Name: Sumra Shahid

Supervisor's Name: Dr. Asimul Islam **Co-Supervisor's Name:** Prof. Faizan Ahmad

Department: Centre for Interdisciplinary Research in Basic Sciences

Topic: Thermodynamics of Protein Folding in the Crowded Environment

ABSTRACT:

The intracellular environment, where protein folds and performs various functions, differs from the dilute buffer solutions often used during *in vitro* experiments. These dilute buffer solutions have been typically assumed to represent the *in vivo* scenario; however, there exists a major difference between the idealized (diluted) conditions and the environment present within cells. It has been estimated that the overall concentration of macromolecules in the cytoplasm ranges from 50 to 400 mg ml^{-1} restricting the space available to each individual molecule and such a cellular condition has been termed as macromolecular crowding. Macromolecular crowding indicates the presence of nonspecific steric repulsion between the molecules and generates the excluded volume effect, where any part of two macromolecules cannot exist in the same place at the same instant of time. Thus, the excluded volume effect should favour the folding reactions where the total volume, which is occupied by the molecules in the cytoplasm, is reduced. Therefore, it is essential to determine how different degrees of macromolecular crowding alter the biophysical properties of proteins. The work in this thesis has focused on how macromolecular crowding affects thermodynamic stability, structure and functional activity of two model proteins, hen-egg white lysozyme and apo form of α-lactalbumin (α-LA). In order to mimic the crowded cellular milieu, synthetic polymers, ficoll 70 and dextran 70 and 40 were used as crowding agents (crowders) in our study. The aim of the study was to perform systematic examination of the relationships between the concentration, shape and size of the crowders (individually and in mixtures) and the thermodynamic and kinetic properties of the proteins using spectroscopic techniques such as UV-Vis absorption spectroscopy and circular dichroism (CD). Thermal denaturation experiments of both the proteins were performed in the absence and presence of crowding agents under different pH values, and it was observed that it is a reversible two-state process at all the experimental conditions. It has been observed that with increasing concentration of each crowding agent, there is an increase in the values of T_m (midpoint of thermal denaturation) and ΔG_D^o (standard Gibbs free energy change of unfolding) without causing a significant change in the values of ΔH_m (enthalpy change at T_m) of both the proteins at

different pH values. There is an unfavorable interaction between peptide backbone and crowding agent which results in the crowder-induced protein stabilization without perturbing their enthalpy of unfolding. Thus, the stabilization of proteins in the presence of crowding agents is entropic in nature.

The extent of stabilization for both the proteins increases with the increasing concentration of the crowding agents and found to be dependent on the shape and size of the crowder at all the pH values. The small sized and rod shaped crowder, dextran 40 resulted in greater stabilization of both the proteins than dextran 70 and ficoll 70 due to its low average molecular mass and large number of molecules leading to highest packing and hence more volume exclusion. The extent of stabilization of both the proteins increases as we move away from their pI values in the presence of each crowding agent which indicates their pH-dependency. The linear expansion of unfolded state of protein in response to increased charged density plays an essential role. It has been observed that mixture of crowding agents stabilizes the proteins more than the sum of the constituent crowding agent due to more volume exclusion by the mixtures of crowders than individually and hence, describes it to be a non-additive effect. We observed that the crowding environment does not alter the structure of the native (N) as well as denatured (D) states of both the proteins at all the experimental conditions. This indicated that the crowding agents stabilized both the proteins without modulating their structures.

Kinetic parameters (K_m and k_{cat}) of lysozyme have been measured in the presence of crowding agents and a relationship has been established between the stability and functional activity of a protein. It has been observed that the values of K_m and k_{cat} of lysozyme decrease with the increasing concentration of each crowding agent and the effect is more noticeable in the case of dextran 40 followed by dextran 70 and then ficoll 70. Moreover, there is a decrease in the functional activity of lysozyme with an increase in its stability in the presence of each crowding agent. It appears stability of the enzyme's active site is raised in the presence of macromolecular crowding resulting in less flexibility and thus less activity which can be associated to the well-established hypothesis of stability-activity trade-off. Our findings are consistent with this hypothesis.

Keywords: Macromolecular crowding, Crowding agent, Thermodynamic stability, Excluded volume effect, Stability-activity trade-off