CHAPTER II

“FACTORS INFLUENCING GLYCATION REACTION IN VITRO”
II.1. Introduction

Protein glycation is a nonenzymatic interaction between reducing sugars including D-glucose, ribose or fructose and proteins (Neglia et al., 1983). The first step of reactions is the interaction between ε-amino group of lysine and glucose (Maillard, 1916), giving rise to an unstable Schiff base derivative of the protein which slowly isomerizes to the more stable ketoamine adduct via the Amadori rearrangement (Gottschalk, et al., 1972; Thorpe and Baynes, 1982). Further cyclization processes lead to intermediate species responsible for protein cross-linking reactions. The ultimate step of such reaction pattern leads to the formation of heterogeneous molecules called “Advanced Glycation End Products” (AGEs) (Reynolds, 1965).

As diabetes is characterized by chronic hyperglycemia, the uncontrolled hyperglycemia may lead to the development of vascular complications including retinopathy, nephropathy, and neuropathy (Brownlee et al., 1984). Many studies have suggested that the formation of AGEs (Advanced Glycation End products) as an important factor associated with the etiology of diabetic complications (Thornalley, 2003a; Goldin et al., 2006). Glycation reaction with lysine and arginine amino acid in protein triggers a cascade of events leading to formation AGEs, which results in alteration of protein structure and function (Thornalley et al., 2003b). Glycation has affected biological activity of many proteins including insulin (Hunter et al., 2003), crystallin (Abraham et al., 2008), glucose-6-phosphate dehydrogenase (Ganea and Harding, 1994), aldehyde reductase (Takahashi et al., 1995), glutathione reductase (Blakytny et al., 1992), Cu-Zn superoxide dismutase (Arai et al., 1987; Oda et al., 1994), HDL (Hendrick et al., 2000), both IgM and IgG (Menini et al., 1992; Lapolla et al., 2000a). Protein glycation has been demonstrated successfully both in vitro and in vivo in several model proteins including haemoglobin, Bovine serum albumin (BSA), Human serum albumin (HSA), Immunoglobulin G (IgG), collagen, insulin etc., and the glycation extent of these proteins is known to differ from protein to protein (Lapolla et al., 2006a). The glycation extent of high molecular mass proteins is more in comparison with low molecular mass proteins. Since, glycation is a non-enzymatic reaction, theoretically all proteins should undergo glycation; but several studies
suggest that certain proteins are more prone to undergo glycation than others depending on the surface-accessible reactive site (Quan et al., 1999). For instance, glycoxidative modifications have been shown to be targeted only to a restricted set of proteins of human peripheral blood lymphocytes (Poggioli et al., 2002). Additionally, 3-deoxyglucosone (3-DG) mediated glycation results in the loss of the activity of the specific enzymes associated with glucose metabolism (Kiho et al., 1996). The specificity of glycation reaction was further strengthened by a recent study showing the intermediate filament vimentin as the major target for the AGE modification in primary human fibroblasts (Kueper et al., 2007). Vimentin glycation is neither based on a slow turnover nor an extremely high intracellular expression level, but remarkably based on its structural properties (Kueper et al., 2007). However, protein structure may not be the sole factor in determining the specificity of glycation. As reported earlier, HbA1c (glycated hemoglobin), the marker of long term diabetes is abundant and has a slow turnover (Krishnamurti and Steffes, 2001). Thus, protein abundance and slow turnover may be equally important factors that may influence glycation reaction. This chapter aims to understand the influence of protein molecular mass and protein abundance on glycation reaction in vitro. Glycation studies were performed with proteins varying in molecular masses ranging from 5 kDa to 150 kDa. These proteins include IgG, HSA, BSA, apomyoglobin, papain and insulin. Further, the glycation extent in these proteins was monitored by MALDI-MS analysis.

II.2. Materials and Methods

II.2.1. Chemicals

Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), Apomyoglobin, Papain, Carbonic anhydrase and Glucose were procured from Sigma-Aldrich (St. Louis, USA), Immunoglobulin G (mass standards) from Applied Biosystems (CA, USA), and Insulin (Biocon Ltd. India) from local Pharmacist.

II.2.2. In vitro Glycation Experiments

To determine the influence of protein molecular mass on glycation reaction, in vitro glycation assay was performed. 10 mg/ml HSA (0.15mM), BSA (0.15mM), and papain (0.42mM), 2mg/ml of insulin (0.34mM) and 1mg/ml of apomyoglobin
(0.05mM) and IgG (0.006mM) were used. All protein samples were prepared in double distilled water. The *in vitro* glycation of proteins was performed by incubating equal volume (50μl) of protein with 1M glucose solution dissolved in 0.2M phosphate buffer, at 37°C for either 3, 6 or 10 days.

To study the influence of protein abundance factor on glycation reaction, different concentrations of albumin (0, 2, 20, and 100mg/ml) and insulin (2 mg/ml) were prepared in 0.2M phosphate buffer (pH 7.4). 10 μl of each protein was used for *in vitro* glycation assay. 0.5M of glucose in 0.2M phosphate buffer pH 7.4 was added to various ratios of albumin and insulin (0: 1, 1:1, 10:1, and 50:1) and then incubated at 37°C for 7 days. The final concentration of insulin was kept constant (1 mg/ml), while the albumin concentrations varied to 0, 1, 10 and 50 mg/ml. Similarly, 10 μl of apomyoglobin was mixed with 10 μl of albumin to have a final concentration of 0, 25, 50 and 100 mg/ml of albumin. The *in vitro* glycation assay was performed in 1:1 ratio of glucose to protein.

### II.2.3. Sample Preparation for Mass Spectrometric Analysis

5 μl of the *in vitro* glycation assay mixture was mixed with 45 μl of freshly prepared sinapinic acid (30% acetonitrile, 0.1% trifluoroacetic acid) and loaded onto the stainless steel MALDI plate by dried-droplet method and incubated for 10 min at 37°C.

### II.2.4. MALDI MS Analysis

The mass spectral analysis was performed on Voyager-DE-STR MALDI-TOF MS (Applied Biosystems, California, USA). A 337nm pulsed nitrogen laser was used for desorption and ionization. An Aquiris 2GHz digitizer board was used for all experiments. MALDI analysis of insulin, apomyoglobin, papain, HSA, BSA and IgG were done using different instrumental settings. For insulin analysis, spectra were acquired in the range of 2500 Da to 7500 Da, on a positive reflector mode with the following settings: an accelerating voltage 25kV, grid voltage 68.5% of accelerating voltage, delayed ion extraction time of 350 ns, low mass ion gate was set to 500 Da. For apomyoglobin analysis, spectra were acquired in the range of 1000 Da to 25000 Da, on a positive linear mode with the following settings: an accelerating voltage...
25kV, grid voltage 93.2% of accelerating voltage, delayed ion extraction time of 1100 ns. Low mass ion gate was set to 1000 Da. For HSA, BSA, papain and IgG analysis, spectra were acquired in the mass range of 10 kDa to 200 kDa, on a positive linear mode with the following settings: an accelerating voltage 25kV, grid voltage 92% of accelerating voltage, delayed ion extraction time of 1500 ns, low mass ion gate was set to 4500 Da. For all spectral acquisition, the laser power was set just above the ion generation threshold to obtain peaks with the highest possible signal to noise ratio. All spectra were acquired with 50 shots in three replications. The spectra were processed for advanced base line correction, noise removal and mass calibration.

II.3. Results and Discussion

Glycation occurs at ε-amino group amino acids including lysine or arginine (Zhao et al., 1997). Protein structure and vicinal amino acids to lysine have known to determine the specificity of glycation reaction (Menella et al., 2006; Kueper et al., 2007). However, several other factors also contribute to the specificity of glycation reaction. For example, a study on hemoglobin suggested that site specificity of glycation is attributed to the protein microenvironment (Nacharaju and Acharya, 1992). In addition, the effect of the buffer system and water activity on the specificity and the rate of glycation has been previously studied (Watkins, et al., 1987; Wu et al., 1980). Therefore, to understand the influence of protein molecular mass on glycation extent, several model proteins of varying mass including insulin, apomyoglobin, papain, HSA, BSA and IgG were used for in vitro glycation studies. The mass of control and glycated proteins after performing MALDI-MS analysis has been shown in Fig 2.1. The increase in molecular mass (ΔM) upon glycation was observed in all the six proteins. The extent of glycation for low molecular mass proteins such as insulin, apomyoglobin and papain was lower compared to the high molecular mass proteins such as BSA, HSA and IgG.
Figure 2.1: MALDI-MS analysis of *in vitro* glycated proteins (A) Insulin (B) Apomyoglobin (C) Papain (D) BSA (E) HSA (F) IgG.

The increase in mass was analyzed for different proteins during glycation reaction (Fig 2.2). $\Delta M$ was less for low molecular mass proteins such as insulin and papain on three different days of glycation whereas for high molecular mass proteins including BSA, HSA and IgG, $\Delta M$ increased with increase in time of glycation reaction. A positive correlation between $\Delta M$ and the molecular mass of proteins on three different days of glycation was observed as shown in Fig 2.3.
Figure 2.2: Increase in mass of six proteins on 3rd, 6th and 10th day of glycation reaction monitored by MALDI-MS.

Figure 2.3: Relationship between protein molecular mass and increase in protein mass (A) day 3 (B) day 6 (C) day 10.

High molecular proteins have more number of lysine and arginine residues; therefore it is obvious to observe an increase in the extent of glycation in larger proteins compared to smaller proteins Fig 2.2. In a recent study, it was observed that the HSA, a large molecular mass protein with 59 lysine residues, is heavily glycated in vivo with 31 glycation sites identified from 38 unique glycated peptides (Zhang et al., 2008a). One of the major reasons of HSA being highly glycated protein is by the
virtue of more number of lysine residues (Garlick and Mazer, 1983; Iberg and Flukiger, 1986). Another possible reason for increased glycation of HSA could be the nature of amino acid present near the lysine residue. It was demonstrated earlier that, the vicinal amino acids including isoleucine, leucine, phenylalanine strongly increases lysine reactivity towards the different carbohydrates (Menella et al., 2006). Therefore, as BSA, HSA and IgG are high molecular mass proteins with more number of lysine and arginine residues facilitating the higher extent of glycation than the low molecular mass protein like insulin and apomyoglobin suggesting the protein molecular mass influences the glycation reaction.

Albumin, the high molecular mass protein is also present in abundance and might compete with low abundant proteins for glycation. For understanding the competitive glycation reaction among proteins, in vitro glycation assay of albumin and insulin in a ratio of 0:1, 1:1, 10:1, and 50:1 was performed. The glycated protein peaks showed an increase in mass of 162 Da with MALDI-MS analysis. The relative intensity of a glycated peak (5970 Da) of insulin was maximum at lowest albumin to insulin ratio (0:2) (Fig 2.4A). Further the relative intensity of a glycated peak of insulin decreased with increasing concentration of albumin (Fig 2.4B-C). Albumin inhibited the insulin glycation by getting itself glycated (Fig 2.5A and 2.5B).
Figure 2.4: Influence of albumin concentration on insulin glycation (A) 0 mg/ml albumin, (B) 1 mg/ml albumin, (C) 10 mg/ml albumin, (D) 50 mg/ml albumin. These spectra were acquired on a positive reflector mode by MALDI-MS analysis.

Figure 2.5: MALDI-MS analysis of (A) unglycated albumin (B) glycated albumin.

Insulin was then replaced with apomyoglobin (0.5 mg/ml) in order to see whether albumin inhibits the glycation of other proteins. Apomyoglobin shows unglycated peak at 16952.44 Da and three glycated peaks at 17114.88 Da, 17276.59
Da, 17438.29 Da respectively, suggesting that at least it has three sites of glycation (Fig 2.6). The intensity of the glycated peaks decreased with increasing albumin concentration. The relationship between the albumin concentration and ratio of absolute intensity of glycated to unglycated peaks showed negative co-relation (Fig 2.7). Inhibition of glycation by albumin was more pronounced in second and third glycated peaks of apomyoglobin than the first glycated peak (Fig 2.7), suggesting that albumin also effects the extent of protein glycation.

![Figure 2.6: Effect of albumin concentration on apomyoglobin glycation](image)

Figure 2.6: Effect of albumin concentration on apomyoglobin glycation (A) 0 mg/ml albumin, (B) 5 mg/ml albumin, (C) 25 mg/ml albumin (D) 50 mg/ml albumin. These spectra were acquired on a positive reflector mode by MALDI-MS analysis. Glycated peaks are labeled.
Figure 2.7: Relationship between ratio of absolute intensity value of glycated and unglycated peaks of apomyoglobin and albumin (HSA) concentration. Blue, red and green curve indicates first, second and third glycated peaks respectively.

Competitive inhibition of insulin glycation was also observed by replacing albumin with 2mg/ml of carbonic anhydrase (Fig 2.8A and 2.8B) and papain (Fig 2.8C and 2.8D).

Figure 2.8: Effect of carbonic anhydrase (CA) and papain on insulin glycation (A) 0 mg/ml CA. (B) 2mg/ml CA. (C) 0 mg/ml papain. (D) 2mg/ml papain. Spectra were acquired on a positive reflector mode by MALDI-MS analysis. Glycated peaks are labeled.
At higher concentration, both carbonic anhydrase and papain were degraded and the degraded products were interfering in MALDI analysis of insulin. Therefore, protein turnover is also an important factor in determining glycation. In this regard, our in vitro experiments strongly suggest that albumin may also have a similar role in vivo in protecting glycation of less abundant proteins. Additionally, previous studies have shown role of glycated albumin in the pathogenesis of diabetic complications by increasing gene expression of interleukin (IL-8) and nitric oxide synthase, as well as increased protein expression of alpha-1 (IV) collagen and fibronectin, the predominant constituents of the expanded extracellular matrix seen in diabetes (Cohen, 2003). However, it has been observed that the patients with hypoalbuminemia ascribed to malnutrition are more prone to develop vascular complications in diabetes (Kaysen, 2001) and is a known predictor of vascular morbidity (Suliman et al., 2003). Similar effects are ascertained with hemoglobin, which is the most abundant protein in the blood. Low hemoglobin level in diabetic patients has been associated with an increased risk of micro vascular complications, cardiovascular disease and end stage renal disease (ESRD). Even in mild anemia, Hb (<13.8 g/dL) increases the risk for progression to ESRD and correction of the same improves performance and quality of life in diabetic patients (Thomas, 2007; Mohanram et al., 2004). These studies suggest that hypoalbuminemia and anemia are strongly associated with diabetic complications.

As mentioned earlier, glycation has affected biological functions of several proteins; however, the functions of albumin and hemoglobin are not easily altered due to their high concentration and slow turnover. These studies combined with above results support our hypothesis that high abundant proteins prevent complications by inhibiting glycation of less abundant proteins. At least in the initial stages of diabetes high abundant proteins like albumin and hemoglobin may protect less abundant proteins from the adverse effects of glycation. In the absence of these proteins the effect of glycation might have been more severe. Therefore, in conclusion both the molecular mass of protein as well as protein abundance is important factors that determine glycation reaction in vitro in addition to the protein structure and its turnover.