Summary:

Investigation of structural and mechanistic features of enzyme has been one of the most favorite areas of research for biochemists. Fundamental aspects revealed by systematic studies have been immensely useful to design therapeutic compounds that alleviate a number of pathophysiological conditions. CN1 and CN2 which belong to the M20 family of metallopeptidases control carnosine concentration in serum as well inside many cells. Changes in activities of these enzymes are responsible for many disease conditions and therefore, studies investigating apparent as well as latent properties of carnosinases are of much important to a biomedical scientist. Very limited structural and biochemical information available on these enzymes prevent the design of competitive or allosteric modulators which can be used for fine tuning the activities of these enzymes under diseased conditions. Besides structure, other important information on mechanism of catalysis are that CN1 hydrolyzes carnosine and carnosine containing peptides more specifically whereas CN2 is promiscuous in nature and acts on dipeptides with varied composition. Carnosine, a pluripotent molecule possesses many properties that are beneficial in nature. Some of the most important and well characterized properties include antioxidant activity, and intracellular buffering capacity, and a good chelator of transition metal ions. Other less well characterized properties include its role in the protection of biomolecules (proteins, lipids, DNA) from benevolent modifications and regulation of blood glucose level. Considering both importance of carnosine metabolism and lack of mechanistic details on these important metallodipeptidases, objectives of thesis were framed to study structural and molecular features of substrate specificity, ligand binding, and in silico screening of potential carnosinase binders.

First, we standardized the expression, and purification of folded human carnosinases. Experimental conditions favorable for purifying functional homodimeric CN1 and CN2 have been fine tuned and it was found that divalent cations, Mn^{2+} in particular has the most stabilizing effect. In addition, activities of carnosinases also increased in the presence of these metal ions. Next, to study and compare kinetic properties of carnosinases, and also other dipeptidases in the future, we developed a fast, reliable, and accurate mass spectrometry based assay for monitoring the rate substrate hydrolysis. This method is label free and devoid of artifacts that arise during OPA based method, and yield comparable results. The developed method here will also be useful to

characterize most of peptidases, including dipeptidases and tripeptidases. In addition, this method can also be used for studying kinetic parameters of peptidase under different solution conditions as well as *in vivo*. Using the ESI-MS method, we showed that CN1 was active without the addition of metal ions while CN2 showed very little activity without metal in assay buffer. Further, the mass spectrometry assay, since it is a rapid assay, was used to confirm suggested allosteric effect of citrate on carnosinases and tripeptide cleavage efficiency of CN2. Next, the structure of human CN2 is resolved at 2.25 Å resolution using X-ray crystallography. CN2 was crystallized with a nonhydrolyzable substrate analogue and also strong inhibitor, bestatin. The structure provided insight into substrate binding mode and catalytic architecture of the CN2. In addition to identifying structural determinants of substrate recognition, we also analyzed structures for CN1 and CN2 to understand structural determinants of specificity and promiscuity of CN1 and CN2. We identified that the Ser423 of CN1 and Gly416 of CN2 might play role in dictating specificity versus promiscuity. Further, we also identified Tyr197 of CN2 to be important residue for inhibitor binding. Overall, the structural details throw light on the latent promiscuous and specific behavior of carnosinases. Using human CN2 structure, we performed in silico screening of small molecule inhibitors. A total of 920 molecules were obtained from searching ZINC data base and identified molecules were docked individually to the active site of CN2. Based on the calculated binding energy, top 20 molecules are profiled which are considered to be potential inhibitors of CN2. The docking results also direct us that the hydrophobic moiety at the N-terminus is essential for a potent carnosinase inhibitor.

To conclude, we have systematically performed biochemical and structural approaches to elucidate the regulatory features of carnosinases in substrate binding and catalysis. We have also identified potential residues which can be crucial in determining inherent differences between the two carnosinases. Future studies can target these residues to obtain further mechanistic details on carnosinase action and site directed mutagenesis of these key residues can provide insight into the specificity switch between the carnosinases. Potential inhibitors identified by docking studies can be validated for their efficiency by binding and activity experiments.