Discussion

Chapter ~ 3

Protein glycation is the non-enzymatic reaction between aminogroups of proteins and reducing sugars. Glycationis commonly recognized as the Maillard reaction (Finot, 2005). In this reaction, the carbonylgroup of a sugar interacts with the nucleophilicamino group of the amino acid, producing N-substituted glycosylamine (Schiff base) which is labileand may undergo two sequential rearrangements,yielding a reasonably stable aminoketose — the Amadori product. The reaction proceeds through a schiff's base adduct, followed by an Amadori rearrangement to yield, a stable ketoamine derivative of the protein (Thorpe and Bayne 1982; Bunn 1981).Non-enzymatic glycosylation of proteins by reducing saccharides such as glucose (glc), fructose (fru) and ribose leads to the formation of fructosamine (Day et al., 1979) and advanced glycation end products (AGEs) (Degenhardt et al., 1998). Certainprotein groups are particularly prone to glycation, they include terminal amino groups andlysine side chains (Frister et al., 1988). Arginineside chains can be glycated as well (Tagami et al., 2000).

Non-enzymatic glycosylation of proteins has been reported in vitro for myofibrillar proteins (Syrovy and Hodny 1993). The present study was designed to study the in vitro glycation of BLC by sugars like glucose, fructose and ribose by means of spectral analysis and to reveal the effects on its anti-papain inhibitory activity. The spectral analysis was made by means of intrinsic fluorescence, AGE specific fluorescence, circular dichroism and UV spectroscopy.

To assess the glycation reaction of BLC, the purified inhibitor (2μM) was incubated with 0.5 M glucose, fructose and ribose for different days of incubation and its antiproteolytic potential was determined. Estimation of protein activity provides a sensitive means to monitor the effect of external environment/ligands on the protein as even minute changes in active site region considerably affects the protein function. It was found that incubation of cystatin with glucose, fructose and ribose resulted in loss of anti-papain inhibitory activity (figure 4.9). Ribose was found to be most active causing the complete loss of activity within 14 days of incubation. Functional inactivation of liver cystatin was also observed for glucose and fructose but to a lesser extent. However, increasing the time of incubation with glucose and fructose also resulted in significant loss of inhibitory activity. Among the three sugars investigated ribose was found to be most reactive. The high reactivity of ribose in glycation reaction has been ascribed to the stability of the open chain of the sugar (Bunn, 1981).

Absorption spectral measurements of liver cystatin in the presence of sugars also provided information related to glycation. The alterations in buffalo liver cystatin induced by three sugars (glucose, fructose and ribose) were also observed with respect to conformational change leading to hyperchromicity. Figure 5.0 shows the UV spectra of liver cystatin upon incubation with sugars for 21 days. The UV spectra of all the three sugars (glucose, fructose and ribose) showed marked increase in absorbance at 280nm. However, the magnitude of alteration was maximum in case of ribose followed by fructose and glucose. Figure 5.0 shows the results of spectral analysis in terms of the change in absorbance at 280nm for varying time periods. Increase in absorbance at 280nm is attributed to protein unfolding and exposure of the chromophoric groups. These results have support from literature (Shaklai et al., 1987).

CD spectral analysis of BLC incubated with glucose, fructose and ribose indicates a change in the secondary structure of the protein due to glycation. The figure 5.7, 5.8 and 5.9 shows the far-UV CD spectra of cystatin when incubated with reducing sugars (glucose, fructose and ribose). There is a decrease in the α-helical content of the BLC in case of all the three sugars (glucose, fructose and ribose). Ribose has a more profound effect on the secondary structure followed by fructose and glucose. In case of glucose (Fig 5.7) there is a decrease in the α helix content as the time of incubation increases. After 7 days of incubation with glucose % α-helix as revealed by negative MRE value at 222 nm is 29% compared with 30.89% α-helix of the native BLC protein. There is a further decrease in the % α-helix after 14 days of incubation with glucose (23.85% α-helix) and 19.36% on 21st day of incubation. After 28 days of incubation with glucose there is further decrease in the % α-helix to 17.23% nevertheless α-helix structure is still retained in case of glucose. Fructose (Fig 5.8) also shows a decrease in the α-helix content as the time of incubation increases. On 7th day of incubation with fructose as revealed by negative MRE value at 222 nm, % α-helix is 27.45% compared to 30.89% α-helix of the native BLC protein. There is again a decrease in the % α-helix after 14 days of incubation with fructose to 21.74% α-helix and 17.45% after 21 days of incubation. When buffalo liver cystatin was incubated for 28 days with glucose there is was a decrease in the % α-helix to 16.56%. Ribose (Fig 5.9) also shows a decrease in the α-helix content as the time of incubation increases. On 7th day of incubation with ribose % α-helix as revealed by

negative MRE value at 222 nm is 26.23% compared to 30.89% α-helix of the native BLC protein. There is a further decrease in the % α-helix after 14 days of incubation with ribose (19.22% α-helix) and 15.17% on 21st day of incubation. After 28 days of incubation with glucose there is further decrease in the % α-helix to 11.31%. However secondary structure is retained in all the cases.

Ribose shows high reactivity towards BLC when incubated with ribose compared to fructose and glucose as revealed by the quenching of fluorescence intensities in figure 5.1. There is a major decrease in the fluorescence intensities after incubations for 3, 7, 14, 21 and 28 days for ribose (fig 5.2) followed by fructose and then by glucose. Similarly, there is a decrease in the % α-helix and ellipticity values in case of ribose incubated for 7, 14, 21 and 28 days with BLC and similar effect is seen by in case of fructose and glucose. Figure 6.0 shows the relative decrease in the % ellipticity values for all the three sugars (glucose, fructose and ribose), where ribose induces more change in the % ellipticity values, fructose shows less change followed by glucose. However, after 28 days of incubation glucose and fructose showed same decrease in % ellipicity values revealing similar degree of secondary structure.

A comparison of the results of intrinsic fluorescence with the far UV-CD confirmed that glycated BLC lost its secondary to some degree while retaining quite a bit of secondary structure but it could probably have lost tertiary structure in presence of these three sugars revealed by the change in the extrinsic fluorescence at 435 nm. The presence of less secondary structure (Figure 6.1) after incubation with all the three reducing sugars shows a presence of molten globule state. Most recently, formation of molten globule-like states has been reported during the progression of glycation reactions *in vitro* (Kikuchi et al., 2003). A key parameter for characterizing molten state is incompactness of this state relative to native form of the protein. The presence of the secondary structure and decrease in the fluorescence intensity reveals formation and a tendency for molten globule like state of BLC especially on 21st day of incubation with glucose, fructose and ribose.

Intrinsic fluorescence of proteins provides considerable information about protein structure and dynamics and has been used extensively to study protein folding and association reactions (Lakowicz 2006). In the present study, fluorescence spectroscopic measurements were undertaken to gain insight in the quenching of

fluorescence at 337nm in all the protein-sugar systems studied. Figure shows the results of the data calculated from fluorescence spectra in terms of the fluorescence intensity at 337 nm for varying time intervals. The magnitude of quenching also varied markedly among the three sugars studied, maximum quenching was observed in case of ribose treated liver cystatin followed by fructose. Minimum quenching was seen in glucose protein system. The marked decrease in fluorescence intensity could be attributed to the perturbations around the microenvironment of the aromatic residues. Loss of tryptophan florescence is ascribed to the destruction of tryptophan and/or modification of the tryptophan microenvironment (Shaklai et al., 1987).

Non-enzymatic glycation reversibly produces Amadori products and over a long period irreversible advanced glycation end products. Figure 6.2 shows that the sugar protein system that were incubated for 21 days revealed an increase in Millaird fluorescence (435 nm) upon excitation at 370 nm. The development of Milliard fluorescence was maximum in case of ribose treated cystatin followed by fructose and glucose respectively. Liver cystatin without sugar, which was incubated for the same duration, did not show any fluorescence around 435 nm upon excitation at 370 nm. Figure 6.3 shows the change in the extrinsic fluorescence of liver cystatin upon incubation with sugars (data calculated from fluorescence spectrum figure 6.2) for varying time periods. Increase in mallaird fluorescence has been shown to be a characteristic of the several glycated proteins and is used as a measure of protein glycation. The increase of fluorescence at 435 nm may be due to fluorescence resonance energy transfer from tryptophan of AGE (Chen et al., 2006). The malliard fluorescence is ascribed to the formation of various new flurophores, whose relative contribution may vary broadly depending on the site, extent of glycation and accompanying autooxidation (Dolhofer-Bliesener and Gerbitz 1990).

Intracellularly the higher reactivity of fructose and its subsequent metabolism may contribute to alterations of cellular proteins and dysfunction of cells. In the case of fructose, the rearrangement of the Schiff base is termed the *Heyns rearrangement*. Two separate Heyns products are then formed, which are obviously different in structure from the Amadori adduct. Heyns compounds have been detected in liver extracts and in human ocular lens proteins (McPherson et al., 1988).

Advanced glycation end products (AGE) formation is accompanied by increased free radical activity that contributes to the bimolecular damage in diabetes (Ahmed 2007; Lyons et al., 1995; Wautier and Guillausseau 2001). AGEs act as mediators and can initiate a wide range of abnormal responses in cells and tisues such as the inappropriate expression of growth factors, alterations in growth dynamics, accumulation of extracellular matrix and initiation of cell death (Stitt 2011; Wautier and Guillausseau 2001) through decreased solubility, elasticity and enzymatic affinities in long living proteins such as collagen (Dunn 1989). In vitro and in vivo studies have shown that AGEs result in irreversible cross-links in long living matrix structural proteins such as type IV collagen, laminin and fibronectin (Wautier and Guillausseau 2001). In addition, the concentration of glycated hemoglobin predicts indictes that glycated haemoglobin also predicts cardiovascular disease in patients with type I diabetes (Nathan et al., 2005). Measurements of glycated hemoglobin is thus an essential component in the management of patients with diabetes. In addition, a large body of evidence has been accumulating that the Maillardreaction is not only implicated in diabetic complicationsbut also in the development of age-related diseasessuch as inflammation (Basta et al., 2002), atherosclerosis (Bucciarelli et al., 2002; Baynes et al., 2004), neurodegenerative disorders (Munch et al., 2002), and in dialysis related amyloidosis (Niwa, 2001) and cancer (Stopper et al., 2003).

Thus, glycation or non-enzymatic glycosylation and subsequent Milliard reactions of protein may contribute to the development of the pathophysiology of diabetes through effects on the structure, function and metabolism of protein.The interaction of sugars with liver cystatin was carried out in vitro under conditions that more or less mimic the in vivo reaction reaction with sugars. The formation of advanced glycation end products (AGE's) found in patients with liver cirrhosis has significant importance in diseased states. The sugar induced alterations in the liver cystatins could shed some light on the role of glycation reaction in various liver diseases.