CHAPTER 1

Fluorescence Study of alpha – Lactalbumin in the presence of Proline/Sugars in Phosphate Buffer, showing Stabilization at pH 7.0
INTRODUCTION
The biomolecules which major class of living organisms is composed of is protein. In comparison to other biomolecules like nucleic acids, lipids or carbohydrates, protein fulfills the largest variety of different functions. Proteins are linear polymers formed from twenty different naturally occurring alpha-L amino acids connected by amide (peptide) bonds. The hydrophobic core of amino acid in protein maintain the typical structural feature of globular protein by preventing the penetration by water. Under so called physiological conditions (i.e. ambient temperature and pressure, low to moderate salt concentration in aqueous solution) most natural proteins predominantly adopt a defined three dimensional structure referred to as the native or folded state. Usually attaining the native structure, in which the linearly connected amino acids are arranged in a defined manner in space, is an absolute prerequisite for protein function. Protein folding is the process by which the amino–acid sequence of protein determines the three-dimensional conformation of the functional protein. Protein folding designates the conformational transition of a protein from its unfolded to folded state. Proteins exist as an equilibrium mixture between their native and denatured states and continuously fold and unfold in vivo. The native structure of proteins is fully encoded in their amino acid sequence (primary structure), thus allowing proteins to fold in the absence of any other factors. However, especially some larger proteins are known to be prone to misfolding and aggregation. These proteins only fold efficiently in the presence of accessory proteins such as chaperons, disulfide bond and peptidyl prolyl isomerases. Chemical Chaperons, which are various low molecular mass compounds, have been shown to stabilize proteins in their native state and to protect them against thermal denaturation and aggregation in vitro.

Native proteins are stabilized by a multitude of non-covalent protein-protein and protein-solvent interactions, most of which are quite weak on their own. The electrostatic interactions, hydrogen bonds and van-der-waals contribute to protein stability, but a major portion result from the so called hydrophobic effect or hydrophobic free energy. At ambient temperature the hydrophobic free energy is mainly entropic
and is thought to result from the ordering of solvent molecules around the protein surface, which is accompanied by loss in orientational freedom. Thus, the hydrophobic effects favour the burial of protein surface in order to minimize the protein-solvent interface. Recent work by the group of Baldwin [16] questioned the energetic role of hydrogen bonds. Taken into account the solvation of hydrogen bonds, these researchers [17- 20] concluded that hydrogen bonds in proteins are strongly stabilizing as long as they are solvent-exposed and thus solvated. On the other hand, hydrogen bonds which are buried within the interior of proteins should contribute only weakly to the stability of the native state since they can not be solvated. Multiple interactions between protein residues well separated in primary structure cause the overall compaction and defined fold of the native protein. Native proteins were found to adopt ordered and defined structures. Several recurring secondary structure elements (alpha-helices, beta-pleated sheets, loops and turns) are arranged in a specific manner in space giving rise to the so-called tertiary structure of proteins [21].

Although native proteins display relatively defined three-dimensional structures, they are far from being static entities but rather comprise ensembles of several inter converting conformers. The temperature factors (B-factors) observed in protein crystals hint at varying mobilities in parts of the protein. Nuclear magnetic resonance (NMR) methods [22, 23], such as relaxation measurements or hydrogen-deuterium exchange [25- 27] are particularly suited to study motions within native proteins [23, 28, 29], ranging from bond vibrations (femto second time scale) and isomerizations of amino acid side chains (pico to nanoseconds) to loop motions (nanoseconds) and local unfolding of protein domains (micro to milliseconds).

Flexibility of native proteins is important for their function as is clearly seen for enzymes, e.g for the prolyl isomerase cyclophilin [30, 31] and in the case of induced fit mechanism of hexokinase [32, 33]. Frauenfelder and coworkers [34] demonstrated that the photodissociation from myoglobin requires motions within the protein. Further evidence was provided by the work of Ansari et al [35] who found that this photo dissociation reaction is drastically slowed in solutions of increased viscosity.
In nature, proteins perform many functions and are considered as "the work horse of life". Thereby proteins evolve and perform their tasks mostly in aqueous environments. The studying of protein in aqueous solution has much wider significance in diverse fields like protein folding, protein engineering, and other interesting phenomenon like solvation dynamics and enzymatic reactions [21, 36, 37]. In general, many investigations of aqueous solution of proteins have stressed the importance of protein-water interactions. Pioneering work studies have also shed light on unique features associated with the ubiquitous nature of water. Undoubtedly, most researchers agree over the putative role of water in shaping the protein energy landscapes. Water as it is commonly perceived, is not simply a filling medium, but plays a crucial role for protein functions. It enhances enzymatic reactions, assists in folding mechanism, and facilitates many reactions of proteins within and outside cells. Therefore, water in proteins has been the subject of intense investigation for a long time [38-42].

Hydrophobic interactions are thermodynamically favored at high temperatures because the ordered structure of water present at room temperature becomes disordered and takes on higher energy states when the system is heated [2]. Right after disulfide bonds and hydrophobic interactions, in order of contribution to structure, electrostatic interactions plays a major role in the folding of proteins, which affects the elasticity and strengths of globular proteins gels.

The pH dictates the total on the protein whereas the ionic strength determines the degree of interaction among the charges because salt interact with charges [2]. When added to protein solution, mono-valent salts will shield charges on the protein. This reduces the electrostatic repulsion on the protein and will prevent it from fully unfolding, which at neutral pH will result in particulate gel formation [43]. This charge shielding phenomena is mainly valid at low salt concentrations [2]. At higher concentrations, the charges on the proteins saturated and the effect of salt falls on the solvent properties, either dielectric constant [2] or solvent quality.

As NaCl concentration is increased up to 0.1 – 0.2 M, the ions primarily interact with the charges on the protein, which enhances the interactions between proteins.
Beyond this concentration, the steady decrease in fracture stress/elasticity is probably caused by changing the solvent properties. This latter observation has also been made by Verheul et al (1998 b) [44].

Osmolytes are known to stabilize proteins against aggregation. Schein described effect of osmolytes on proteins and on the solvent properties of water [45]. Many potential stabilizing co-solutes for proteins have been investigated [45] that mainly affect the solvent properties of water as related to protein polarity and protein diffusion. Osmolytes as solvent additives can effect protein affinity for the hydrophobic surfaces of enzymes, as well as protein stability and solubility. Any mechanism offering generalize protection of protein against denaturation is of fundamental importance to their folding, stability, and function and is of major practical interest in biotechnology, evolutionary biology and biochemistry [46]. The theory that osmolytes became associated with particular proteins through natural selection implies that particular physicochemical properties of the stabilizing organic osmolytes solutions were selected for their ability to protect macromolecules and other components of the organism [47, 48].

Osmolytes are classified as ‘compatible osmolytes’ including polyol and free amino acids and ‘counteracting osmolytes’ such as trimethyl amine N-oxide (TMAO) [46]. Compatible osmolytes protect proteins subjected to threatening conditions such as extreme temperature fluctuations, excessive dryness or high salt environments, while counteracting osmolytes protect cellular proteins against urea inactivation [49, 46]. Compatible and counteracting osmolytes may have different mechanisms for protecting proteins because the relevant environmental stresses vary [47].

Osmolytes maintain adequate catalytic rates of proteins, a high level of regulatory responsiveness and a precise balance between stability and flexibility of the structure (tertiary conformation, subunit assembly and multi protein complexes) [50]. Therefore, we suggested that osmolytes might have more important physiological functions for maintaining life development and evolution. It was suggested that osmolytes like dimethylsulfoxide and proline prevented exposure of creatine kinase [51] and hence they are not only energy substrates for metabolism and organic components in vivo, but also exert an important physiological function
for maintaining adequate rates of enzymatic catalysis and for stabilizing the protein's secondary and tertiary conformations.

The unfolding of proteins by reagents such as urea or guanidium chloride has long been considered to arise because of the favourable interaction of these reagents with the normally buried interior segments of a protein, thereby stabilizing the unfolded form relative to the folded one [52]. In like manner stabilization of folded protein structures by osmolytes such as sucrose is thought to result from unfavourable interactions with interior residues of the proteins there by producing a relative destabilization of the unfolded form [53]. It is possible to treat both of the above processes as two aspects of the same phenomenon, differing from one another only in the sign of the free energy of interaction [54].

Addition of sugars to an aqueous solution of the protein resulted in an unfavourable free-energy change [55] and the effect was shown to increase with an increase in protein surface area, explaining the protein stabilizing action of these sugars and their enhancing effect of protein association.

Proteins contain only three residues that have the property of intrinsic fluorescence. These chromophores form the following series: tryptophan > tyrosine > phenylalanine according to their quantum yield. The fluorescence of tryptophan is most commonly used for analysis of proteins since the quantum yield of phenylalanine fluorescence is extremely low and tyrosine fluorescence is strongly quenched in the majority of cases. Quenching of tyrosine fluorescence can be due to ionization, location energy transfer to tryptophan [55]. Application of intrinsic fluorescence to the study of protein conformational analysis relies on the fact that the parameters of tryptophan emission (intensity and wavelength of maximal fluorescence) depend essentially on environmental factors, including solvent polarity, pH, and presence and absence of quenchers [55]. For example, a completely solvated tryptophan residue (e.g. free tryptophan in water or tryptophan in an unfolded polypeptide chain) has a maximum fluorescence in the vicinity of 350nm, whereas embedding this chromophore into the non-polar interior of a compact globular protein results in a characteristic blue shift (stokes shift) of its fluorescence maximum (stokes shift) by as much as 30-40nm [56-58]. This means
that the value of $\lambda_{\text{max}}$ of tryptophan fluorescence contains some basic information about whether the given protein is compact or not under the experimental conditions. For this reason, the analysis of intrinsic protein fluorescence is frequently used for the study of protein structure and conformational change.

To some extent, the information obtained from dynamic quenching of intrinsic fluorescence is similar to that obtained from studies of deuterium exchange, since it reflects the accessibility of defined protein groups to the solvent. However, in distinction from the deuterium exchange, this method can be used to evaluate the amplitude and time scale of dynamic processes by using quenchers of different size, polarity and charge. In one example of this approach, it has been shown that the rate of diffusion of oxygen which is one of the smallest and most efficient quenchers of intrinsic protein (fluorescence) within a protein molecule is only two to four times slower than in an aqueous solution [59, 60].

Furthermore, oxygen was shown to affect even those tryptophan residues that, according to X-ray structural analysis, should not be accessible to the solvent. These observations clearly demonstrated the presence of substantial structural fluctuations in proteins in the nanosecond time scale [60, 61]. Acrylamide is one of the most widely used quenchers of intrinsic protein fluorescence [62]. Acrylamide, like oxygen is a neutral quencher but with a much larger molecular size. This size difference results in a dramatic decrease in the rate of protein fluorescence quenching over that of oxygen [63, 62]. This decrease is due to the inaccessibility of the globular protein interior to the acrylamide molecule. Thus, acrylamide actively quenches only the intrinsic fluorescence of solvent exposed residues. As applied to conformational analysis, acrylamide quenching was shown to decrease by two orders of magnitude as unstructured polypeptide chains transitioned to globular structure [63, 64]. Importantly the degree of shielding of tryptophan residues by the intermolecular environment of the molten globule state was shown to be close to that determined for the native globular proteins, whereas the accessibility of tryptophans to acrylamide in the pre-molten globule state was closer to that in the unfolded polypeptide chain [65]. The fluorescence quenching by acrylamide of the single tryptophan residue in the beta to subunit of tryptophan synthase was to verify
the presence of a conformational transition induced by interaction with the cofactor; pyridoxyl 5' phosphate [66].

Simultaneous application of quenchers of different size, polarity and charge (oxygen, nitrite, methyl vinyl ketone, nitrate, acrylamide, acetone, methyl ethyl ketone, succinimide, etc.) could be more informative since it may yield information not only about protein dynamics but also about peculiarities of the local environment of chromophores. The information on the local environment of chromophores could be also retrieved from simple quenching experiments. In an example of simultaneous application of multiple quenchers, the heterogeneous fluorescence of yeast 3-phosphoglycerate kinase was resolved into two approximately equal components, one was accessible and another one was inaccessible to the quencher succinimide [66]. The fluorescence of the inaccessible component was shown to be blue shifted and exhibited a heterogeneous fluorescence decay which had a temperature dependence and steady-state acrylamide quenching properties typical of a single tryptophan in a buried environment. This component was assigned to the buried tryptophan W333. The presence of succinimide greatly simplified the fluorescence, allowing the buried tryptophan to be studied with little interference from the exposed tryptophan [55].

Useful information about the mobility and aggregation state of macromolecules in solution can be obtained from analysis of fluorescence polarization or anisotropy. If excited light is polarized and passed through a protein solution, fluorescence will be depolarized or remain partially polarized. The degree of fluorescence depolarization results from the following factors that characterize the structural state of the protein molecules:

1) Mobility of the chromophores, (strongly dependent on the density of the environment).
2) Energy transfer between similar chromophores [55, 67-72].

Further more the relaxation time of tryptophan residues determined from polarized luminescence data are a reliable indicator of the compactness of the polypeptide chain. The relaxation times of tryptophan residues determined by fluorescence polarization for alpha lactalbumin [73, 74] and carbonic anhydrase B
[75] showed high degree of protein compactness in both the native and the molten globule states.

In many ways, fluorescence quenching is an ideal probing technique from both an interpretive and experimental point of view. Because exposure can be reported by mere collision with the excited state of tryptophan before it fluoresces, the quencher does not have to be in constant contact with the fluorophor, as is the case with solvent perturbation and related techniques [76, 73]. Consequently, a much lower total concentration of the probing agent is needed by the fluorescence technique. Since the technique is basically a fluorescence experiment, only a very small amount of protein is required (less than a milligram). In contrast to chemical modification studies [77], quenching with acrylamide leads to ground-states molecules, so that the protein is not permanently damaged, and the reaction itself does not induce a structural change in the protein.

Alpha lactalbumin is a small (14 KDa), highly abundant calcium-binding protein from milk, which, after decades of intensive study, become one of the best characterized proteins in protein science [76]. One of the reasons for the high interest in the study of alpha lactalbumin is its ability to convert under mild denaturing conditions into the equilibrium molten globule state, representing what is now considered a general intermediate in protein folding [73]. The study of alpha lactalbumin is very interesting from several point of view, alpha lactalbumin has a single high affinity Ca\(^{2+}\) site, [77, 78], and it is frequently considered as a simple model Ca\(^{2+}\) binding protein, alpha lactalbumin forms several partially folded intermediate states. At acidic pH and in the apo-state at elevated temperatures, alpha lactalbumin is the classic "molten globule" [73, 79]. A wide range of experimental studies has been undertaken to gain insight into the molten globule state of alpha lactalbumin [80, 81], a species that has recently gained considerable attention through successful clinical trials as a key component of an anti tumor compound [82]. It was found that some form of alpha lactalbumin can induce apoptosis in tumour cell [83, 84] and also posses bacteriacidal activity [85, 86] which suggest that it fulfills many important protective biological functions. Finally, it has been recently shown that alpha lactalbumin is able to form amyloid fibrils at low
pH, where it adopted the classical molten globule like conformation [87]. Alpha lactalbumin has the lowest denaturation temperatures of the whey proteins, which varies only slightly when pH is varied. The denaturation temperatures are 59 °C, 61 °C, 62 °C for pH 3.5, pH 6.5 and pH 7.5, respectively [88,89]. However, it is the only whey proteins that is thermo reversible (90 > %) (i.e, it renatures upon cooling) [88].

In the present work, an attempt has been made to study:

i) The stabilization effect of proline with its varying concentration on alpha-lactalbumin in terms of lambda maximum (λmax) of fluorescence.

ii) To measure stability effect of sugars [fructose, maltose, and sucrose (with its varying concentration)] on stability of alpha lactalbumin in terms of lambda maximum (λmax) of fluorescence.

iii) The interaction of reducing (fructose and maltose) and non reducing (sucrose) sugars with alpha-lactalbumin.

iv) To compare the extent of stabilization of protein by two types of sugars (reducing and non reducing) in the presence and absence of proline.
EXPERIMENTAL
MATERIALS AND METHOD:

Alpha – lactalbumin and proline were obtained from sigma chemical Company. All other chemicals were of analytical reagent grade. Fructose, Sucrose, and Maltose were obtained from Merck Company. For sample preparation, the 0.1M aqueous solutions of both monobasic and di-basic sodium phosphate (purchased from E. Merck) were mixed in different proportions to prepare phosphate buffers of pH 7.0. The pH of these solutions was measured by digital pH meter (Elico Pvt. Ltd. Hyderabad, model T-10). The $2.828 \times 10^{-7}$ M of alpha-lactalbumin were prepared in 0.1 M phosphate buffer at pH 7.0 and the concentration of alpha-lactalbumin was kept constant in all the system under study. Two set of solutions were prepared. In first,

1. Three solutions of alpha-lactalbumin and buffer were prepared each in the absence of proline with different sugars (fructose, maltose, and sucrose) with varying concentration (0.063M, 0.375M & 0.750M).

2. Three solutions of alpha-lactalbumin each in the presence of proline (0.063M, 0.375M & 0.750M) with different sugars (fructose, maltose & sucrose) with varying concentration (0.063M, 0.375M & 0.750M).

Fluorescence measurement were performed on a Hitachi spectrofluorometer, Model F- 2500, part no. 251-0098 with a 150W Xenon lamp and slit width of 10 nm, using a 1.0cm quartz cell. The excitation wave length of alpha lactalbumin was performed at 280nm with emission wave length ranges from 300 to 400nm.
RESULT AND DISCUSSION
RESULT AND DISCUSSION:

Fluorescence spectra of alpha-lactalbumin in presence of phosphate buffer at pH 7.0 were recorded. The lambda maximum ($\lambda_{\text{max}}$) of native protein was found to be 329nm. In the presence of proline with protein and buffer the lambda max ($\lambda_{\text{max}}$) decreases [blue shift (shows the stabilization effect)] at all of its three concentration i.e at 0.063 M (shift from 329 to 326.5nm), 0.375 M (shift from 329 to 327.5nm), and 0.750 M (shift from 329 to 328.5nm), but comparative increase in lambda max [red shift (shows the destabilization effect)] with corresponding increase in concentration of proline was observed from Table 1.1.

With the absence of proline but with the presence of individual sugars (fructose, sucrose and maltose) with its varying concentration (0.063M, 0.375M and 0.750 M) with alpha-lactalbumin and buffer (Table 1.1) the increase in lambda max (red shift) was observed in all of the three sugars at all of its concentration (mentioned above) compared to native protein.

Now with the addition of proline (corresponding concentrations i.e 0.063M, 0.375M and 0.750M) in the presence of individual sugars (fructose, sucrose and maltose) with its varying concentration (0.063M, 0.375M, 0.750M) with alpha lactalbumin and buffer (Table 1.2) the decrease in lambda max ($\lambda_{\text{max}}$) was observed as compared to the system in which proline is absent [Table 1.1] in all of the sugars-proline at all of its concentration, except fructose-proline in which increase in wavelength was observed by the addition of proline at two of its higher concentration, i.e at 0.375M and 0.750M, the decrease in lambda max ($\lambda_{\text{max}}$) was observed more in lower concentration range of sugars - proline i.e at 0.063M. At 0.063M of proline - sugars (fructose, sucrose and maltose) lambda max ($\lambda_{\text{max}}$) shift to the lower wavelength (blue shift) compare to the native protein (from 329 to 327.5nm in case of fructose - proline, from 329 to 328nm in case of sucrose-proline, and from 329 to 327.5nm in case of maltose-proline) showing the stabilization effect in the presence of proline (Table 1.2).
TABLE 1.1 Experimental values of lambda max ($\lambda_{\text{max}}$) of intrinsic fluorescence of proline / fructose /sucrose /and maltose - alpha-lactalbumin – buffer system at pH 7.0 versus concentration of respective systems.

<table>
<thead>
<tr>
<th>Concentration of cosolvent (mol/l)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>proline</th>
<th>fructose</th>
<th>sucrose</th>
<th>maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.063</td>
<td>326.5</td>
<td>330.0</td>
<td>336.0</td>
<td>341.5</td>
</tr>
<tr>
<td></td>
<td>0.375</td>
<td>327.5</td>
<td>341.5</td>
<td>335.5</td>
<td>335.0</td>
</tr>
<tr>
<td></td>
<td>0.750</td>
<td>328.5</td>
<td>336.5</td>
<td>331.0</td>
<td>332.5</td>
</tr>
</tbody>
</table>

Native protein in buffer $\rightarrow$ 329nm

Cosolvents $\rightarrow$ proline, fructose, sucrose, maltose.
TABLE 1.2 Experimental values of lambda max ($\lambda_{\text{max}}$) of intrinsic fluorescence of proline / proline - fructose / proline - sucrose / and proline - maltose - alpha- lactalbumin – buffer system at pH 7.0 versus concentration of respective systems.

<table>
<thead>
<tr>
<th>Conc. of cosolvent (mol/l)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>proline</th>
<th>proline - fructose</th>
<th>Proline - sucrose</th>
<th>Proline - maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.063</td>
<td>326.5</td>
<td>327.5</td>
<td>328.0</td>
<td>327.5</td>
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<tr>
<td></td>
<td>0.375</td>
<td>327.5</td>
<td>342.5</td>
<td>327.0</td>
<td>330.0</td>
</tr>
<tr>
<td></td>
<td>0.750</td>
<td>328.5</td>
<td>354.0</td>
<td>330.0</td>
<td>332.0</td>
</tr>
</tbody>
</table>

Native protein in buffer $\rightarrow$ 329 nm

Cosolvents $\rightarrow$ proline, fructose, sucrose, maltose.
We observed that the presence of proline gives blue shift in lambda max \( \lambda_{\text{max}} \). Bovine alpha-lactalbumin contains four tryptophan residues at positions 26, 60, 104 and 118. It has been reported that of these four tryptophans, the one at position 60 is part of a loop and is exposed to the solvent in the native state and it contributes only 7% to the total fluorescence of the protein [90]. The tryptophan residues at position 104 and 118 are a part of the 3_{10} Helix and 26 is a part of \( \alpha \)-helix. These three residues are essential for the formation of MG (Molten globule) like structure and MG state was observed in our case in Figure 1.1 and 1.2 both.

The tendency of polar groups on the surface of proteins to bind the water molecules tightly in aqueous solutions, generally known as water of hydration, and if this hydration is maintained in a concentrated solution of solvent additives (cosolvent, cosolute or osmolyte), a difference in the concentration of additives result in preferential hydration such that excess water accumulates near the protein.

Preferential binding is the adhering of the additives (cosolvent) with the protein molecule at a level at which concentration of cosolvent in the vicinity of the protein exceeds the concentration of the additives in bulk solution. If there is an excess of additive or ligand in the protein domain relative to the bulk solvent composition, it is described as preferential binding [93, 94].

Intrinsic fluorescence of protein was observed alone,

A) In the presence of proline with its varying concentration (0.063M, 0.375M, 0.750M),

B) In the absence of proline with varying concentration of sugars (0.063M, 0.375M, 0.750M) and

C) In the presence of proline with varying concentration of sugars (0.063M, 0.375M, and 0.750M). The fluorescence spectra of protein gives the characteristics lambda maximum \( \lambda_{\text{max}} \) of alpha lactalbumin at 329nm, which is consistent with that obtained in the literature (Fasman)[99].
Figure 1.1 Plots of $\lambda_{\text{max}}$ (nm) of intrinsic fluorescence of proline/ fructose, sucrose, and maltose-alpha-lactalbumin-buffer system at pH 7.0 versus varying concentration of respective systems.
Figure 1.2  Plots of $\lambda_{max}$ of intrinsic fluorescence of proline in the presence of proline with fructose, sucrose, maltose-alpha-lactalbumin-buffer system pH 7.0 versus varying concentration of respective systems.
A) At 0.063 M of proline with protein and buffer (Table 1.1) there is a blue shift from 329nm to 326.5nm. At 0.375 M of proline there is again blue shift from 329nm to 327.5nm and in the presence of 0.750 M of proline there is a shift of the lambda max ($\lambda_{\text{max}}$) from 329nm to 328.5nm (blue shift), indicating stabilization. We observed that, however at individual concentration of proline lambda max ($\lambda_{\text{max}}$) shows blue shift (stabilization) with respect to that of protein but with increasing concentration of proline (Figure 1.1 a) the fluorescence spectra of the protein gives the comparative red shift (destabilization). It means that proline shows destabilization effect at its higher concentration. This may be due to the higher concentration of proline at which sufficient hydration of the protein capacity decreases and it may be expected that some amount of proline may come into the vicinity of protein and may bind to its surface, showing a small comparative red shift in the fluorescence spectra of protein with proline with its varying concentration. This is taken by the fact that at high osmolyte (cosolvent) concentrations (above about 1 M (molar) sufficient hydration of the protein molecules is no longer possible (in accordance with calorimetric data (Ravindra and Winter, 2003 [91] and Ravindra and Winter, 2004 [92]) and the effect may be reversed.

B) With the absence of proline and in the presence of sugars (fructose, sucrose, and maltose) with protein and buffer the spectra shows red shift (shifting of smaller wavelength to larger wavelength) compare to the native protein at all concentration ranges of sugar. By observing the Figure [1.1( b)], it can be reported that, However, with increasing concentration of fructose lambda max ($\lambda_{\text{max}}$) shifted to larger wavelength (red shift) i.e lambda max ($\lambda_{\text{max}}$) shifted from 330nm (at 0.063M) to 341.5nm (at 0.375M), with the further increase in concentration of fructose (0.750M) $\lambda_{\text{max}}$ shifted to lower wave length (blue shift) i.e 336.5nm. Similar is the case with sucrose [Figure 1.1 c] lambda max ($\lambda_{\text{max}}$) shifted from 336nm (at 0.063M) to 331nm (at 0.750M) at its final concentration. In maltose also same lowering in wave length (blue shift) was observed with the corresponding increase in concentration [figure 1.1 d].This reflects the stabilization behaviour of sugars. However, by comparing with the native protein red shift was observed instead of blue shift at all of its (sugar)
Figure 11(a): Fluorescence emission spectra of 2.828 \times 10^{-2} M \alpha\text{-}lactalbumin at pH 7.0 at room temperature in buffer (\lambda_{\text{max}} = 329 \text{ nm}) (A) and in the presence of varying concentration of proline: [proline-protein buffer, 0.633 M (\lambda_{\text{max}} = 326.5 \text{ nm})] (B), [proline-protein buffer, 0.375 M (\lambda_{\text{max}} = 327.5 \text{ nm})] (C) and [proline-protein buffer, 0.750 M (\lambda_{\text{max}} = 328.5 \text{ nm})] (D).
Figure 1.1(b): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$=329nm) (A) and in the presence of varying concentration of fructose: [fructose-protein-buffer, 0.063M ($\lambda_{\text{max}}$=330nm)] (B), [fructose-protein-buffer, 0.375M ($\lambda_{\text{max}}$=341.5nm)] (C) and [fructose-protein-buffer, 0.750M ($\lambda_{\text{max}}$=336.5nm)] (D).
Figure 1.1(c): Fluorescence emission spectra of $2.82 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$=329nm) (A) and in the presence of varying concentration of sucrose: sucrose-protein-buffer, 0.03M ($\lambda_{\text{max}}$=336nm) (B), sucrose-protein-buffer, 0.375M ($\lambda_{\text{max}}$=335.5nm) (C), and sucrose-protein-buffer, 0.75M ($\lambda_{\text{max}}$=331.0nm) (D).
Figure 1.1(d): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$=329nm) (A) and in the presence of varying concentration of maltose: [maltose-protein-buffer, 0.063M ($\lambda_{\text{max}}$=341.5nm)] (B), [maltose-protein-buffer, 0.375M ($\lambda_{\text{max}}$=335.0nm)] (C) and [maltose-protein-buffer, 0.750M ($\lambda_{\text{max}}$=332.5nm)] (D).
concentrations. A pronounced red shift of tryptophan fluorescence does not necessarily reflect a considerable increase in its mobility but in some cases native proteins with a fixed but relatively polar environment of tryptophan residues also shows a characteristic red shifted spectrum [95]. The phenomenon of giving red shift does not necessarily indicate the destabilization effect of protein but there is a possibility of accumulating relatively hydrophobic molecule in to apolar regions of proteins, thus facilitating the quenching of buried tryptophans [96]. Therefore, in our case, sugars in the presence of protein shows red shift does not necessarily indicate that it is solvent exposed. This is evident that lambda max (λ_max) cannot be relied upon to predict the exposure of a residue. Other factors such as specific interactions between the indole ring and polar groups on the protein, or the presence of water molecules in the protein's interior may play a part in determining the position of the fluorescence spectrum [100].

The fluorescence maxima λ_max of tryptophanyl residues in native proteins commonly ranges from about 325 to 350nm. It has been speculated that their may be some relationship between the λ_max and the exposure of tryptophan in a protein [101]. This is based on the fact that denatured and unfolded proteins have a red (~ 350nm) emission, and that in solvents of low dielectric constant, such as dioxane, the λ_max of indole is blue shifted (~ 320nm) and the theory is that surface residues that are exposed to water would fluoresce red, and those buried in a relatively apolar interior region of a protein would appear blue. Alternatively, a tryptophan might fluoresce blue if it would sandwiched in a rigid portion of a protein matrix, even if its microenvironment were not particularly apolar [100]. Therefore in the present case of sugars with protein the shifting to longer wave length compared to native protein does not necessarily reflect that it is solvent exposed. However with increasing concentration of sugars in all of the three cases (fructose, sucrose, and maltose) comparative blue shift was observed [Figure 1.1 (b), Figure 1.1 (c), and Figure 1.1 (d)] showing stabilization effects at its higher concentration. By observing the Figure 1.1 and Figure 1.1[(e), (f) & (g)], of the three sugars (fructose, sucrose, and maltose) the lowering in wave length
Figure 1.1(e): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer($\lambda_{\text{max}}$-329nm) (A) and in the presence of different sugars (0.063M): [Fructose-protein-buffer, 0.063M ($\lambda_{\text{max}}$-330.0nm)] (B), [Sucrose-protein-buffer, 0.063M ($\lambda_{\text{max}}$-336.0nm)] (C), and [Maltose-protein-buffer, 0.063M ($\lambda_{\text{max}}$-341.5nm)] (D).
Figure 1.1(f): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{max}$=329nm) (A) and in the presence of different sugars (0.375M): [Fructose-protein-buffer, 0.375M ($\lambda_{max}$=341.5nm)] (B), [Sucrose-protein-buffer, 0.375M ($\lambda_{max}$=335.5nm)] (C), and [Maltose-protein-buffer, 0.375M ($\lambda_{max}$=335.0nm)] (D).
Figure 1.1(g): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$=329nm) (A) and in the presence of different sugars (0.750M): [fructose-protein-buffer, 0.750M ($\lambda_{\text{max}}$=336.5nm)] (B), [Sucrose-protein-buffer, 0.750M ($\lambda_{\text{max}}$=331.0nm)] (C), and [Maltose-protein-buffer, 0.750M ($\lambda_{\text{max}}$=332.5nm)] (D).
was observed more in the case of sucrose (336.0nm to 331.0nm) with its increasing concentration (0.063M to 0.750M) followed by maltose (341.5nm to 332.5nm) at the same concentration range. Presence of sucrose shifts the conformational equilibria towards the most compact protein species within native state ensembles which can be explained by preferential exclusion of sucrose from the protein surface leading to stabilization [97].

C) In the presence of sugars with proline the spectra shows blue shift (the higher wavelength shifted to lower wavelength) as compared to native protein ($\lambda_{max}$, 329 nm) in all of the three sugars at its lower concentration (0.063M). By observing the spectra of fructose - proline [figure 1.2(a)] it can be seen that lambda maxima shifted from 329nm to 327.5nm (blue shift) at the lower concentration of fructose and proline (0.063M) indicating stabilization. The spectra of sucrose - proline [figure 1.2 (a)] also showed a lowering in wave length (blue shift) i.e $\lambda_{max}$ shifted from 329nm to 328nm at its lower concentration (0.063M) indicating stabilization. Similar is the case with maltose and proline [figure 1.2 (a)] same decrease in wave length was observed (329nm to 327.5nm) at the same concentration (0.063M) reflecting the stabilization. But at their higher concentration (0.375M and 0.750M of sugars -proline) comparative red shift (destabilization) was observed [Fig 1.2(b & c)]. This may be due to the effect of proline, due to the increase in concentration of proline sufficient hydration of protein decreases (result in destabilization) and red shift was observed. This is taken from the similar fact which we have given above for the proline alone [Figure 1.1 (a)] that with the increase in cosolvent concentration sufficient hydration of the protein is no longer possible and the effect of stabilization may get reversed [91,92]. By observing the Figure 1.3 [system in the presence of fructose and fructose - proline], Figure 1.4 [system in the presence of sucrose and sucrose - proline], and figure 1.5 [system in the presence of maltose and maltose - proline] we can report that the system in which proline is absent shows comparative lowering in wavelength (blue shift) [downward shift of the curve] with corresponding increase in concentration of respective sugars but in the presence of proline similar increase in concentration of
Figure 1.2(a): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$=329nm) (A) and in the presence of proline (0.063 M) alone and proline with different sugars (0.063 M): [proline-protein-buffer, 0.063M ($\lambda_{\text{max}}$=326.5nm)] (E), [fructose-proline-protein-buffer, 0.063M ($\lambda_{\text{max}}$=327.5nm)] (B), [sucrose-proline-protein-buffer, 0.063M ($\lambda_{\text{max}}$=328.0nm)] (D), [maltose-proline-protein-buffer, 0.063M ($\lambda_{\text{max}}$=327.5nm)] (C).
Figure 1.2(b): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer ($\lambda_{\text{max}}$-329nm)(A) and in the presence of proline (0.375 M) alone and proline with different sugars (0.375M): [proline-protein-buffer, 0.375M ($\lambda_{\text{max}}$-327.5nm)] (E), [fructose-proline-protein-buffer, 0.375M ($\lambda_{\text{max}}$-342.5nm)] (B), [sucrose-proline-protein-buffer, 0.375M($\lambda_{\text{max}}$-327.0nm)] (D), [maltose-proline-protein-buffer, 0.375M($\lambda_{\text{max}}$-330.0nm)] (C).
Figure 1.2(c): Fluorescence emission spectra of $2.828 \times 10^{-7}$ M alpha-lactalbumin at pH 7.0 at room temperature in buffer($\lambda_{\text{max}}$-329nm) (A) and in the presence of proline (0.750 M) alone and proline with different sugars (0.750M): [proline-protein-buffer, 0.750M ($\lambda_{\text{max}}$-328.5nm)] (E), [fructose-proline-protein-buffer, 0.750M($\lambda_{\text{max}}$-354nm)] (B), sucrose-proline-protein-buffer, 0.750M($\lambda_{\text{max}}$-330.0nm] (D), [maltose-proline-protein-buffer, 0.750M($\lambda_{\text{max}}$-332.0nm)] (C)
Figure 1.3: Plots of $\lambda_{\text{max}}$ (nm) of fructose in the absence and presence of proline-alpha-lactalbumin-buffer system at pH 7.0 versus concentration of respective systems.
Figure 1.4: Plots of $\lambda_{\text{max}}$ (nm) of intrinsic fluorescence of sucrose in the absence and presence of proline-alpha-lactalbumin-buffer system at pH 7.0 versus concentration of respective system.
Figure 1.5: plots of $\lambda_{\text{max}}$ (nm) of intrinsic fluorescence of maltose in the absence and presence of proline - alpha-lactalbumin- buffer system at pH 7.0 versus concentration of respective system.
sugars and proline shifted the wave length to the higher side (red shift) [upward shift of the curve], thus showing the destabilization effect [Figure 1.3, 1.4 & 1.5] at higher concentration of sugars-proline. Hence, proline is responsible for showing red shift at higher concentrations of sugars-proline. It may be concluded that proline acts as a good stabilizer at its lower concentration (0.063M) at which proline shows a greater stabilization effect as compared to three sugars (fructose, sucrose, and maltose).[Table 1.1] but at its higher concentration it may act as a denaturant. But all over we have seen that native structure of alpha-lactalbumin stabilizes more in the presence of proline, as, at all of its concentration (0.063M, 0.375M,& 0.750M) blue shift (shifting of larger wave length to smaller wave length) was observed compared to the native protein [figure 1.1 (a)] and also presence of proline in sugars shifted the wave length to lower side (blue shift) at lower concentration of sugars – proline(0.063M) compared to the native protein [figure 1.2(a)]. The stabilization effect of proline also confirmed by the fact that proline stabilizes the secondary and tertiary conformation of protein (creatine kinase) against denaturation. Hence, act as a stabilizer [98].
CONCLUSION
CONCLUSION:

A) It is concluded from Figure 1.1 that,

a) A comparative red shift instead of blue shift was observed in the presence of proline - protein and buffer with increasing concentration of proline may be due to the increase in concentration of proline at which the sufficient hydration of the protein capacity decreases and the effect of stabilization may get reversed. It indicates that the proline acts as a stabilizer at low concentration, but at its higher concentration small destabilization may result compare to the stabilization at lower concentration.

b) The fructose with protein and buffer shows the destabilization at all of its concentration. But lambda max (λ_{max}) of fructose with protein and buffer shows a comparative blue shift with increasing concentration of fructose. This shows the stabilization effect of sugars.

c) The value of lambda max (λ_{max}) of sucrose with protein and buffer at all of its concentration is higher than the native protein, but comparative blue shift was observed with increasing concentration of sucrose. This may be due to the increase in steric exclusion with the increase in concentration of sucrose result in preferential hydration.

d) The addition of maltose with protein – buffer solution also shows comparative red shift at lower concentration (0.063M) than at higher concentration (0.375M and 0.750M), reflecting the destabilizing phenomenon at lower concentration but the comparative blue shift is also found in lambda max (λ_{max}) at its higher concentration. This may be due to the same increase in steric exclusion with the increase in molecular weight and concentration effect of maltose, more possibility of increase in preferential hydration.

This means that the stability of protein in presence of sugars increases with its concentration.

B) Figure 1.2 shows that

a) Proline with lactalbumin and buffer solution indicates stabilization at all of its three concentrations compared to the native protein but comparative red shift was observed with the corresponding increase in concentration of proline [Table 1.2 and
Figure 1.1(a)]. This may be due to decrease in hydration capacity of protein with increasing concentration of proline.

b) The presence of proline with fructose (reducing sugar) indicate good stabilization of protein at 0.063M of proline - fructose compared to the native protein, but again an abnormal red shift was observed at two of its higher concentration (i.e at 0.375M and 0.750M). This may be due to the same decrease in hydration capacity of protein due to increase in concentration of proline and fructose.

c) The presence of proline with maltose (reducing sugar) indicate stabilization of protein at 0.063M of proline - maltose compared to the native protein, but again abnormal red shift was observed by maltose and proline in lambda max ($\lambda_{max}$) at its two higher concentration (i.e at 0.375M and 0.750M) may be due to decrease in hydration capacity of protein with increase in concentration of proline and maltose.

d) While the presence of proline with sucrose [Table 1.2] reflects stabilization of protein at two of its lower concentration (i.e at 0.063M and 0.375M) compared to the native protein. Thus we have seen that stabilization activity of sucrose is more as compared to fructose and maltose. This may be due to the fact that presence of sucrose shifts the conformational equilibria towards the most compact protein species within native state ensembles, by preferential exclusion of sucrose from the protein surface. With the increase in concentration of sucrose due to its larger size, capacity of steric exclusion increases which in turn increases the preferential hydration of protein and due to this much less increase in wavelength (red shift) was observed (329nm to 330nm) compared to native protein as given by fructose-proline (329nm to 354nm) and maltose-proline (329nm to 332nm) at the same concentration (0.750M).

C) The pattern of lambda max ($\lambda_{max}$) versus concentration of cosolvents in non reducing sugar sucrose and reducing sugars maltose and fructose is shown to be some what similar, but the shape of these curves are slightly different for fructose (reducing sugar) [Figure 1.1&1.2].

In the absence and in the presence of proline:

In the case of sucrose (non reducing sugar), in the absence and presence of proline (figure 1.1 and 1.2), the lambda max ($\lambda_{max}$) was at lower position
in all of its concentration compared to lambda max in the case of fructose and maltose (reducing sugars).

More decrease in lambda max ($\lambda_{\text{max}}$) was observed in case of sucrose (non reducing sugar) as compared to maltose and fructose (reducing sugars) with the increase in concentration of sugar/sugar-proline.

Finally we can conclude that the stabilization effect was observed by fluorescence technique to be highest in case of proline as blue shift was observed ($\lambda_{\text{max}}$ shifted to lower wavelength) at all the concentration of proline compared to native protein [Table 1.1 and Figure 1.1(a)] and also by observing the Figure [1.3, 1.4, 1.5] in which the curve shows that the presence of proline keeps its position lower (lower wavelength, showing blue shift) as compared to the curve of the protein sample without any proline indicate stabilization effect of proline. A slight different behaviour was observed in fructose at two of its concentration (i.e at 0.375M & 0.750M), where curve shifted upwards (increase in $\lambda_{\text{max}}$) in the presence than in the absence of proline.
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