015, 0.0023, 0.0038, 0.0053, 0.0069, 0.0084, 0.099, 0.0114, 0.013 and 0.0144 μM)

m_D are shown in Table 1 at different pH. Then the stability of insulin calculated by Eq. 3 (Manning *et al*., 1989; Shortle and Meeker, 1986):

$$\Delta G^{\circ}(H_2O) = \Delta G_1^{\circ}(H_2O) + \Delta G_2^{\circ}(H_2O) \quad (3)$$

Surfactants of polysorbate group increased the helix content and helix dipole in human insulin and thereby affect an increase in stability. This corresponds to an increase in the free energy of stabilization for each of the surfactants. The observation of the noticeable increase in intrinsic fluorescence and far UV-CD data of human insulin at various polysorbate concentrations

Table 1: Thermodynamic parameters of human insulin at different media

pH	Surfactant	$-mD_1$ (kJ mol^{-1})	$-mD_2$ (kJ mol^{-1})	ΔG°_1 (kJ mol^{-1})	ΔG°_2 (kJ mol^{-1})	ΔG° (H_2O) (kJ mol^{-1})
3	Polysorbate 20	0.0010	0.0009	22.880	12.676	35.5560
7	Polysorbate 20	0.0009	0.0013	20.122	18.632	38.7540
10	Polysorbate 20	0.0008	0.0006	18.233	7.9835	26.2165
3	Polysorbate 40	0.0008	0.0008	16.146	10.884	27.300
7	Polysorbate 40	0.0007	0.0008	15.613	12.032	27.6450
10	Polysorbate 40	0.0009	0.0003	18.872	2.4886	21.3606
3	Polysorbate 60	0.0014	0.0008	25.753	10.765	36.5180
7	Polysorbate 60	0.0011	0.0017	22.993	12.700	35.6930
10	Polysorbate 60	0.0013	0.0004	26.022	3.5805	29.6025
3	Polysorbate 80	0.0011	0.0017	24.430	25.669	50.0990
7	Polysorbate 80	0.0009	0.0010	20.041	45.573	65.6140
10	Polysorbate 80	0.0013	0.0011	26.010	15.164	41.1740

cannot be used alone to predict accessibility of tyrosine residues. Although, insulin is a multi-tyrosine protein, hence it is difficult to determine exactly the contribution of each tyrosine to the fluorescence emission. It could be concluded that upon addition of polysorbate group surfactants the human insulin becomes more stable. Polysorbate 80 more than the others increased the free energy and the α-helix content of human insulin structure.

CONCLUSION

Since, polysorbates (especially polysorbate 80) increase the stability of human insulin, they could be considered in formulation of proteins drugs. However, detailed interpretation of these results calls for further investigations.

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