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A COMPREHENSIVE INVESTIGATION OF THE INTERRELATIONSHIP BETWEEN FLUORESCENCE AND UV-DIFFERENCE SPECTROSCOPY OF DENATURATION OF OVALBUMIN BY UREA AND β-BME

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ABSTRACT

The structural thermodynamic and functional aspects of ovalbumin of chicken egg, unfolding induced by urea and βME(β-mercaptoethanol) has been studied at pH 7.0. Ovalbumin belongs to the **Serpin** class of protein. We have shown that the transition from native to denatured induced by urea and βME passes through essential unfolding of the protein. The phenomenon of denaturation of ovalbumin has been studied in terms in λ_{max}, fluorescence intensity, change in Gibbs free energy at zero denaturant concentration, ΔG_D (H_2O) using the LEM (Linear Extrapolation Method). The fluorescence intensity (specially tryptophan fluorescence intensity) should be a minimum (10%) decrease on addition of 1M urea and maximum (97.7%) decrease on addition of 1N βME and 9M urea mixture in ovalbumin. Intensity quenching due to environmental change (or substantial conformational change) The chemical deaturation leading to exposure of tyrosine residues was studied with UV-difference spectroscopy as function of concentration of urea and βME. The study showed that ovalbumin was highly denatured in presence of urea and βME. The UV-difference spectra were evaluated to calculate Gibbs free energy change, $\Delta G_D(H_2O)$, using the linear extrapolation method, which reflects the stability difference between native and denatured species The study should that ovalbumin was highly denatured in presence of urea and βME due to disruption of hydrogen bonds as well as intra- and interchain disulfide bonds indicating the flexibility of ovalbumin increase on addition of βME, so it becomes susceptible to digestion.

KEYWORDS: Interrelationship, Fluorescence, UV-Difference Spectroscopy, Denaturation, Ovalbumin, Urea and β-BME

INTRODUCTION

Fluorescence spectroscopy is one of the most widely used spectroscopic technique because of its acute sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes. The fluorescence measurements in case of macromolecules can give information about conformation, binding sites, solvent interactions, degree of flexibility, intermolecular distances and the rotational diffusion coefficient of macromolecules. The intrinsic fluorescence of aromatic amino acids in proteins have been used in a number of studies, however, the fluorescence unfolding /refolding profiles, because they arise from structural changes local to particular tryptophan residues, have revealed three state behavior [1,2]. Three major fluorescence parameters can be monitored as indicators of protein structure. These three parameters are the intensity of the emission, and the average wavelength of the emission, and the polarization.

Dynamic quenching of fluorescence results from the excited state encounter of the tryptophan with a quenching

moiety, such as a disulfide, an amine, or other electron-accepting group on a neighbouring amino acid [3]. The average energy of emission,ν_g or the intensity (Fi) weighted average of the inverse wavelengths (ν_i) scanned in a spectrum, is indicative of the solvent exposure of the tryptophan.

$$\bar{\nu}_g = \sum_{i=\nu_1}^{\nu_n} Fi\nu i \; \sum_{i=\nu i}^{\nu_n} Fi$$

Solvent relaxation around the excited state dipole of the tryptophan leads to a lowering of the energy of the excited state and thus to a less energetic, or red shift emission. The magnitude of the shift to the red observed on denaturation depends on the extent to which the tryptophan residue is buried in the native protein and exposed on denaturation [4], whereas observations on the blue edge can yield an apparent loss in intensity. The protein fluorescence spectra are usually dominated by tryptophan absorbance and emission, in part because tryptophan has the highest extinction coefficient of the three and in part because resonance energy transfer from phenylalanine and tyrosine to tryptophan frequently occurs. 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, temperature, pH changes and pressure [5, 6]. The fluorescence properties of tryptophan residues in particular are sensitive to perturbations of protein structure. The intrinsic tryptophan residues in ovalbumin were excited at 295nm and 280nm and emission spectrum was recorded at a wavelength range from 320-450nm. All measurements were carried out at a constant temperature of 25^oC [7].

There are number of denaturants were known for proteins e.g. Urea, guanidine hydrochloride, and like compounds are believed to cause the most complete unfolding (Tanford 1968). The linear extrapolation method (LEM) is a frequently used procedure for obtaining the difference in stability between the native (N) and denatured (D) states of a protein [8-10]. Protein stability is expressed as a Gibss free energy change, ΔG_D (H_2O), obtained upon linear extrapolation of cooperative unfolding free energy data at zero denaturant concentration, $\Delta G_D(H_2O)$, is said to represent the free energy difference between N and D species in the absence of denaturant.[11-12].

A simple way for ΔG_D to be a linear function of denaturant concentration would be for the respective free energies of the native and denatured states (G_N and G_D) to also be linear functions of denaturant concentration and the intersection point defines $C_{1/2}$ the denaturant concentration at the mid point of the N→D transition (Tm). Denaturants most certainly shift the N→D equilibrium, but they can also change the thermodynamic behaviour of the individual N and D ensembles non linearly with denaturant concentration.

In present study, we want to compare the stability of ovalbumin in the presence of different denaturants in terms of their unfolding free energy changes, by fluorescence studies. This free energy value obtained by fluorescence studies to be compared by $\Delta G_D(H_2O)$ obtained from UV-difference spectral studies.

The mechanism and the identity of protein conformations involved in a protein folding process can be studied by monitoring UV-spectrophotometer changes in the quaternary, tertiary and secondary structures. As previous data shows that ovalbumin contain a single tryptophan residue, four cysteine sulfides, and one cysteine disulfide [13].

The spectra of proteins are usually shifted towards longer wavelength (bath chromic or red shift), when compared with the corresponding spectra of their constituent amino acids mixed in the correct proportions [14-17]. These spectral shifts provide the basis of employing UV-spectroscopy to study protein structural transitions. The peaks arises from multiple contributions from tryptophan (291-294 nm), tyrosine (286-288 nm), phenylalanine (245-270 nm) and cysteine

(264-268 nm), in the 245-294 nm region. The molar absorbance of phenylalanine is very much lower than tryptophan and tyrosine and phenylalanine has very little absorbance above 275 nm. The hypsochromic shift was reported for denaturation of ovalbumin protein [18].

In this work, UV-spectroscopy has been used to study the proteins unfolding process by chemical denaturation specially the denaturation of ovalbumin by urea in the presence of βME and βME alone to observe the structural changes from folding to unfolding. The free energies have been calculated by using of linear extrapolation method (LEM) [19-21].

In the present study, we want to compare the stability of ovalbumin in the presence of different denaturants in terms of their unfolding free energy changes, by fluorescence studies. This change in free energy value obtained by fluorescence studies was compared to $\Delta G_D(H_2O)$ obtained from UV-difference spectral studies.

EXPERIMENTAL PROCEDURES

Materials

Purified, crystallized and Lyophilized chicken egg ovalbumin were obtained from Sigma was used without further purification. Sodium phosphate, Urea and β-mercaptoethanal (βME) were purchased from Merck chemicals. Sodium phosphate buffer concentration was always 0.1M and pH 7.0 was measured by digital pH meter (Elico Pvt. Ltd. Hyderabad, Model T-10). All other reagents that were used analytical grade. All solutions were prepared in doubly distilled water. Samples for all techniques measurements were filtered and the exact concentration of the protein was determined spectrophotometrically using an extinction coefficient ε, (3.218×10^4 dm^3 M^{-1} cm^{-1}) excitation at 280nm of ovalbumin at pH 7.0 in a sodium phosphate buffer. pH of all samples were determined by pH mater before measurements. A few pinch of sodium azide was add to the buffer to prevent the growing of microbes.

Methods

Fluorescence Spectroscopy. Fluorescence emission spectra were obtained on a F-2500 FL Spectrophotometer (Hitachi, Japan with 400001 ROM version) at 25°C in a 1cm path length quartz cell. The emission was recorded from 300-400 mm with 10nm and 5nm slit widths for excitation and emission respectively. The excitation wavelength for both the tryptophan and tyrosine, of ovalbumin was 282nm. The protein concentration was 9.45μM for all fluorescence measurements.

UV-difference spectroscopy. UV–absorption spectra were carried out on a UV−VIS spectrophotometer (Model Cintra-5, G.B.C., Australia) at 25°C in a 1cm path length quartz cell. For denaturation studies, absorbance of protein solutions in the presence and absence of denaturants were recorded at 280 nm against their respective blanks. The absorbance spectra were recorded at each wavelength in the range of 240-360 nm. The protein samples were prepared by using a fixed protein concentration of 14.6 μM, and the denaturants were added in varying concentrations.

Theory

We want to test the data in terms of the fraction unfolded (F_D) by Fluorescence spectroscopy calculated from the equation:

$$F_D = \frac{(F_{obs} - FN)}{(F_U - F_N)} \quad (1)$$

Where fobs is the observed value of the signal at a given denaturant concentration and F_N and F_U are the values of native and unfolded protein, respectively The values of F_N and F_D were obtained by linear extrapolation of pre and post-transition regions by LEM model.

The equilibrium constant (K_D) between the native and denatured states at a given denaturant concentration was calculated from

$$K_D = \frac{F_D}{(1 - F_D)} \quad (2)$$

The values of K_D thus obtained were then used for the calculation of Gibbs free energy change ΔG_D, using the standard relationship.

$$\Delta G_D = -RT \text{ in } K_D \quad (3)$$

Where R is the gas constant (0.002Kcal/mol), T is the absolute temperature. The Gibbs free energy of stabilization $\Delta G_D(H_2O)$ was obtained by linear extrapolation of the plot between ΔG_D and denaturant concentration to zero denaturant concentration. If a standard two state model is assumed, the denaturant transitions were fitted to the equation:

$$\Delta G_D = \Delta G_D (H_2O) - m_G [D] \quad (4)$$

Where $\Delta G_D(H_2O)$ and ΔG_D are Gibbs free energy of the folding in water and at a denaturant concentration D, respectively. m_G is the slope of the transition (proportional to the increase in solvent accessible surface area upon the transition from the native to denatured state), and [D] is the denaturant concentration, T_m (proportional to the increase in solvent-accessible surface area upon the transition from the native to denatured state) [22,23].

RESULTS

The intrinsic fluorescence of ovalbumin in phosphate buffer, pH 7.0 was measured at 25ºC temperature, at an excitation wavelength of 280nm. The fluorescence emission spectral studies were carried out to monitor denaturation of ovalbumin in the presence of varying urea concentration from 0-9M, as shown in Figure 1.1. The denaturation profile showed a prominent red shift in the emission wavelength from 335nm for the native ovalbumin to a wavelength value of 349.5nm for the maximally denatured protein at 9M urea concentration.

The denaturation of ovalbumin by urea results in a gradual decrease in fluorescence intensities. However, sharp changes in intensity are observed above 5M urea concentration and a 57% decrease in intensity was observed (Figure 1.2). Similarly denaturation of ovalbumin was monitored under varying concentration of βME (0-1N) (Figure 1.3). No significant red or blue shift was observed in the emission wavelength. A sharp decrease was observed in fluorescence intensity of ovalbumin denatured in varying concentration of βME. About 81.2% decrease in total intensity was observed. Conclusively that fluorescence emission maximum shift showed irregular pattern with a slight change in λE_{max} during denaturation and no significant shift in $\Delta\lambda E_{max}$ was observed with gradual decrease in the fluorescence intensity.

It is well known that during denaturation the addition of βME disrupts the disulfide bridges in ovalbumin, which are responsible for its stability in the fluctuating extracellular environment [24], and addition of urea breaks the hydrogen bonds, which affect the structure and topology of the protein. In order to study the combined denaturation effects of both

denaturants βME and urea on ovalbumin is shown in Figure 1.5. The fluorescence spectra of ovalbumin on the addition of varying concentration of βME (0–1N) and urea (0–9M) showed a significant red shift in the fluorescence emission maximum from 335nm (native ovalbumin) to 343.5nm (Figure 1.6) showed that sharp change was observed in the presence of both urea and βME (Figure 1.7) and indicates the change in emission maxima, $\Delta\lambda E_{max}$, was 14.5nm with increasing urea concentration. A significant red shift in wavelength was seen at concentration higher than 4M. The steep positive change in emission wavelength was observed above 6M urea concentration. As the urea concentration increases gradually, about 57% decrease in intensity was observed (Figure 1.8). About 97.7% of decrease in total intensity was observed in presence of the two denaturants with a corresponding red shift from 335nm to 343.5nm.

Figure 1.9 shows denaturation of ovalbumin as functions of changing urea concentrations (1M and 9M) with a constant concentration of βME (0.01N). There is no red shift at 1M urea but a decrease in fluorescence intensity is observed. When concentration of urea was increased up to 9M, a significant red shift in wavelength from 335nm to 348.5nm was observed with decrease in fluorescence intensity of about 55.5%.

Similarly, Figure 1.10 shows fluorescence spectra at fixed concentration of βME (1N) with varying urea concentration (1M and 9M). No red shift in emission wavelength was observed at βME (1N) and urea (1M) but decrease in fluorescence intensity (of about 87.9%) at 9M. and a significant red shift from 335nm to 343.5nm was observed along with a 97.7% decrease in fluorescence intensity. The comparison of Figures 1.7 and 1.10 indicates that the increase in emission wavelength depends on urea concentration only while decrease in intensity of fluorescence of ovalbumin depends upon concentration both urea as well as βME, as the increase of urea concentration was responsible for the exposure of more tryptophan residues. Whereas βME concentration was found to have greater influence in decreasing fluorescence intensity is observed in Figures 1.8 and 1.10.

The fluorescence intensity as function of concentration of denaturants (urea, βME and urea + βME) has been plotted in Figures 1.2, 1.4 and 1.6 respectively. We have used linear extrapolation method (LEM) for evaluating the Gibbs free energy change, $\Delta G_D(H_2O)$, during transition of native to denatured protein upto zero concentration of urea. The method involves conversion of the observed equilibrium constants evaluated in the transition region to free energy data, ΔG_D. The ratio of fraction of folded to unfolded protein during urea denaturation was plotted against urea concentration as shown in Figure 1.11.

The pre-and post denaturation baselines in urea-induced denaturation were found to be linear over a significant urea concentration range, as shown in Figure 1.2 making non-linear least square analysis rather easy and resulting in a ΔG_D (H_2O) (by equation 1.3) value of 4.81 Kcalmol^{-1} as shown in Figure 1.12.

The value of slope m_G of the curve (equation. 1.4) was obtained as 0.776 kcalmol^{-1}M^{-1}(Figure 1.12). This value is an experimental measure of the dependence of ΔG_D on denaturant concentration and it indicates the number of freshly exposed groups (of protein) to solvent in the unfolded state at 9M urea. The denaturant concentration of urea at the mid point of transition, T_m was 6.25M (Figure 1.11).

The structure-function relationship and unfolding behaviour of ovalbumin was studied using difference spectroscopy. The absorbance spectrum of ovalbumin under native and denatured conditions showed significant differences in either shape or in magnitude of absorbance. So, chemically induced denaturation of ovalbumin at pH 7.0 was studied with UV-difference spectroscopy as functions of concentration of denaturants.

Two denaturants, urea and βME have been used for denaturation studies. This was accomplished by measuring a difference spectrum in 9 M urea (Figure 2.1), 9 M urea and 0.05 N βME (Figure 2.2), 9 M urea and 1 N βME (Figure 2.3), in 0.5 N βME (Figure 2.4) and in 1 N βME (Figure 2.5). A deep trough at 286 nm was observed in the difference spectrum of ovalbumin in 9 M urea as shown in Figure 2.1 and therefore, this wavelength was selected for monitoring the spectral changes and indicated the exposed tyrosine residues.

The UV-difference spectra did not show a trough at 286 nm but instead a peak was observed at 252 nm on the addition of 0.05 N βME to the ovalbumin in 9 M urea solution and also showed significant changes in between 244 to 288 nm (Figure 2.2). On increasing concentration of βME from 0.05 N to 1 N for the ovalbumin solution in the presence of 9 M urea, a smaller and less significant peak at 254 nm, a prominent trough at 266 nm, and a significant peak at 282 nm were obtained as shown in Figure 2.3. Some wiggles were shown in Δε from 240 to 290 nm.

Similarly, under same experimental conditions, the UV-difference spectrum of ovalbumin in the presence of 0.5 N βME denaturant (Figure 2.4) showed a significant peak at 266 nm. The increase in Δε values occurred from 250 to 266 nm. The maximum obtained was observed quite close to peak reported for phenylalanine and cysteine residues. When the concentration of βME was increased further from 0.5 N to 1 N, a significant trough at 266 nm was observed as shown in Figure 2.5

The denaturation curves (Figure 2.6) can be divided into three regions on the basis of change in the physical parameters. These three regions are:

Pre-transition region in which the physical parameter, Δε, of folded protein changes slowly with significant changes with denaturant concentration.

Transition region showing major variation of the physical parameter, Δε, shows as unfolding proceeds

Post-transition region, which shows slow changes in the physical parameter, Δε, of the protein during unfolding Denaturation curve shows that denaturation of ovalbumin started at 3 M urea and was complete at 8.5 M urea concentration.

The comparison of Figures 2.7 and 2.8 shows that denaturation of ovalbumin in presence of 0.05 N βME started at 2 M urea and was complete at 6 M urea concentration, while in the presence of 0.5 N βME it commenced at 1 M urea and was complete at 6 M urea concentration (post transition region is gradual in this case than in presence of 0.05 N βME).

The magnitudes of chemically induced denaturation were compared in Figure 2.9, which shows highest value for urea and 0.5 N βME mixtures. The fraction of denatured ovalbumin in urea (0-9 M) derived from UV-difference spectral measurement is shown in Figure 2.10 and the ratio of fraction of folded-denatured of protein as function of urea concentration is shown Figure 2.11. This gave an indication of the fraction of molecules present in the native and denatured state at a particular urea concentration in buffer solution. A least-square fit of data gave the following equation:

$$\Delta G_D = \Delta G_D (H_2O) - m_G [D]$$

The value of ΔG_D (H_2O) was also calculated from the intercept of the given plot and found to be 4.05 k.cal.mol^{-1} (Figure 2.12) the denaturant concentration of urea at the midpoint of transition (T_m) is 5.25 M (Figure 2.11) The slope, m_G, of the plot is 0.669 kcal.mol^{-1}.M^{-1} which is used for the measuring the steepness of the curve.

The physico-chemical characteristics of ovalbumin have been elucidated to gain insight into the structure-function relationship in view of its stability towards denaturants. A physical description consists of determine the protein's size, shape and related parameters. A biological description of a protein may entail pinpointing the various central mechanisms regulating its function.

As we know the spectrum is determined by the polarity of the environment of the tryptophan, tyrosine and phenylalanine residues and by their specific interactions. Therefore, the changes in the micro- environment around these residues affect intrinsic fluorescence of different forms of the ovalbumin molecules. Basically, the fluorescence emission maximum suffers a red shift when the chromophores become more exposed to solvent and the quantum yield of fluorescence decreases when the chromophores interact with quenching agents either in a solvent or in the protein itself [25-28]. The maxima of fluorescence emission spectra of native ovalbumin, excited at 280nm was found to be 335±1nm, indicating that the excitable chromophore (s) is in a substantially hydrophilic environment and a rigid conformation as in the case of some other proteins–Tray [29] and ervatamin [30]. Our data for the fluorescence spectrum of unfolded ovalbumin in 9M urea, showed a shift in λE_{max} from 335nm to 349.5nm along with a decrease in fluorescence intensity of about 42-44% are shown in Figure (1.1, 1.8) and Table 1. It is observed that urea did not cause any structural perturbations, and the red shift in the wavelength maximum was the result of more tryptophan residues of the protein being exposed to a polar environment which is characteristic of unfolding. Besides this, the decrease in fluorescence intensity could be the result of a decreased distance between tryptophan and specific quenching groups, such as protonated carboxyl, protonated imidazole, deprotonated ε-amino groups and tyrosine, which consequently resulted in quenching of tryptophan fluorescence [28]. Each of the three amino acids (phenylalanine, tyrosine and tryptophan) have quite different emission spectra, phenylalanine showed maxima emission at 282nm, tyrosine at 303nm, and tryptophan at 348nm. Although most proteins contain all three types of residues, protein fluorescence spectra are usually dominated by tryptophan absorbance and emission, in part because tryptophan has the highest extinction coefficient of the three, and in part because resonance energy transfer from phenylalanine and tyrosine to tryptophan frequently occurs [30]. In Figure 1.1 the first transition (2-4M urea) was characterized by a small shift in emission maximum from 335nm to 337nm in the intermediate state, indicative of less solvent-exposed tryptophan residues, while in the second transition (4-9M urea) there is a large shift in emission maximum from 337 nm to 349.5nm, indicating complete solvation of the exposed tryptophan residues.

The fluorescence spectra of ovalbumin in 1NβME did not show any effective red or blue shift from the λE_{max} value for native protein 335 ± 1nm, while a 81.29% decrease in fluorescence intensity was achieved (Figure 1.8), Table 2, indicating that no tryptophan residues of ovalbumin were exposed to polar environment and so ovalbumin shows irregular changes in emission wavelength . Ovalbumin an extracellular proteins of known sequence contains one s-s bridge and four cysteins. The disulfide bridges can perform a variety of functions, although their major role appears to be to add stability in the fluctuating extracellular environment. When monitoring the extent of denaturation of ovalbumin at high concentration of βME, it must be noted that in 1NβME, leads to the partial denaturation of ovalbumin due to disruption of intermolecular and in tramolecular disulfide bonds [31]

It was reported earlier that the denaturation of ovalbumin by sulfhydryl compound, β-mercapto ethanol (βME) results in changes in the structure, by cleavage of disulfide bonds (S-S) of cysteine amino acids [32-35]. The ease with which SH-group containing compound, βME can reversibly oxidize disulfide structures has two experimental applications. First, we frequently add βME to reaction mixtures to prevent oxidation of essential SH-group of ovalbumin protein. In

these instances, the added SH-compound acts as an “antioxidant”, and prevents ovalbumin from undergoing oxidation [36]. The second application involves the intentional cleavage of disulfide bonds in ovalbumin by treatment with βME. In ovalbumin protein, we reduce disulfide bonds to sulfhydryl groups, reduction of each disulfide bond is coupled to oxidation of an added SH-compound, βME to its corresponding disulfide (Scheme 1) [36, 37].

It is known that the cleavage of disulfide bonds are possible in two ways i.e. (a) by cleavage of interchain disulfide bond forms between two cystine residues located in the different polypeptide chains and (b) other by cleavage of intrachain disulfide bonds forms in the same polypeptide chain. Both types of disulfide bonds broken by reduction to sulfhydryl groups Breaking of intra or interchain disulfide bonds by reduction produces a compound with new ionizable group (-SH) [36].

As a matter of fact, when ovalbumin was denatured at 9M urea in the presence of 1NβME, the fluorescence emission spectrum showed red shift from 335nm to 343.5nm with a 97.7% decrease in fluorescence intensity, indicating that greater exposure of tryptophan in presence of urea & βME and lesser with 9M urea alone after disruption of disulfide bonds of ovalbumin. A few tryptophans are quenched by new groups in the solvent formed in the presence of βME. The presence of urea (5-9M) and βME (0.08-1N) indicating complete solvation of the exposed of tryptophan residues with a decrease in fluorescence intensity of 50.22% and complete reduction of disulfide bonds as shown in Figure (1.5, 1.7 and 1.10).

It was observed that (a) 54% decrease in fluorescence intensity takes place when concentration of urea was increased from 1M to 9M in the presence of 0.01NβME (Figure 1.9), (b) while 79% decrease in fluorescence intensity takes place when concentration of Urea was increased from 1M to 9M in the presence 1NβME (Figure 1.10). The comparison of figure 1.9 and 1.10 shows that complete exposure of tryptophan occurred due to the affect of high urea concentration and disruption of disulfide bonds by βME in the former case and the higher βME concentration in the latter case caused quenching of tryptophan in addition to the rupture of disulfide bonds.

In the presence of urea, ovalbumin has a structure less random coil with maximum flexibility and therefore minimum polarization. Hence, a fully denatured molecule containing S-S bonds will not have minimum polarization until the S-S bonds are broken. On the addition of βME not only S-S bonds are broken but decrease in polarization also takes place. However, it is believed that a completely denatured state occurs at 9M urea and in the presence of 1NβME (Figure 1.10) and Table 3 when λE_{max} was shifted from 335nm to 349.5nm.

In this respect it is noteworthy that we provide solely the values of the denaturant concentration at the mid point of the transition curve. When applying the linear extrapolation model, LEM is a well established method to describe the equilibrium stability of globular proteins, mid point of the transition curve could be the correct parameter to characterize the stability, the unfolding Gibbs free energy change which depends linearly on denaturant concentration [38, 39]. The sigmoidal change of the unfolded/folded fraction was indicative of a two-state unfolding of ovalbumin (Figure 1.11) and the concentration of urea at which the protein was half folded, termed as transition mid point, Tm, was found to be 6.25M. In case of the denaturant urea, non-linear least squares analysis was applied to give the free-energy, $\Delta G_D(H_2O)$ value of unfolding of ovalbumin as 4.81Kcal mol^{-1} (Figure 1.12). The ovalbumin sensitivity parameter to urea, m_G was calculated to be 0.776 Kcal $mol^{-1}M^{-1}$ and found to be low in comparison to the value calculated from the Myers equation [40]. A linear positive relationship exists between m_G and the surface area exposed to solvent upon unfolding (ΔASA, Accessible Surface

area). The ΔASA of ovalbumin in the unfolded form was lower than expected, suggesting that ovalbumin is not fully unfolded but retains at least local hydrophobic cluster upto 8M urea. Aune and Tanford have shown that m_G is proportional to the numbers and types of groups that are freshly exposed to solvent when the protein unfolds [41].

Finally we conclude that red shift was maximum in the presence of 9Murea alone and minimum in the presence of 1NβME, which indicate that maximum exposure of tryptophan residues to polar environment occurred in presence of 9M urea and minimum exposure in case of 1NβME.(Table 1) in presence of βME no tryptophan residues were exposed to polar environment instead specific quenching takes place during rupture of disulfide bridge[42]. Decrease in fluorescence intensity was highest in presence of 1NβME and 9Murea, which indicate that maximum changes in distance (or structure) occurred in presence of both 1N βME and 9Murea.

The chemically-induced unfolding of ovalbumin was monitored in the presence of denaturants-urea and βME using UV-absorption spectroscopy. In the present work, the maximum absorbance of native ovalbumin was observed near 280 nm. The UV-difference spectra of ovalbumin in 9 M urea, pH 7.0 in the range of 240-360 nm is shown in Figure 2.1. In this case urea induced difference spectra showed that larger changes around the aromatic residues, probably reflect the unfolding of globular proteins. Two minima were observed: one at 246 nm and other at 286 nm. A deep trough was observed at 286 nm and therefore, this wavelength was selected for monitoring the difference spectral change and indicated the change in environment of tyrosine residue upon unfolding of ovalbumin. A small and broad peak in the region 260-266 nm was assigned to phenylalanine [43]. The contributions of each aromatic side chain to the difference spectrum of a protein were quite characteristic. The contribution of each may be gained from the difference spectra of phenylalanine, tyrosine and tryptophan. In ovalbumin, the molar ratio of phenylalanine: tyrosine: tryptophan is 20:9:3 [44]. The absence of a trough in the 292-294 nm region indicated that either tryptophans were exposed or their contribution was quenched by tyrosines and phenylalanine's. Because ovalbumin contains three folds more tyrosine residues and seven times more phenylalanine residues as compared to nearly the tryptophan's [44]. In the present data the peaks obtained due to tryptophans were found in the region 292-294 nm, that of tyrosine in the region 285-288 nm [45, 46] and those found below 270 nm were due to phenylalanine [43].

As we know that the Δε values are proportional to number of aromatic residues affected under a given set of contributions, the magnitude of Δε is a quantitative indication of the fraction of given type of aromatic residues. The denaturation by urea, which occurs as a consequence of altered solvent properties rather than by specific binding of urea with the protein, serves to preserve the solubility of the denatured protein. Urea interacts with proteins in two ways, by hydrogen bonding to the protein backbone, and by preferred solvation of hydrophobic groups. The backbone interaction was thought to be caused by one urea molecule replacing, thus freeing and increasing the entropy of two water molecules in the immediate solvation sphere around the solute while still providing hydrogen bonds to both solvent and solute [47].

The effect of urea (9 M) in the presence of βME (0.05 N on the UV-difference spectra of ovalbumin is shown in Figure 2.2. A prominent peak was obtained in the 254-256 nm region while no peak or trough was seen in the region from 270 to 294 nm. It was observed that high the phenylalanine content of ovalbumin were responsible for the appearance of more prominent changes in the 254-270 nm region, with the disruption of disulfide bonds (i.e. reduction of S–S bonds) and thus exposure of cysteine residues, while tyrosines and tryptophans were quenched by the larger proportions of phenylalanine residues and reduced residues of disulfide bonds formed in the presence of βME.

A significant “wiggles” were observed in the 250-270 nm region during denaturation of ovalbumin by urea (9 M) in the presence of higher concentrationof βME (1 N) (Figure 2.3), indicating the high content of phenylalanine on exposure of solvent to tyrosine, tryptophan and phenylalanine residues [48]. There was no peak near 291 to 294 nm, indicating quenching of tryptophan’s by phenylalanine, tyrosine and reduced residues formed after breaking of disulfide bonds, contribution of cystine peptide residues and cysteine residues.

The βME denatured ovalbumin (Figure 2.4) showed a characteristic peak at 266 nm indicating exposure of reduced cysteine residues by cleavage of cystine bridges in between intrapeptide chains and S-S bonds of interpeptide chains of protein. These results showed partial denaturation of ovalbumin in βME (1 N) took place. At higher concentration a prominent changes were obtained in the 250-266 nm region with a trough at 266 nm (Figure 2.5) indicating exposure of cysteine residues to a larger extent. In other words on increasing the concentration of βME from 0.5 to 1 N, the cysteine residues were increased in comparison to cystine peptide residues.

Denaturation effect at different concentrations of urea (0-9 M) has been studied as function of concentration of urea (Figure 2.6) indicating an intermediate formation and it was observed that urea initiate the denaturation of ovalbumin at calcium loaded positions at the low concentration of urea (1 M). As indicated in Figure 2.9 the denaturation of ovalbumin in phosphate buffer at pH 7.0 started at 3 M in case of pure urea and was complete at 8.5 M, while denaturation started at 2 M in case of urea and 0.05 N βME and at 1 M in case of urea and 0.5 N βME, which indicates that the disulfide bridges play a major role in denaturation of ovalbumin in comparison to hydrogen bonds. The comparison of the different curves (Figure 2.9) indicates the magnitude of denaturation was highest in case of urea and highest concentration of βME as shown by curve 3.

In the present study, special importance was given to observe the distribution and correlations of disulfide bonds during denaturation of ovalbumin by βME in the presence of urea and βME alone. Our data is in accordance with the general pattern. It was reported earlier that disulfide bonds were the strongest interactions in protein structures [49], and their number negatively correlated with the content of aliphatic hydrophobic residues in the protein chain [50]. These finding suggest that the stability of ovalbumin mainly depends on disulfide bonds in the native state.

It is reported in the previous literature that hen egg ovalbumin had four thiol groups and one disulfide bond [51], and a single polypeptide chain of 385 amino acid residues and six cysteine residues. Only two cysteine residues (Cys^{73} and Cys^{120}) form a disulfide bond in the native state [52, 53]. Tatsumi *et al.* (1994) reported that the egg white protein undergoes extensive sulfhydryl/disulfide exchanges producing all the 15 possible disulfide isomers with one disulfide and four sulfhydryl under highly denaturing conditions and near neutral pH conditions [54].

The denaturation of ovalbumin in the presence of urea was shown in Figure 2.6. By using the linear extrapolation method, the stability of the folded form of a protein relative to unfolded form in the limit of zero concentration of denaturant was evaluated. The sigmoidal curve of the unfolded fraction was indicative of a two-state unfolding of ovalbumin (Figure 2.10), and the concentration of urea at which the protein was half folded was found to be 5.25 M (Figure 2.11). In case of denaturant urea non-linear least squares analysis was used to calculate Gibbs free energy change, ΔG_D (H_2O) value of unfolding of ovalbumin was 4.05 $k.cal.mol^{-1}$ (Figure 2.12), which is comparable to the value 4.81 $k.cal.mol^{-1}$ found by fluorescence spectral studies.

The parameter showing sensitivity of ovalbumin of denaturant (urea), m_G was calculated and found to be 0.669

k.cal.mol^{-1}.M^{-1}, close to the value found by fluorescence spectrum, a value rather low compared with the value expected from the Myers equation [55]. Myers *et al*. [55] had indicated that a linear positive relationship exist between m_G and the surface area exposed to solvent upon unfolding (ΔASA, Accessible surface area). Thus, the ΔASA of ovalbumin in the unfolded form is lower than expected value suggesting that ovalbumin was not fully unfolded but retains at least local hydrophobic clusters and disulfide bonds even upto 8 M urea concentration and complete unfolding was observed at 9 M urea. Similar results were also reported for some other proteins, which retain residual structures in 9 M urea at neutral pH [56-58]. For globular proteins, the two requirements may act in opposite directions. Bastolla *et al.* (2004) reported that stability against misfolded states found by lattice simulations has been indicated as the upper limit on protein hydrophobicity [59].

Finally, we can conclude that difference absorbance spectra were able to show the internal interactions of protein with solvent and denaturant, and that such spectra were the results of (i) changes in the environment around a given chromophore induced as a result of some perturbations, such as ligand binding due to exposure to solvent, (ii) and changes due to breaking hydrogen bondsand disulfide bridges.

The present study shows that ovalbumin was highly denatured in presence of urea and βME, which lead to disruption of hydrogen bonds as well as intra- and interchain disulfide bonds. The urea-denatured ovalbumin had been partially folded with the presence of disulfide bonds, but after addition of βME, complete denaturation takes place due to rupture of both hydrogen and disulfide bonds.

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APPENDICES

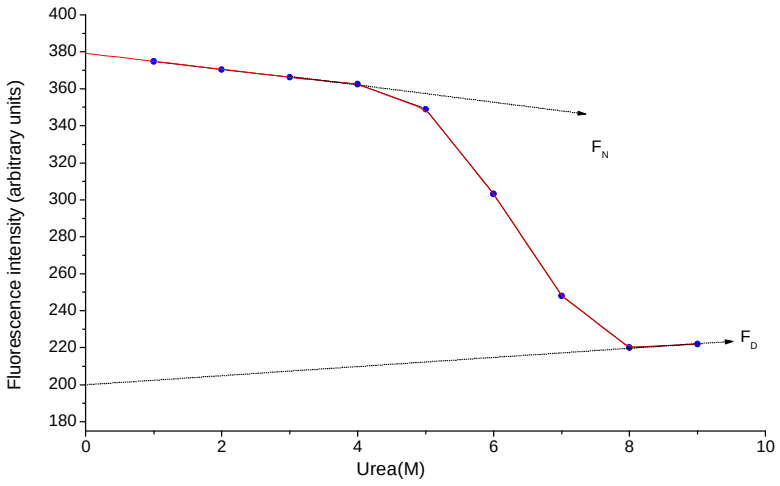

Figure (1.2): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0,under the effect of urea(0-9M). Excitation wavelength was 280nm.

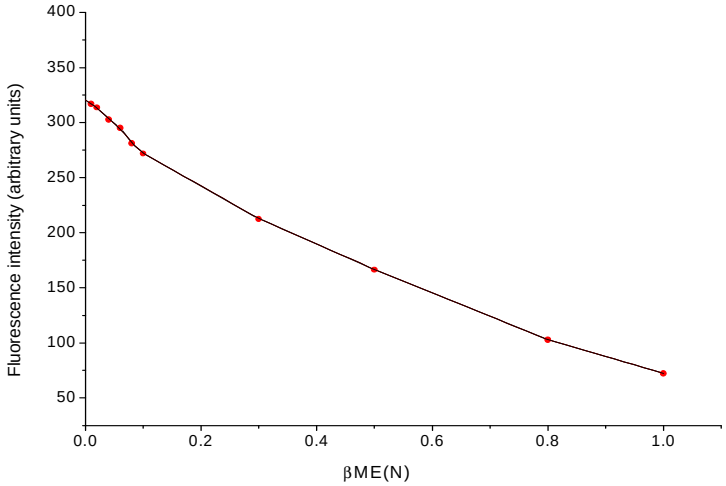

Figure (1.4): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0,under the effect of βME(0-1N). Excitation wavelength was 280nm.

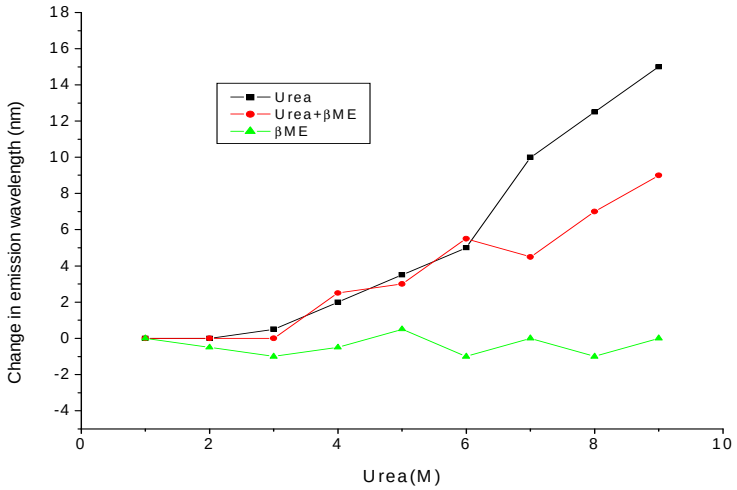

Figure (1.7): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0, under the effect of urea(0-9M), urea+βME(0-9M+0-1N)and βME(0-1N) respectively. Excitation wavelength was 280nm.

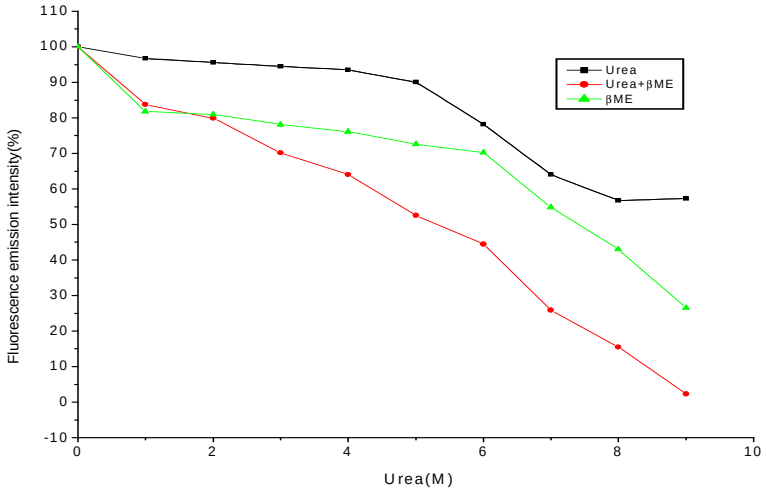

Figure (1.8): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0, under the effect of urea(0-9M), urea+βME(0-9M+0-1N) and βME(0-1N) respectively.Excitation wavelength was 280nm.

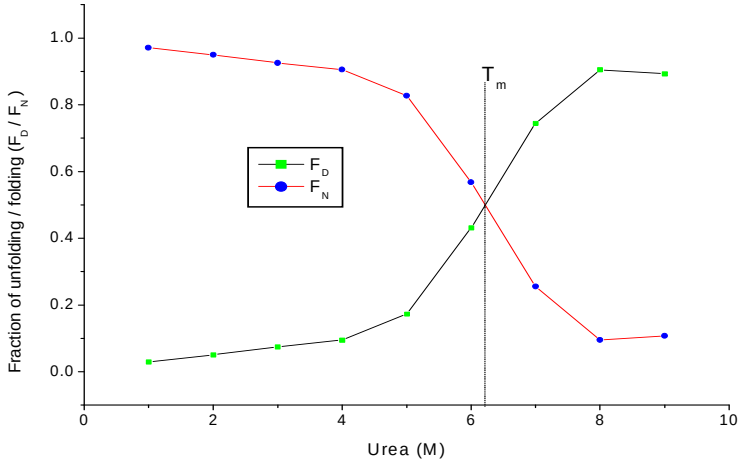

Figure (1.11): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0, under the effect of urea(0-9M). Excitation wavelength was 280nm.

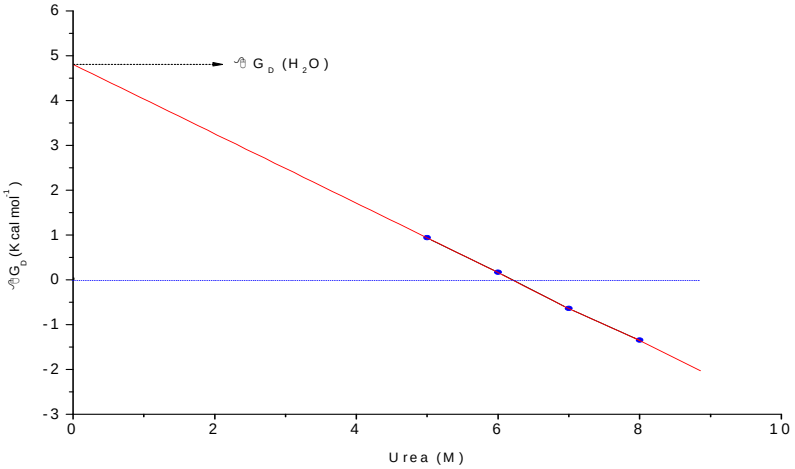

Figure (1.12): Intrinsic fluorescence spectra of ovalbumin in sodium phosphate buffer at pH 7.0, under the effect of urea(0-9M).Excitation wavelength was 280nm.

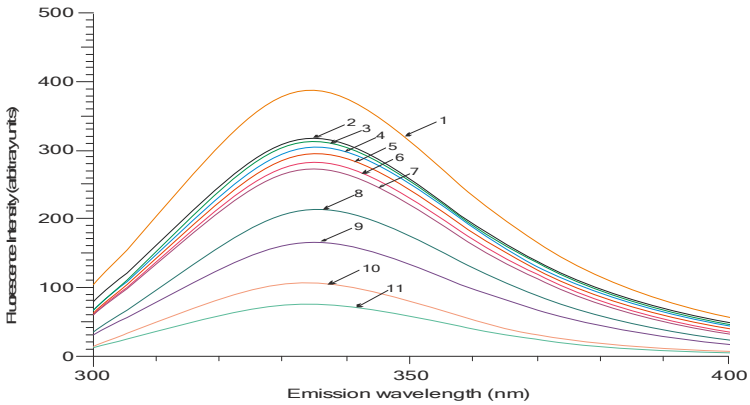

Figure (1.3): Intrinsic fluorescence spectra of native and denatured ovalbumin (0.425 mgml)$^{-1}$ in sodium phosphate buffer at pH 7.0 under the effect of zero (curve 1), 0.01N (curve 2), 0.02N (curve 3), 0.04N (curve 4), 0.06N (curve 5), 0.08N (curve 6), 0.1N (curve 7), 0.3N (curve 8), 0.5N (curve 9), 0.8N (curve 10), 1N (curve 11), concentration of βME. Excitation wavelength was 280nm.

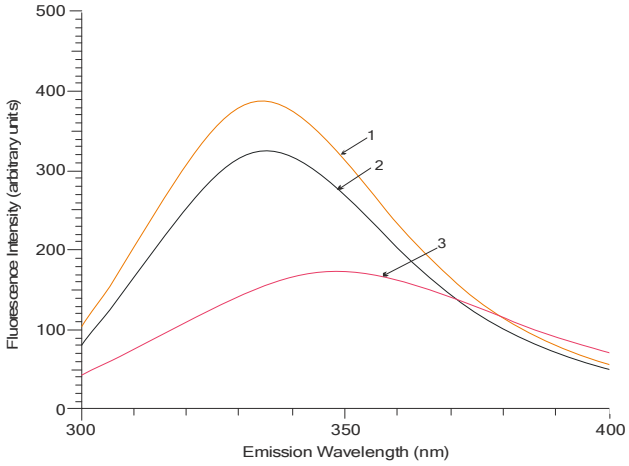

Figure (1.9): Intrinsic fluorescence spectra of native and denatured ovalbumin (0.425 mgml)' in sodium phosphate buffer at pH 7.0 under the effect of zero (curve 1), 0.01N+1M (curve 2), 0.01N+9M (curve 3), concentration of ME & urea respectively. Excitation wavelength was 280nm.

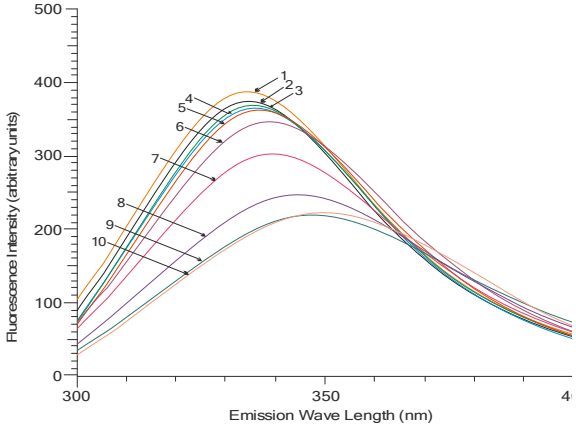

Figure (1.1): Intrinsic fluorescence spectra of native and denatured ovalbumin (0.425 mgml)' in sodium phosphate buffer at pH 7.0 under the effect of zero (curve 1), 1M (curve 2), 2M (curve 3), 3M (curve 4), 4M (curve 5), 5M (curve 6),6M (curve 7), 7M (curve 8), 8M (curve 9), 9M (curve 10), concentration of Urea. Excitation wavelength was 280nm.

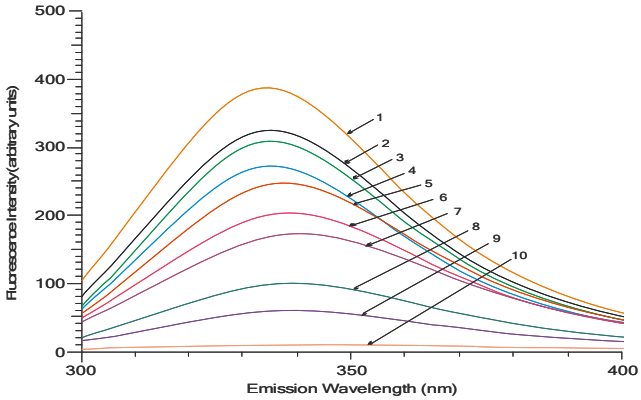

Figure (1.5): Intrinsic fluorescence spectra of native and denatured ovalbumin (0.425 mgml)' in sodium phosphate buffer at pH 7.0 under the effect of zero (curve 1), 0.01N+1M (curve 2), 0.02N+2M (curve 3), 0.04N+3M (curve 4), 0.06N+4M (curve 5), 0.08N+5M (curve 6), 0.1N+6M (curve 7), 0.3N+7M (curve 8), 0.5N+8M (curve 9), 1N+9M (curve 10), concentration of ME & urea respectively. Excitation wavelength was 280nm.

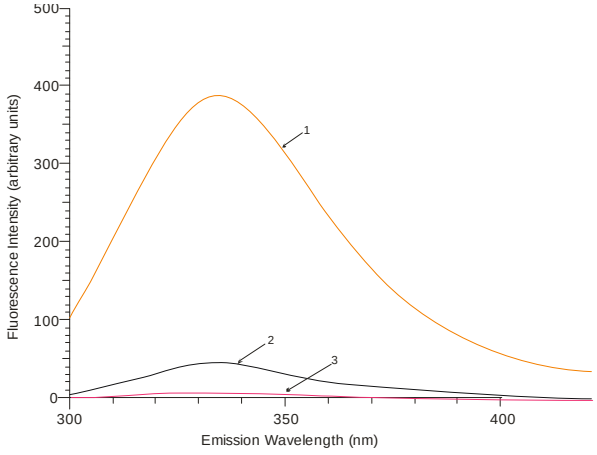

Figure (1.10): Intrinsic fluorescence spectra of native and denatured ovalbumin (0.425 mgml $^{-1}$) in sodium phosphate buffer at pH 7.0 under the effect of zero (curve 1), 1N+1M (curve 2), 1N+9M (curve 3), concentration of βME & urea respectively. Excitation wavelength was 280nm.

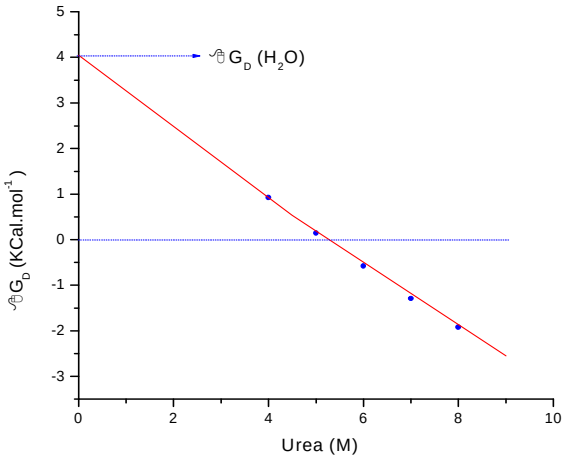

Figure (2.12): Linear extrapolation of free energy (ΔG_D) of denaturation of ovalbumin in sodium phosphate buffer at pH 7.0 as a function of urea concentration.

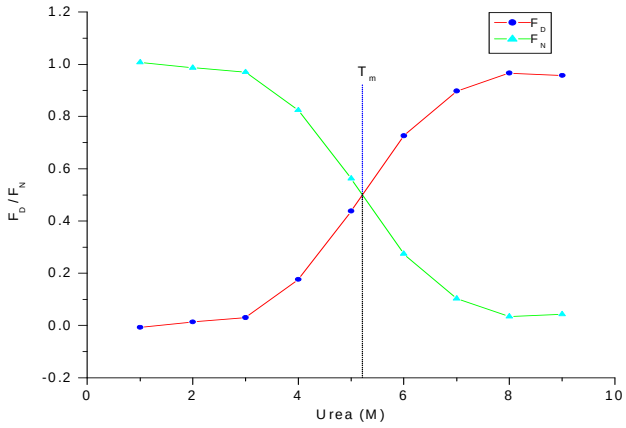

Figure (2.11): Fraction of denatured and natured, F_D / F_N, of ovalbumin in sodium phosphte buffer at pH 7.0 as a function of urea concentration.

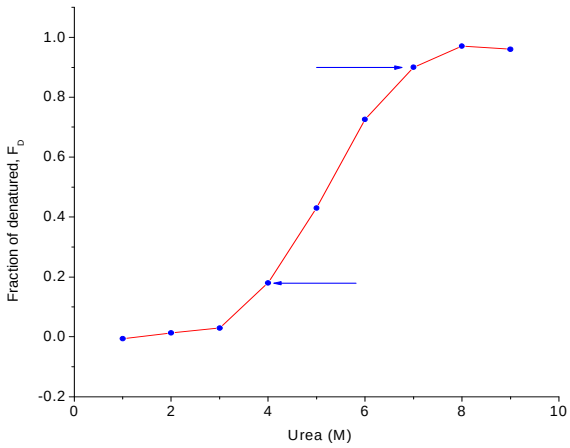

Figure (2.10): Fraction of denatured, F_D, of ovalbumin in sodium phosphate buffer at pH 7.0 as a function of urea concentration.

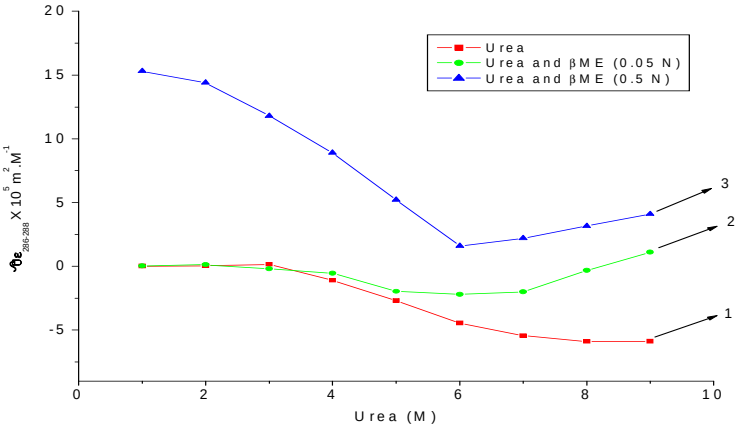

Figure (2.9): Denaturation effect of urea (0-9 M) curve 1, urea (0-9 M) and βME(0.05 N) curve 2, urea (0-9 M) and βME (0.5 N) curve 3 on ovalbumin in sodium phosphate buffer at pH 7.0.

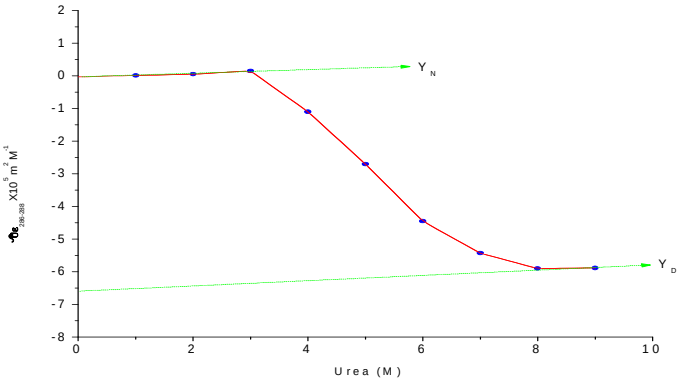

Figure (2.6): Denaturation effect of urea(0-9 M) on ovalbumin in sodium phosphate buffer at pH 7.0.

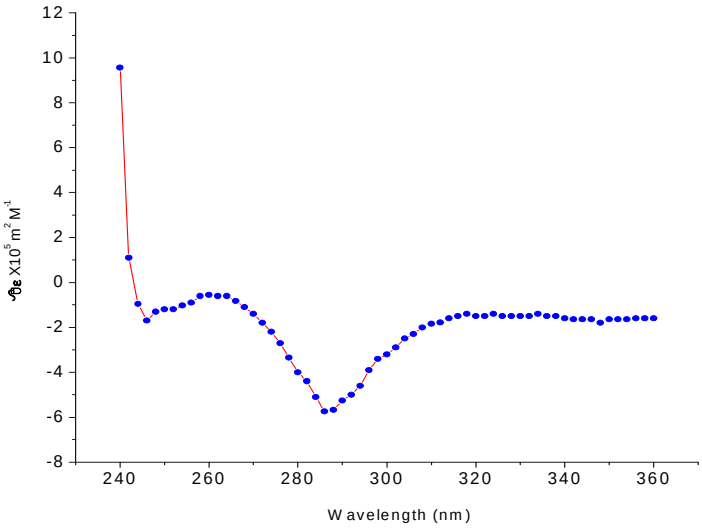

Figure (2.1): Effect of urea(9 M) on ovalbumin in sodium phosphate buffer at pH 7.0.

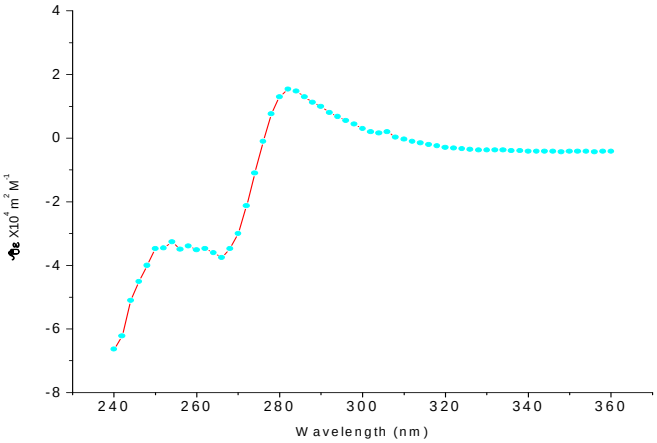

Figure (2.3): Effect of urea(9 M) and βME(1 N) on ovalbumin in sodium phosphate buffer at pH 7.0.

Table 1: The Values of Wavelength Maxima of Fluorescence Emission Spectra, λE_{max}, Fluorescence Intensity, Change in Fluorescence Emission Maximum, $\Delta\lambda E_{max}$ and % of Fluorescence Denatured Fraction of Ovalbumin in Presence of Urea

Conc. of Urea (M) in Ovalbumin	λE_{max} (Nm)	Fluorescence Intensity (Arbitrary Units)	$\Delta\lambda E_{max}$ (Nm)	% of Fluorescence Denatured Fraction of Protein
0	335.0	387.5	-	0.00
1	335.0	374.9	0.0	3.25
2	335.0	370.5	0.0	4.39
3	335.5	366.2	0.5	5.5
4	337.0	362.5	2.0	6.45
5	338.5	348.9	3.5	9.96
6	340.0	303.2	5.0	21.75
7	345.0	248.1	10.0	35.97
8	347.5	220.0	12.5	43.23
9	349.5	222.1	14.5	42.68

Table 2: The Values of Wavelength Maxima of Fluorescence Emission Spectra, λE_{max}, Fluorescence Intensity, Change in Fluorescence Emission Maximum, $\Delta\lambda E_{max}$, and % of Fluorescence Denatured Fraction of Ovalbumin in Presence of βME

Conc. of βME (N) in Ovalbumin	λE_{max} (Nm)	Fluorescence Intensity (Arbitrary Units)	$\Delta\lambda E_{max}$ (Nm)	% of Fluorescence Denatured Fraction of Protein
0.01	335.0	317.0	0.0	18.19
0.02	334.5	313.9	-0.5	19.0
0.04	334.0	302.8	-1.0	21.86
0.06	334.5	295.2	-0.5	23.82
0.08	335.5	281.4	0.5	27.38
0.1	334.0	272.1	-1.0	29.78
0.3	335.0	212.8	0.0	45.08
0.5	334.0	166.6	-1.0	57.01
0.8	335.0	103.0	0.0	73.42
1.0	334.0	72.5	-1.0	81.29

Table 3: The Values of Wavelength Maxima of Fluorescence Emission Spectra, λE_{max}, Fluorescence Intensity, Change in Fluorescence Emission Maximum, $\Delta\lambda E_{max}$, and % of Fluorescence Denatured Fraction of Ovalbumin in Presence of Urea and βME

Conc. of Urea (M) and βME (N) in Ovalbumin	λE_{max} (Nm)	Fluorescence Intensity (Arbitrary Units)	$\Delta\lambda E_{max}$ (Nm)	% of Fluorescence Denatured Fraction of Protein
0+0	335.0	387.5	0.0	0.0
1+0.01	335.0	324.5	0.0	16.26
2+0.02	335.0	309.5	0.0	20.13
3+0.04	335.0	271.8	0.0	29.86
4+0.06	337.0	248.2	2.0	35.95
5+0.08	338.0	203.5	3.0	47.48
6+0.1	340.5	172.2	5.0	55.56
7+0.3	339.5	100.4	4.5	74.09
8+0.5	342.0	59.75	7.0	84.58
9+1.0	343.5	8.9	8.5	97.7