Effect of Sugars on the Thermal Stability of Hen Lysozyme both in the Absence and Presence of Denaturants

Thesis Submitted

In partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

IN

CHEMISTRY

Submitted by:

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Under the supervision of

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JULY, 2013

Certificate

*This is to certify that the thesis entitled "**Effect of sugars on the thermal stability of hen lysozyme both in the absence and presence of denaturants"** being submitted in the partial fulfilment of requirements for the award of degree of **Master of Science in Chemistry** submitted in **the School of Chemistry and Biochemistry, Thapar University, Patiala** is a bonafide work carried under the supervision of **Dr. Rajesh Kumar,** Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala and that no part of this project has been submitted for the award of any other degree.*

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CANDIDATE'S DECLARATION

I hereby declared that the work presented in this thesis entitled ***"Effect of sugars on the thermal stability of hen lysozyme both in the absence and presence of denaturants"*** *submitted in the partial fulfilment of requirements for the award of degree of* ***Master of Science in Chemistry*** *submitted in* ***the School of Chemistry and Biochemistry, Thapar University, Patiala*** *is an authentic record of my own work carried out under the supervision and guidance of* ***Dr. Rajesh Kumar, Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala*** *and refers other researcher's work which are duly listed in the reference section.*

The matter embodied in this thesis has not formed the basis for the award of any other degree of this or any other university.

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ACKNOWLEDGEMENT

I take this opportunity to express my gratitude to the people who have been involved in the successful completion of this project. I would like to extend my sincere thanks to all of them.

I am highly indebted to my supervisor Dr. Rajesh Kumar, Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala, for their guidance and constant supervision as well as for providing necessary information regarding the project and also for his support in completing the project. It is indeed my privilege to work under him. I would like to express my special gratitude to Head, School of Chemistry and Biochemistry for utilizing all the necessary research facilities required for this project and thanks for giving me such attention and time.

I take this opportunity to thank the research fellow Ms. Rishu Jain, Mr. Sandeep Kumar and Mr. Rajesh Kumar for their help and moral support. My thanks and appreciations also go to my colleagues and all the teaching and non-teaching staff members in developing the project and people who have willingly helped me out with their abilities.

In the end, I wish to express my deep sense of gratitude to my family, for supporting and encouraging me at every step of my work. It is power of their blessings, which has given me the courage, confidence and zeal for hard work.

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Regards,

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1.0 Introduction

Both theoretical and experimental research in protein folding is proceeding at an explosive rate worldwide [1-10]. The process of protein folding, while critical and fundamental to virtually all of biology, in many ways remains a mystery. To perform biological function, an unfolded polypeptide chain folds to three-dimensional native conformation. However, sometimes proteins do not fold properly and misfold. Protein misfolding is the major cause of most of the human diseases such as, cancer, diabetes, lysosomal storage diseases, cancer cystic fibrosis and neurodegenerative diseases such as Alzheimer, Parkinson's and Huntington's and prion diseases [11-12]. Specialized proteins called chaperones assist in the *in vivo* folding of nascent polypeptides in the cell and inhibit their misfolding. The protein folding studies are important in designing protein with novel biological functions, understanding the structure, function relationships of the proteins and rational engineering of the existing proteins.

All biological processes depend on proteins being stable and in the appropriate folded conformation. It is important to know how proteins fold into their biologically active states, and how these active states are stabilized. The complete characterization of any protein requires stability determination and the forces which lead to stability and correct folding. Several factors, such as electrostatic interactions, hydrophobic interactions, hydrogen bonding, and conformational entropy are accountable for the folding and stability of native proteins. The roles of electrostatic [13-17], hydrophobic [18-19] and hydrogen bonding [20-21] interactions in determining the stability of proteins are well recognized, but the relative contributions of each of these factors vary from protein to protein and with the solution conditions to which the protein is exposed [13,19].

Sugars such as, sucrose, trehalose, glucose, and maltose have been used for many years as stabilizing agents (kosmotropic cosolvents) for the maintenance of the biological activity of macromolecules [22-29]. These sugars generally protect proteins against the thermal and chemical denaturation and counteract the various stress conditions that an organism encounters [30-31]. Among these, compatible sugar trehalose, has been found to be particularly efficient [28-29]. Chemical denaturant such as GdnHCl typically unfold the proteins when present at higher concentrations. GdnHCl unfolds the proteins through directly binding to peptide groups of protein [32-34]. Though, the effects of sugars and denaturants on the heat perturbation of proteins are extensively studied [25-28, 32-34], however, the effect of sugars on the heat perturbation of native protein in the presence of denaturants is rarely studied. In the present work, we analyzed the effect of sugars on the thermal stability of hen egg white lysozyme both in the absence and presence of denaturants by monitoring the wavelength shift with temperature increase in the fluorescence emission spectrum (ex: 290) of lysozyme at pH 4.5. Thermal experiments revealed stabilizing effect of sugars. Among sugars, the thermal effects were larger in the presence of trehalose while it is least in the presence of glucose. This stabilizing effect of sugars also counteracts the destabilizing effect of GdnHCl.

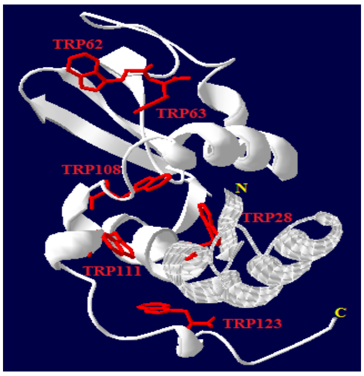

Figure 1. Ribbon schematic of hen egg white lysozyme (PDB accession ID: 1AKI. The tryptophan residues with their side chains and marked according to their position and three letter symbol.

Hen egg white lysozyme is a small globular protein of about 129 amino acids folded in helical and β-sheet domains. In case of intrinsic photoluminescence (PL) experiment of lysozyme, to minimize the excitation of tyrosine residue, the excitation wavelength was set at 290 nm, corresponding to the maximum absorption band of tryptophan [35].

2.0 Materials and Methods

Hen egg white lysozyme (HEWL) was purchased from calbiochem and was used without further purification. GdnHCl was from USB. Sugars (trehalose, sucrose, glucose, and maltose) and sodium acetate were from Sigma. All experiments were done in 50mM sodium acetate buffer at pH ~4.5.

2.1 Thermal unfolding of native lysozyme (pH 4.5) in the presence of different concentrations of sugars

To determine the effect of sugars on thermal unfolding of lysozyme, the protein samples (~6μM) were prepared in 50 mM sodium acetate buffer that contained different concentrations of sugar (trehalose, sucrose, maltose, and glucose). PL measurements were performed on fluorescence spectrometer (PerkinElmer LS 55) equipped with a temperature controlled cell holder and an optical cuvette of 1 cm path length. Thermal unfolding of lysozyme was followed by measuring intrinsic emission fluorescence (excitation 290 nm) in the wavelength range of 280 to 480 nm and temperature from 10 to 90 °C. Pelteir controlled heating rate was 1°C/min. Slits were set as 6 nm for both excitation and emission.

2.2 Thermal unfolding of native lysozyme (pH 4.5) as a function of GdnHCl both in the absence and presence of sugars

To investigate the effect of denaturant on thermal unfolding of lysozyme, the protein samples (~6μM) were prepared in 50 mM sodium acetate buffer that contained different concentrations of GdnHCl. To determine the effect of sugars on the GdnHCl dependent thermal stability of protein, the protein samples were prepared in 50 mM sodium acetate buffer that contained 1.0 M sugar (trehalose, sucrose, maltose, and glucose) and different concentrations of GdnHCl. Thermal unfolding measurement parameters are same as described in section 2.1.

3.0 Results

3.1 Effect of sugars on the thermal denaturation of native lysozyme

To determine the effect of sugars on the thermal denaturation of native lysozyme, we recorded a series of intrinsic tryptophan PL-monitored thermal-denaturation curves for lysozyme in the presence of varying concentration of sugars (trehalose, maltose, glucose, and sucrose) at pH 4.5. Figure 2 shows the representative temperature dependent fluorescence emission spectra of lysozyme in the absence of sugar at pH~4.5. Figure 3a shows the PL-monitored temperature-induced $\lambda_{\max}$ shift of lysozyme in the absence and presence of different concentrations of trehalose (0.0, 0.5, 1.0 and 1.5 M) at pH~4.5. At low temperatures, the $\lambda_{\max}$ not change significantly. Starting from 60°C, the tryptophan luminescence band shows a progressive red shift which ends at a temperature depending on the concentration of sugar used. Further, the signal intensity decreases with increase in the temperature, which is probably due to the thermal activation of nonradioactive channels, which quench the luminescence from the excited electronic state [35].

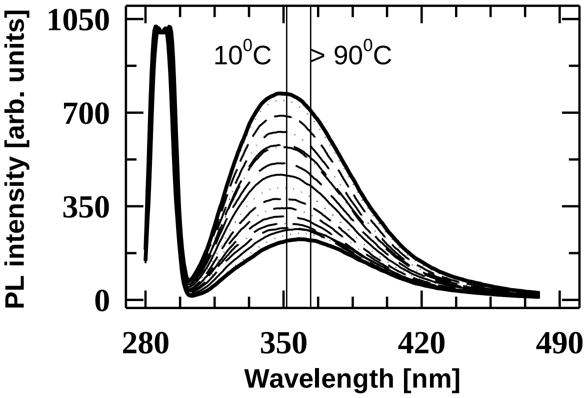

Figure 2. Steady state PL spectra of lysozyme excited at 290 nm and measured at different temperatures. The bold black lines indicate the spectra at the lowest (10 °C) and the highest (90 °C) temperature. The vertical solid lines indicate the position of the band peaks as a guide to the eyes.

The data presented in Figure 3a were normalized according to equation (1)

$$\text{Fraction Unfolded} = \frac{\lambda_{\max,\,(\mathrm{obs})} - (m_{\mathrm{pre}}T + c_{\mathrm{pre}})}{(m_{\mathrm{post}}T + c_{\mathrm{post}}) - (m_{\mathrm{pre}}T + c_{\mathrm{pre}})} \qquad (1)$$

where, $\lambda_{\max\ (\mathrm{obs})}$ is the observed $\lambda_{\max}$, T is the temperature, m_{pre} and c_{pre} are slope and intercept, respectively, of the pre-transition baseline, and m_{post} and c_{post} are slope and intercept of the post- transition baseline in the presence of a given concentration of sugar.

Clearly, as the concentration of the trehalose is increased in the reaction medium, the thermal unfolding curve shift toward higher temperature (Figure 3b). The thermodynamic parameters associated with the temperature-induced unfolding of lysozyme in the presence of trehalose and other sugars were obtained by nonlinear least squares analysis of the temperature dependence of the $\lambda_{\max}$. In the analyses, a two-state denaturation process was assumed when fitting the data to the van't Hoff equation [36]:

$$\lambda_{\max,\,(obs)}\,(\mathrm{T}) = \frac{\left(m_{pre}T + c_{pre}\right) + \left(m_{post}T + c_{post}\right) exp\left[\frac{-\Delta H_{\mathrm{m}}}{RT}\left(\frac{1}{T_{\mathrm{m}}} - \frac{1}{T}\right)\right]}{1 + exp\left[\frac{-\Delta H_{\mathrm{m}}}{RT}\left(\frac{1}{T_{\mathrm{m}}} - \frac{1}{T}\right)\right]} \qquad (2)$$

Where, $\lambda_{\max\ (\mathrm{obs})}$ (T) is the observed variable parameter, ΔH_{m} is the enthalpy at the transition temperature T_{m}, R is the gas constant and T is the absolute temperature.

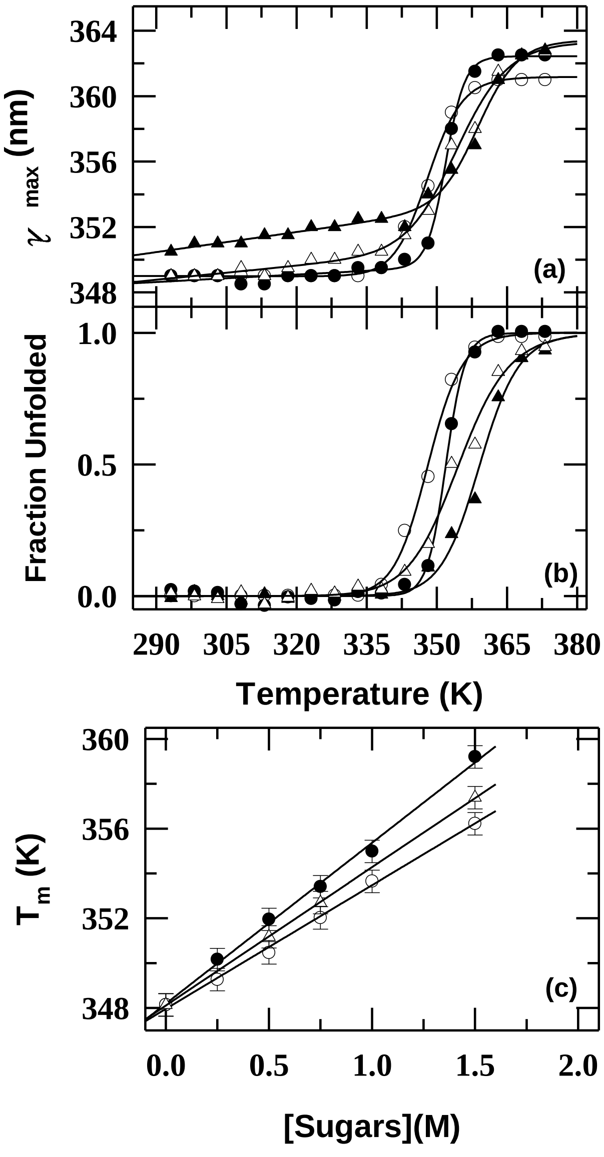

Figure 3 (a) PL-monitored temperature-induced $\lambda_{\max}$ shift of lysozyme in the presence of different concentration of trehalose (0.0M (o), 0.5M (●), 1.0M (Δ), and 1.5M (▲)), 50mM sodium acetate buffer, pH~4.5. (b) Panel (b) shows the normalized temperature-induced denaturation curves of lysozyme in the presence of different concentration of trehalose (0.0M (o), 0.5M (●), 1.0M (Δ), and 1.5M (▲)). The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation 2. (c) Variation of T_{m} with sugars concentration (trehalose (•), sucrose (Δ), glucose (o)). The solid lines in panel (c) represent the linear fit to the data.

The resulting midpoint transition temperature, T_{m}, for unfolding of lysozyme was plotted as a function of sugar concentration in Figure 3c. As sugar concentration is increased, the T_{m} increases linearly. The increase in the value of T_m is more pronounced in case of trehalose and least for glucose (Figure 3c).

3.2 Effect of GdnHCl on thermal denaturation of native lysozyme

Figure 4(a) shows the results of λ_{max} analysis on the steady-state PL of lysozyme in the presence of different concentrations of GdnHCl (0.0, 0.5, 1.0, 1.5 and 2.0 M) at pH ~4.5. The data shown in Figure 4a were normalized by using the equation (1). The fraction of unfolded protein was plotted as a function of temperature in Figure 4b. Figure 4b clearly showed that with increasing the concentration of the GdnHCl in the reaction medium, the thermal denaturation curve shift toward the lower temperatures. Thermodynamic parameters (*i.e.*, T_m and ΔH_m) associated with the temperature-induced unfolding of lysozyme in the presence of different concentrations of GdnHCl were obtained by fitting the data to the van't Hoff equation (2). The resulting, T_m, for unfolding of lysozyme was plotted as a function GdnHCl concentration in Figure 4c. As GdnHCl concentration is increased, the T_m decrease linearly, which suggests that GdnHCl decrease the thermal stability of protein.

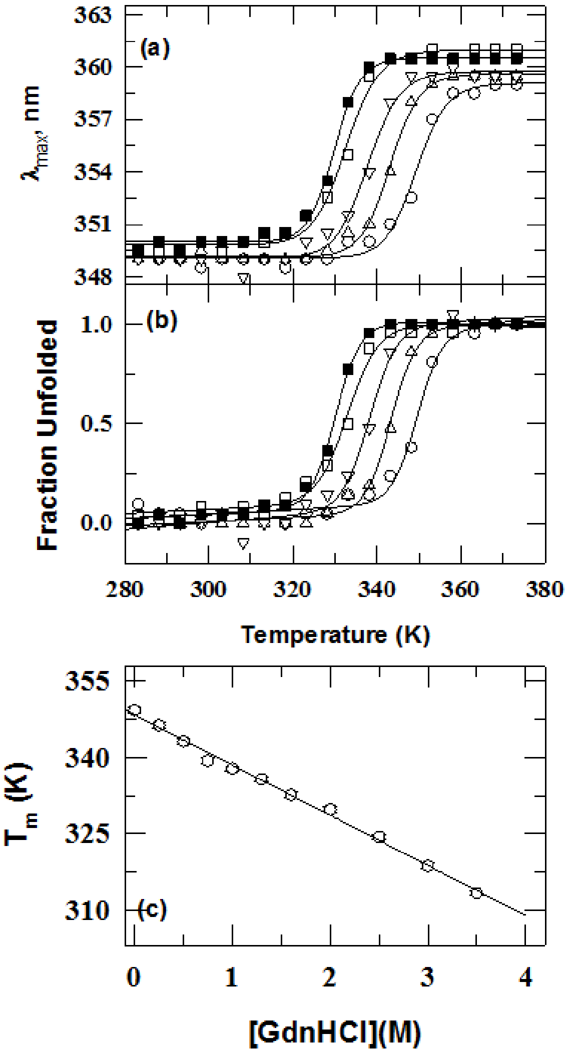

Figure 4. (a) PL-monitored temperature-induced λ_{max} shift of lysozyme in the presence of different concentrations of GdnHCl (0.0 (o), 0.5 (Δ), 1.0 (∇), 1.5 (□), 2.0 M (■)) in 50 mM sodium acetate buffer, pH~4.5. (b) Panel (b) show the normalized thermal-induced denaturation curves of lysozyme in the presence of different concentrations of GdnHCl (0.0 (o), 0.5 (Δ), 1.0 (∇), 1.5 (□), 2.0 (■)). The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation (2). (c) Variation of T_m with GdnHCl concentration. The solid lines in panel (c) represent the linear fit to the data.

3.3 The effect of sugars on the GdnHCl-linked thermal stability of native lysozyme

Figure 5 (a) shows the results of λ_{max} analysis on the steady-state PL of lysozyme in the absence and presence of 2.0 M GdnHCl, at pH 4.5. The Figure 5(a) also shows the results of λ_{max} analysis on the steady-state PL of lysozyme in the presence of 2.0 M GdnHCl and 1.0 M of sugar (trehalose, glucose, sucrose, maltose). The data presented in Figure 5b were normalized according to equation (1). Figure 5b clearly showed that the thermal unfolding curve shifts toward lower temperatures in the presence of GdnHCl. However, with the inclusion of 1.0 M sugar, the GndHCl-induced shifts toward lower temperatures become less pronounced (Figure 5b).

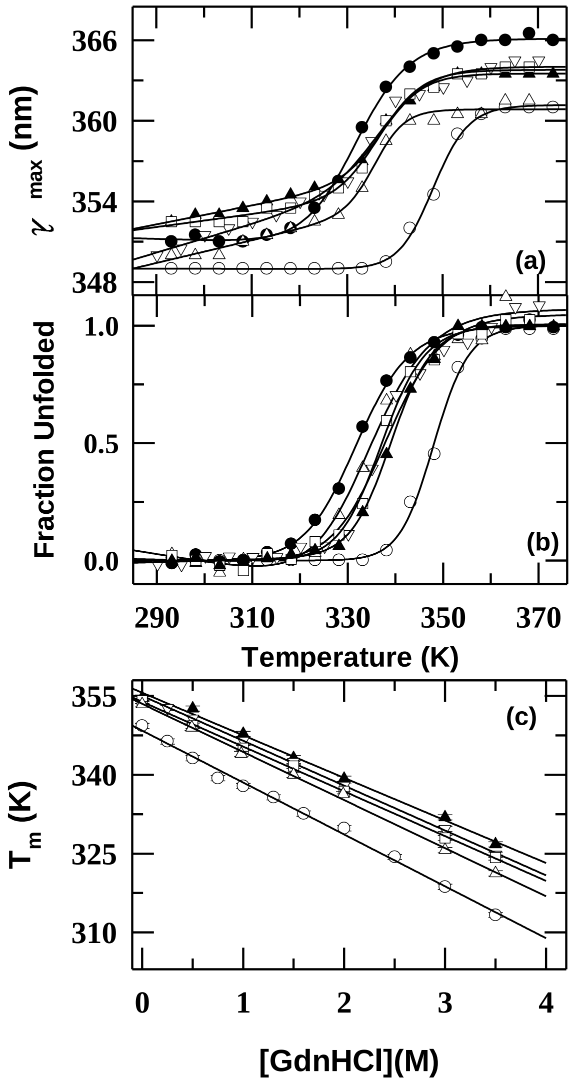

Figure 5 (a) PL-monitored temperature-induced λ_{max} shift of lysozyme in the presence of 0.0 M GdnHCl (○), 2.0 M GdnHCl (●) and 2.0 M GdnHCl with 1.0 M sugar (trehalose (▲), sucrose (∇), maltose(□), glucose (Δ)), in 50 mM sodium acetate buffer, pH~4.5. (b) Panel (b) show these thermal-induced denaturation curves of lysozyme in the normalized form. The solid lines in panels (a) and (b) represent the non-linear least–squares fits to the equation (2). (c) The variation of T_m with GdnHCl concentration both in the absence (○) and presence of 1.0 M sugar (trehalose (▲), sucrose (∇), maltose (□), glucose (Δ)). The solid lines in panel (c) represent the linear polynomial fit to the data.

The thermodynamic parameters (T_m and ΔH_m) associated with temperature-induced unfolding of lysozyme (Figure 5a and Figure 5b) were obtained by fitting the data to the van’t Hoff equation (2). Figure 5c shows the variation of T_m with the GdnHCl concentration both in the absence and presence of 1.0 M sugar (trehalose, sucrose, maltose, and glucose). Figure 5c clearly shows that the T_m decreases linearly with increase in the concentration of GdnHCl. However, the decrease in T_m by GdnHCl is less pronounced in the presence of sugars. This finding indicates that the sugars (trehalose, maltose, glucose, and sucrose) counteract the destabilizing effect of denaturants.

4.0 Discussion

The present work discusses the effect of sugars on the thermal stability of native lysozyme both in the absence and presence of GdnHCl. The possible explanations for the observed effect of various sugars on the thermal stability of native lysozyme in the presence of GdnHCl are also discussed.

4.1 Sugars modulates the thermal stability of native lysozyme

The results obtained from thermal unfolding of native lysozyme in the presence of various sugars (trehalose, maltose, glucose and sucrose) indicate that these sugars increase the thermal stability of native lysozyme. It is also observed that the thermal stability of native lysozyme is increased more for trehalose and least for glucose. Few earlier studies on the effect of sugars on thermal unfolding of other proteins have also shown that sugars increase the thermal stability of proteins. Lee et al (1975, 1981) have also shown that the stabilizing effect of sugars on proteins originates from the preferential hydration of proteins [24-26]. Recentely, Miyawaki (2009) analysed the effect of sugars on thermal unfolding of various proteins, and showed that water activity also plays a major role in stabilization of proteins in the presence of sugars [37].

4.2 GdnHCl decreases the thermal stability of native lysozyme

Thermal denaturation of lysozyme strongly depends on the GdnHCl concentration, and it is accompanied by a red-shift of the PL emission band, associated with the solvent exposure of tryptophan 108. The addition of GdnHCl to the native protein decreases progressively the thermal stability of the protein [Figure 4b]. Several models suggest that the denaturants destabilize the proteins by migrating into the interior of the protein and forming hydrogen bonds to atoms in the backbone [38]. Another source of structural destabilization can be the preferential interaction of protein with solvent components at high concentration of additives. For example, conformational changes induced by GdnHCl are linked to the binding of GdnHCl to the protein [39-40].

4.3 Sugars counteracts the destabilizing effect of GdnHCl

The denaturing action of GdnHCl is presumed to be mainly based on their ability to bind to the protein [41-43]. The data shown in Figure 4a,b and Figure 5a,b clearly indicate that the thermal stability of native lysozyme decreases in the presence of GdnHCl. Further, the data in Figure 5c clearly indicate that the inclusion of sugars results in the counteraction of the destabilizing action of the GdnHCl. Timasheff et al (1985, 1994) have reported that osmolyte counteracts the denaturing effect of denaturant mainly by altering the balance between preferential binding of denaturant and preferential exclusion of osmolytes, resulting in the increased hydration of the protein [44-45]. Similarly, in case of lysozyme, it is expected that the addition of sugars alters the balance between the preferential binding of GdnHCl and preferential exclusion of these sugars, resulting in the increased hydration of the protein and helps in the counteraction of the destabilizing action of the denaturant.

5.0 Conclusion

The present work showed the effect of sugars on the thermal stability of native lysozyme (pH 4.5) both in the absence and presence of sugars. The thermal stability of the protein was found to increase in the presence of sugar. Among the sugars used (trehalose, sucrose, glucose and maltose), the thermal stability of the protein is increased more for trehalose and least for glucose. The stabilizing effect of sugars is mainly due to preferential hydration of protein or the preferential exclusion of the sugars [45]. In contrast to sugars, GdnHCl has been found to decrease the thermal stability of the protein. The destabilizing action of

GdnHCl is attributted mainly to their ability to bind to the protein [41-43]. The effect of sugars on the thermal stability of lysozyme in the presence of GdnHCl is also examined. The inclusion of sugars results in the counteraction of the destabilizing effect of the GdnHCl. This may be attributed to the fact that the addition of sugars alters the balance between the preferential binding of GdnHCl and preferential exclusion of sugars, resulting in the increased hydration of the protein and thus helps in the counteraction of the destabilizing action of the GdnHCl. It is also observed that the counteraction effect of sugars on the destabilizing effect of the GdnHCl is more pronounced for trehalose and least for glucose.

6.0 References

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