PYRIDOXAMINE PROTECTS AGAINST GLUCOSE-INDUCED PROTEIN DAMAGE

By

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This thesis is dedicated to my family for their love, support, and encouragement.

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LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End Products
ALEs	Advanced Lipoxidation End Products
BSA	Bovine Serum Albumin
CEL	Carboxyethyllysine
CML	N^{ε}-carboxymethyllysine
DG	Deoxyglucosone
ELISA	Enzyme-Linked Immunosorbent Assay
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
LC-ESI/MS/MS	Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry
GLA	Glycoaldehyde
GO	Glyoxal
GOLD	Glyoxal lysine-lysine dimmer
HAS	Human Serum Albumin
HNE	Hydroxynonenal
HTRP	Hydroxytryptophan
KYN	Kynurenine
LDL	Low density lipoprotein
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry
MDA	Malondialdehyde

MGO	Methylglyoxal
MOLD	Methylglyoxal lysine-lysine dimmer
MS	Mass Spectrometry
3OH-KYN	3-Hydroxykynurenine
PM	Pyridoxamine
RNA	Ribonucleic Acid
RNase	Ribonuclease A
ROS	Reactive oxygen species
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Xcorr	Correlation Score
z	Peptide charge state

CHAPTER I

INTRODUCTION

Statement of Problem

Nonenzymatic glucose modification of proteins occurs by the free aldehyde group of glucose modifying the side-chain of lysine residues at the ε-amino group and the N-terminal α-amino groups on proteins. This reaction, known as the Maillard protein glycation pathway, leads to the reversible formation of a Schiff base followed by a practically irreversible rearrangement to an Amadori intermediate. The Amadori intermediate then undergoes several cycles of condensations and oxidative fragmentations to produce irreversible heterogeneous compounds referred to as advanced glycation end products (AGEs) (Brownlee 1995). One of the most common AGEs found in tissues is N^{ε}-carboxymethyllysine (CML). Another pathway of protein modification by glucose involves the autooxidation of glucose or the Schiff base to form low weight carbonyl compounds such as glyoxal (GO), methylglyoxal (MGO), and glycolaldehyde (GLA), which can modify lysine as well as arginine residues (Voziyan and Hudson 2005).

AGEs have been implicated in the pathogenesis of diseases, such as diabetes, atherosclerosis, neurodegeneration, and in ageing. The elevated levels of glucose found in diabetes can lead to the acceleration of glycation reactions, which have been found to affect both the cardiovascular and renal systems (Brownlee, Cerami et al. 1988; Brownlee, Cerami et al. 1988). In human diabetic kidneys, glycated collagen IV was

found (Raabe, Hopner et al. 1998), and in atherosclerosis, AGEs have been found on low density lipoproteins (Imanaga, Sakata et al. 2000). AGEs have also been reported to play a role in Alzheimer's disease and in ageing (Thorpe and Baynes 1996; Sasaki, Fukatsu et al. 1998).

Although the chemical structures of many AGEs have been determined and new AGEs are continuously being discovered, how glycation reactions affect protein function is not understood. Baynes and colleagues studied the model protein ribonuclease to determine the sites of Amadori and CML formation. In this study, they point out the need for an understanding of how glycation affects protein function. Currently, it is unknown how the sites of glycation and AGE formation are related, nor is it known what effect different protein environments have on the formation of AGEs (Brock, Hinton et al. 2003). Assays do exist to detect the presence of particular AGEs, which can serve as surrogate markers of glycation reactions *in vivo.* However, it is not known if these modifications are benign or if they do in fact affect protein function and are directly involved in pathogenic mechanisms.

Because of the role of AGEs in many diseases, it has been important to find inhibitors to the formation of AGEs. Pyridoxamine (PM) is an *in vitro* inhibitor of the formation of AGEs by blocking the reaction at the Amadori intermediate (Booth, Khalifah et al. 1996). The exact mechanism of how PM functions as a post-Amadori inhibitor is unknown. However, it has been determined that PM scavenges reactive carbonyl products of glucose degradation along with interfering with the catalytic role of redox metal ions in glycoxidative reactions (Voziyan, Metz et al. 2002). *In vivo* PM prevented the development of nephropathy and retinopathy in the streptozotocin rat

model of diabetes (Degenhardt, Alderson et al. 2002; Stitt, Gardiner et al. 2002). Phase II clinical trials of PM in diabetic nephropathy have been completed. The *in vivo* mechanism of action of PM remains unknown, although it is assumed to act as a post-Amadori inhibitor and as a scavenger of reactive aldehydes.

The main objective of this study was to use different model proteins to gain insight into the question of how glucose modifications alter protein functionality and how PM prevents the loss of function in these reactions. The model proteins ribonuclease A (RNase), lysozyme, bovine serum albumin (BSA), and ubiquitin were utilized in these studies. Lysozyme was focused on more intensely to address the following questions: (1) what is the variety of modifications that form; (2) what structural features of the protein environment promote the formation of modifications, i.e. residue exposure, specific motifs; (3) what are the requirements for PM protection against the formation of modifications, i.e. residue exposure, specific motifs. The results of this study provide insight into the damaging effects of glucose modifications on protein function, which includes CML formation on lysine residues and the oxidation of tryptophan residues, along with the degradation and cross-linking of proteins. PM provided protection against CML formation and the oxidation of tryptophan residues in addition to preserving protein integrity and functionality. The results of this study advance the knowledge of glycation reactions and PM protection against protein damage induced by glucose modifications.

Advanced Glycation End Products

Chemistry of AGE Formation

Since AGEs have been implicated in many diseases, the chemistry of how AGEs form has been extensively investigated. AGEs form as a result of the Maillard reaction. This reaction involves the aldehyde group on glucose, or other reducing sugar, modifying the ε-amino side-chain of lysine residues or the N-terminal α-amino groups of polypeptides. This leads to the reversible formation of a Schiff base followed by a practically irreversible rearrangement to a more stable ketoamine known as the Amadori intermediate, or fructosyllysine. The Amadori intermediate then undergoes several cycles of condensations and oxidative fragmentations to produce irreversible heterogeneous compounds referred to as AGEs (Brownlee 1995). Many AGEs, comprised of different chemical structures, have now been detected in animal and human tissues, such as the most prominent AGE N^{ε}-carboxymethyllysine (CML), pentosidine (a lysine-arginine crosslink), and glucosepane (Fig 1) (Ahmed, Thorpe et al. 1986; Hayase, Nagaraj et al. 1989; Sell and Monnier 1989).

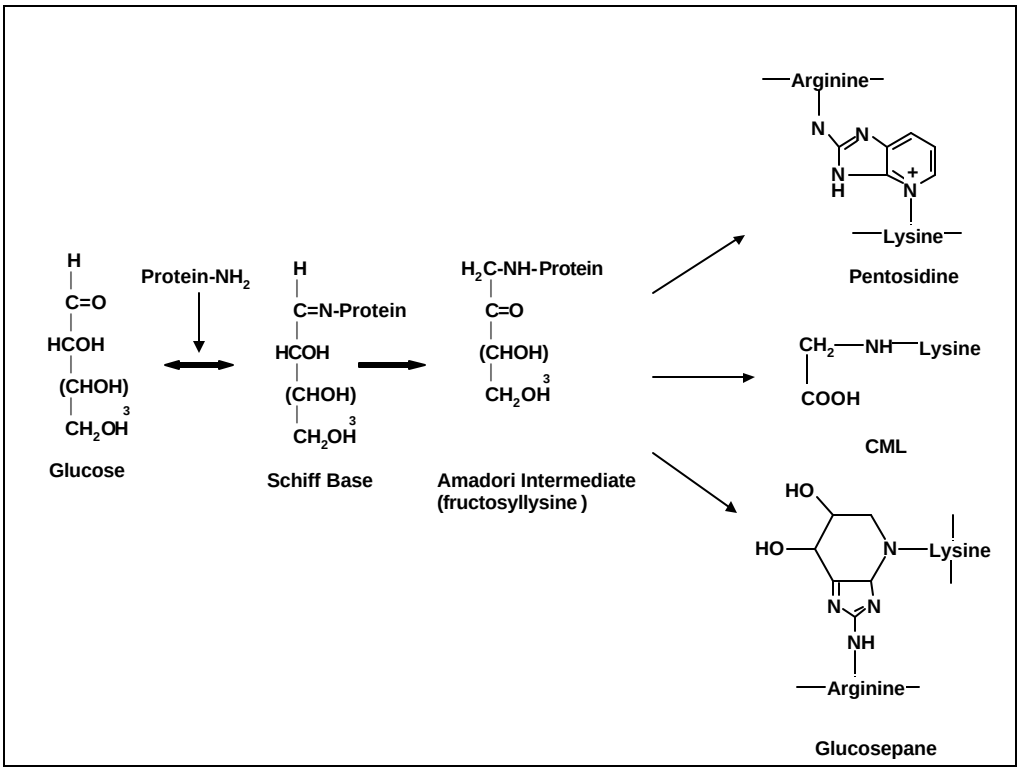

Figure 1. **Maillard Glycation Pathway**.

AGEs can also form from another pathway of protein modification by glucose, which involves the autoxidation of glucose or the Schiff base, particularly in the presence of transition metals, to form low weight carbonyl compounds such as glyoxal (GO), methylglyoxal (MGO), and glycolaldehyde (GLA). These reactive carbonyl compounds can modify both α- and ε-amino groups as well as the guanidinium group of arginine. This is unlike glucose, which reacts mainly with amino groups (Voziyan and Hudson 2005). These carbonyl compounds have been found to cross-link proteins via MOLD (methylglyoxal lysine-lysine dimer) and GOLD (glyoxal lysine-lysine dimer) (Ahmed, Brinkmann Frye et al. 1997; Degenhardt, Thorpe et al. 1998; Odani, Shinzato et al. 1998). The carbonyl compounds also modifiy arginine residues forming products such as hydroimidazolone (Thornalley 2005).

A variety of AGEs have been identified (Fig 2), and as mentioned above, they arise as result of different pathways. For instance, the Amadori intermediate can undergo non-oxidative rearrangement and hydrolysis reactions to form products such as 1-deoxyglucosones and 3-deoxyglucosones (1DG, 3DG), which both contain the carbon skeleton of glucose (Thorpe and Baynes 2003). The AGEs CML, N^{ε}-carboxyethyllysine, and pentosidine require oxidative fragmentation of the carbon skelton of glucose, while the formation of pyrraline is a non-oxidative process. Other AGEs like verperlysines and crosslines are fluorescent and appear to derive directly from glucose, maintaining the intact carbon skelton of glucose (Thorpe and Baynes 2003).

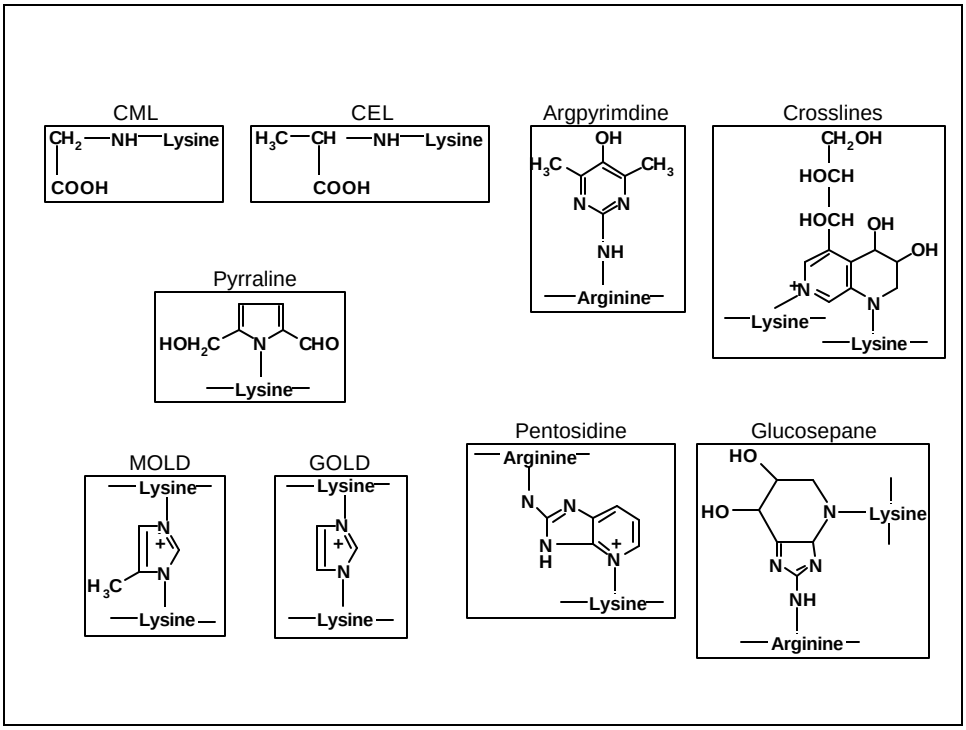

Figure 2. **AGE Structures.** The structures of some of the known AGEs are seen above.

While AGEs form from carbohydrate precursors such as glucose, advanced lipoxidation end products (ALEs) form from the modification of proteins by polyunsaturated fatty acids in lipoproteins. Malondialdehyde (MDA) and hydroxynonenal (HNE) form adducts on lysine (MDA-Lys, HNE-Lys) (Onorato, Jenkins et al. 2000). The products CML and CEL may arise from both carbohydrate and lipid modifications of proteins (Metz, Alderson et al. 2003).

Reactive oxygen species (ROS) can be generated from the formation of both AGEs and ALEs. The autoxidation of glucose or the oxidative degradation of the Amadori intermediate can lead to the formation of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical. Lipid peroxidation produces the ROS peroxyl and alkoxyl radical. ROS cause damage to amino acid side chains in proteins, such as the oxidation of cysteine to sulfonate, the conversion of methionine to sulphoxide, and the hydroxylation of aromatic amino acids. Oxidation of tryptophan residues occurs producing products such as hydroxytryptophans (HTRP), kynurenine (KYN), and 3-hydroxykynurenine (3OH-KYN) (Finley, Dillon et al. 1998). ROS also play a role in diseases like diabetes and atherosclerosis by triggering pathogenic signaling (Li and Shah 2003). Thus, the three major pathways of protein damage by glycation reactions are the (1) Amadori pathway, (2) reactive carbonyl species pathway, and (3) reactive oxygen species pathway (Fig 3).

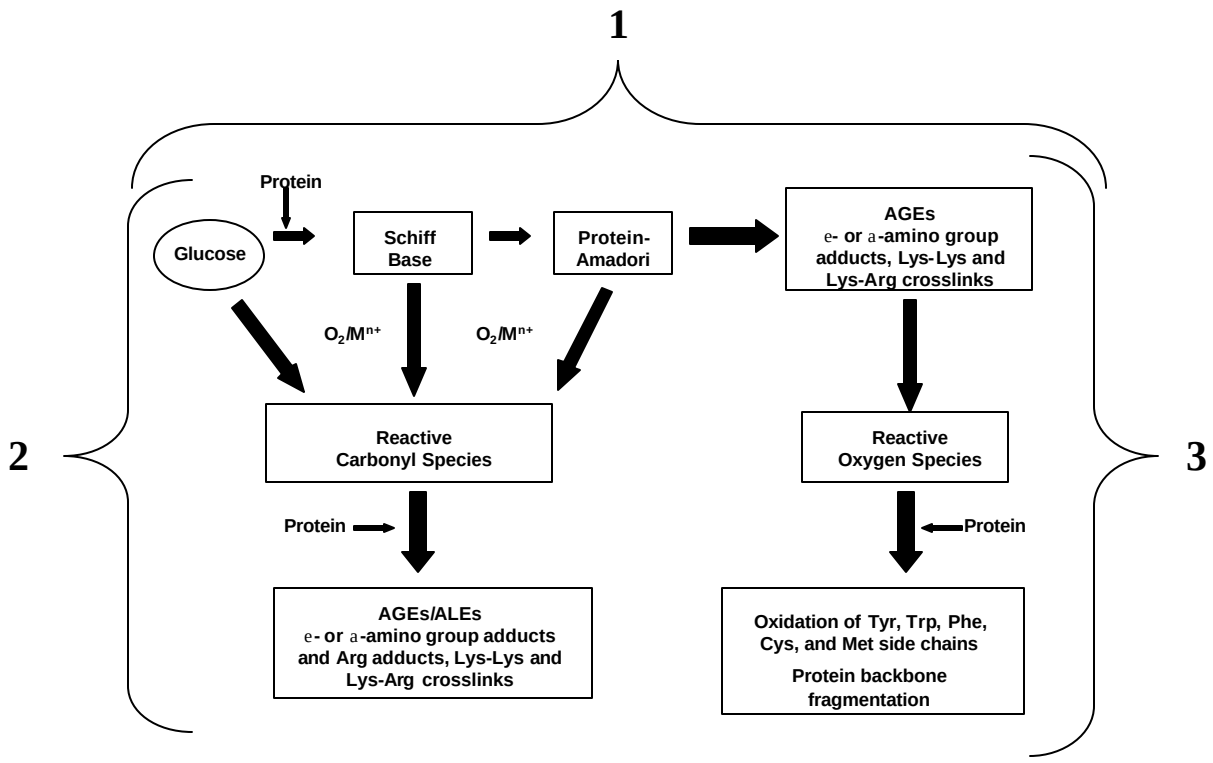

Figure 3. **Pathways of Protein Damage by Glycation Reactions**. The following pathways are represented above: (1) the Amadori pathway, (2) the reactive carbonyl species pathway, (3) the reactive oxygen species pathway.

Physiological Complications of AGEs

AGEs have been implicated in many diseases, such as diabetes, atherosclerosis, neurodegeneration, and in ageing. Diabetes is characterized by high levels of glucose, which leads to many physiological complications such as diabetic nephropathy. High glucose levels lead to the formation of AGEs, which alter the structure and function of proteins like the extracellular matrix proteins. The non-enzymatic glycation of proteins is a slow process and has been found to occur on long-lived proteins such as collagen IV, which is a major component of the renal extracellular matrix. This results in diminished glomerular filtration rates, which is evident in the high levels of AGEs found in both plasma and tissues (Raabe, Molsen et al. 1996; Heidland, Sebekova et al. 2001).

Other complications associated with diabetes are eye diseases such as retinopathy. AGEs have been found in retinal vessels in diabetics and are associated with the microvascular dysfunction in the retina (Stitt, Gardiner et al. 2002). AGEs are also involved in altering the cornea in diabetic patients. There is an increased risk for the development of cataracts in diabetics, which is caused by protein modifications in the lens of the eyes leading to loss of vision (Jain, Lim et al. 2002).

Accelerated atherosclerosis is another major complication associated with diabetes. Elevated levels of AGEs have been found in diabetic patients with coronary heart disease, and immunohistochemical analyses have detected AGEs in atherosclerotic lesions. AGEs have been found on low density lipoproteins (LDL) and contribute to the altered clearance of LDL from the body. Studies have also shown that glycated LDL can be taken up by macrophages leading to foam cell formation, which is characteristic of atherosclerotic lesions (Basta, Schmidt et al. 2004; Brown, Dean et al. 2005).

AGEs have also been reported to play a role in neurodegenerative diseases such as Alzheimer's disease. Alzheimer's is characterized by the presence of neurofibrillary tangles and plaques made of tau proteins and amyloid β proteins. Immunohistochemical analyses have identified AGEs in the neurofibrillary tangles and plaques from Alzheimer's patients. In fact, glycation of amyloid β proteins increases aggregation *in vitro*. The results of these studies suggest that AGEs may contribute to the neuronal dysfunction found in Alzheimer's disease (Sasaki, Fukatsu et al. 1998).

The process of ageing is not well understood, but AGEs may contribute to ageing. AGEs accumulate on long-lived proteins and the formation of AGEs produces reactive oxygen species. These two factors are linked to ageing. The levels of AGE biomarkers,

such as CML and pentosidine, increase with age. With age, there is also an increased risk of cataracts due to protein modifications in the lens. Age-dependent stiffening of the tendons occurs due to collagen modifications (Baynes 2000).

Pyridoxamine

Because of the role of AGEs in many diseases, it has been important to find inhibitors to the formation of AGEs. In a search for inhibitors of AGEs, the Hudson laboratory discovered that pyridoxamine (Fig 4), a natural intermediate of vitamin B_6 metabolism, is an *in vitro* inhibitor of the conversion of the Amadori intermediate to AGEs, such as CML (Booth, Khalifah et al. 1997; Khalifah, Baynes et al. 1999). *In vivo*, pyridoxamine prevented the development of renal disease, inhibiting albuminuria and creatinemia in the streptozotocin-diabetic rat (Degenhardt, Alderson et al. 2002). Pyridoxamine was also found to inhibit dyslipidemia in the streptozotocin-diabetic rat. Decreases in CML and CEL were observed along with decreased cross-linking of skin collagen (Metz, Alderson et al. 2003). PM is currently in on the FDA "fast track" for Phase III clinical trials for the treatment of diabetic nephropathy. Other preclinical data suggest that PM may also be a candidate for the treatment of diabetic retinopathy, hyperlipidemia, and kidney stone disease in addition to diabetic nephropathy (Voziyan and Hudson 2005). Therefore, PM has been established to prevent some of the physiological complications of AGEs. However, the exact mechanism of how PM functions is not entirely understood.

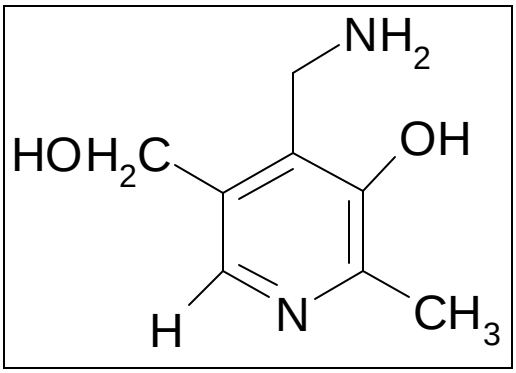

Figure 4. **Structure of Pyridoxamine.**

Studies have been conducted in order to better understand the mechanism of PM. As mentioned previously, reactive low molecular weight carbonyl products can form from glucose autoxidation and through the degradation of the Schiff base intermediate. They can also be produced from lipid peroxidation reactions. Experiments were carried out to determine if PM can react carbonyl compounds such as glyoxal and glycoaldehyde. The results showed that PM protects proteins from carbonyl stress by trapping these carbonyl compounds thus preventing the formation of AGEs (Voziyan, Metz et al. 2002).

Further studies revealed that PM prevents the Amadori intermediate from converting to AGEs by interfering with the catalytic role of redox metals. PM can form complexes with transition metals with particular preferences for Cu^{2+} and Fe^{3+}. The aminomethyl and phenol moieties on PM bind to the metal, so PM structural analogs lacking these moieties were studied to determine how they compared to PM in the inhibition of CML formation. The structural analogs showed no inhibition, while an analog that possessed both required moities did show inhibition. Additional studies have demonstrated that PM does not form adducts with the Amadori intermediate. Therefore,

the results of these studies show that PM inhibits CML formation by interfering with the metal ions involved in the glycoxidative reaction (Voziyan, Khalifah et al. 2003).

PM also shows some effectiveness against reactive oxygen species. By inhibiting the post-Amadori reactions from occurring, PM is also inhibiting the ROS that are generated from those reactions. Studies have found that PM repressed the accumulation of the hydroxyl radical, $^{\bullet}OH$, in the Fenton reaction (Voziyan and Hudson 2005) and the superoxide radical, $O_2^{\bullet-}$, in red blood cells treated with glucose (Jain and Lim 2001). Malondialdehyde levels, a marker for free radical lipid peroxidation, were reduced in streptozotocin-diabetic hamsters treated with PM (Takatori, Ishii et al. 2004). The results of these studies suggest that PM is protecting against ROS. Therefore, the mechanism of action of PM includes the following: (1) inhibiting the Maillard reaction at the Amadori intermediate, (2) scavenging of reactive carbonyl compounds, and (3) trapping reactive oxygen species (Fig 5). Though these studies do provide important insight into the mechanism of PM, there still remain unanswered questions, in particularly involving AGE formation in diverse protein microenvironments.

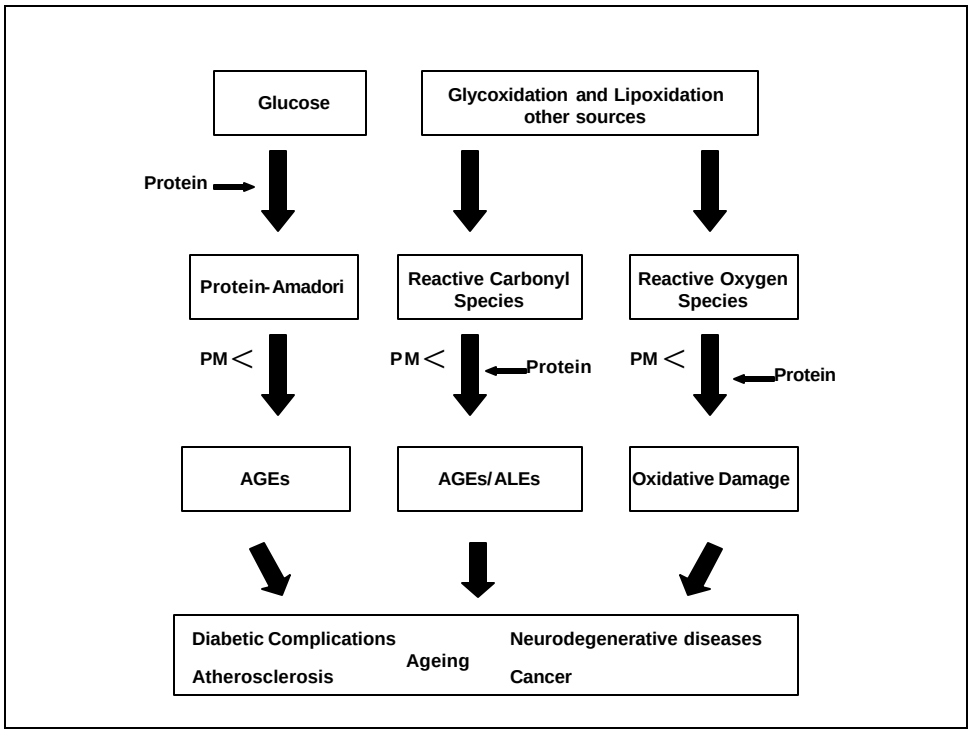

Figure 5. **PM Inhibition of AGE Formation**. The following pathways are represented above: the Amadori pathway, the reactive carbonyl species pathway, the reactive oxygen species pathway. PM inhibition of the reactions has the potential to prevent or delay the development of diseases that arise from these pathways.

Model Protein Systems

The main objective of this study was to use different model proteins to gain insight into the question of how glucose modifications alter protein functionality and how PM prevents the loss of function in these reactions. The model proteins ribonuclease A (RNase), lysozyme, bovine serum albumin (BSA), and ubiquitin were utilized in these studies.

While studies have been conducted on the mechanisms of glycation reactions, there still remain many unanswered questions. For instance, it is unknown what protein modifications arise as a result of lysine microenvironments. Studies were carried out with RNase to determine how protein environment affects glycation, but the focus was

only on the Amadori and CML modifications (Brock, Hinton et al. 2003). As mentioned earlier, there are many possible AGEs; therefore, there exists a need to more thoroughly study how the microenvironments of lysine residues affect the formation of modifications. This particular study will address these questions by using a set of proteins with a particular focus on lysozyme.

In addition to determining what varieties of modifications are formed during glycation reactions, the factors that influence the formation of the specific modifications also need to be determined. For instance, residue exposure, neighboring residues, and structural characteristics, such as the presence of certain motifs, may influence the type of modification that forms. Past studies have been conducted on peptides to determine how certain neighboring residues affect the reactivity of amino groups to glycation. Results indicate that hydrophobic and charged residues located near lysine residues increase the reactivity of those lysines to glycation (Mennella, Visciano et al. 2005). While these results are true for peptides, it is unknown how the microenvironment of lysine residues within an intact protein affects the reactivity of those residues to glycation. This question is addressed in this study by characterizing the glycation of model proteins.

As mentioned previously, studies have revealed that PM works by trapping reactive carbonyl compounds, interfering with the catalytic role of redox metals, and reacting with ROS. However, there is still much to be learned about the mechanism of PM in specific protein environments. For instance, certain structural features may be required for PM inhibition. The experiments conducted in this study address how different protein environments affect PM protection of AGE formation.

Since AGEs have been implicated in many diseases, it is important to study glycation reactions under physiologically relevant conditions. Many past studies have been conducted using extremely high concentrations of glucose. While these studies have provided information about glucose modifications, more relevant insight can be gained by investigating the modifications that arise as a result of physiological concentrations of glucose, which has been the focus of this particular study.

RNase

The ribonuclease A protein used in these experiments comes from bovine pancreas. It functions to catalyze the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent nucleotide. The molecular weight of RNase is 13,700 Daltons, and it contains ten lysine residues, K-1, 7, 31, 37, 41, 61, 66, 91, 98, 104. Residues located in the active site of RNase are His-12 and His-119 (Worthington Biochemical).

Baynes and co-workers have used RNase A as a model protein system in the study of glycation. Recent work has utilized ESI-LC-MS to study the specific sites of glycation on RNase in an effort to learn more about how glycation affects proteins. RNase samples were incubated with 0.4 M glucose at 37°C for 3, 7, and 14 days, and then digested with trypsin. ESI-LC-MS analysis revealed that Lys-1, 7, 37, and 41 were the main sites of Amadori formation and Lys-7, 37, and 41 were the main sites of CML formation. The work also revealed that Amadori is the main precursor of CML formation (Brock, Hinton et al. 2003). We will utilize these results, along with our data, to analyze how PM affects the glycation process in RNase. Since the three dimensional structure of

RNase has been solved, structural requirements needed for PM action can be assessed (Carlisle, Palmer et al. 1974).

Lysoyzme

The lysozyme protein used in these experiments comes from hen egg whites and is known as lysozyme "c". Lysozyme functions to hydrolyze the β-1, 4 glucosidic linkages between N-acetylmuramic acid and N-acetylglucosamine, which occur in microorganisms such as *Micrococcus lysodeikticus*. The molecular weight of lysozyme is 14,388 Daltons, and it contains six lysine residues, K-1, 13, 33, 96, 97, 116. Residues located in the active site of lysozyme are Asp-52, Asp-101, and Glu-35 (Worthington Biochemical).

The glycation of lysozyme has been examined in a few previous studies. Kislinger and colleagues studied the glycation of lysozyme by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). The samples contained lysozyme along with high levels of glucose (100, 250, 500 mM) and were incubated at nonphysiological temperatures (50°C) for 1, 4, 8, and 16 weeks. Samples were digested with endoproteinase Glu-C followed by MALDI-TOF mass spectrometry analysis. The results of the study revealed two peaks in the spectra of samples incubated with glucose that were consistent with the formation of an Amadori product and CML (Kislinger, Humeny et al. 2003). Another study involved studying the glycation of lysozyme by ESI-mass spectrometry. Lysozyme samples were incubated with glucose in a 1:1 molar ratio of ε-amino group:sugar carbonyl groups at 50°C for 1, 2, 5, 10, and 14 days. Samples were subjected to a tryptic digestion. The results of this particular study

found that all six lysine residues were involved in glycation dependent upon incubation time. The order of reactivity of lysine residues is as follows: Lys-97 > -33 > -1 > -13 > -116 > -96 (Yeboah, Alli et al. 2004). Further studies need to be conducted in order to determine how the protein microenvironment affects the formation of AGEs on lysozyme. These issues will be addressed in this study. Also, by utilizing PM in these glycation reactions, more knowledge can be gained about how PM affects the glycation process. Again, the three dimensional structure of lysozyme has also been solved, so structural requirements needed for PM action can be assessed (Blake, Koenig et al. 1965).

BSA

Another model protein used in our experiments is bovine serum albumin. The molecular weight of BSA is 66,430 Daltons, and it contains fifty-nine lysine residues (Sigma). BSA functions to transport fatty acids that are insoluble in circulating plasma. BSA has also been studied in glycation reactions. Lapolla and colleagues set up glycation experiments which involved incubating BSA with 0.5 M glucose at 37°C for 0, 15, 39, 60, and 90 days. The samples were subjected to digestion with proteinase K followed by both MALDI-MS and HPLC/ESI-MS in order to identify AGE peptides. It was determined that a complex set of AGE peptides are generated that have molecular masses in the range of 300-3500 Daltons (Lapolla, Fedele et al. 2001). Baynes and colleagues also studied BSA in glycation reactions. From those studies, it was concluded that a dominant AGE that forms is CML (Reddy, Bichler et al. 1995). While the three dimensional structure of BSA has not yet been solved, the structure of human serum albumin (HSA) has been determined, and BSA shares 76% sequence homology with

HAS (Huang, Kim et al. 2004). Again, further knowledge about the mechanism of PM and the structural requirements necessary for the inhibition of AGEs can be gained by studying BSA glycation.

Ubiquitin

The human ubiquitin used in these experiments has a molecular weight of 8,565 Daltons, and it contains seven lysine residues, K-6, 11, 27, 29, 33, 48, 63 (Sigma). Ubiquitin, a highly conserved protein, functions in the degradation of many proteins in eukaryotic cells. Proteins are targeted for degradation through the covalent binding of the selected proteins and ubiquitin at lysine residues (Ciechanover 1998). Past studies have used ubiquitin as a model protein to study the antiglycation effects of polyamines spermine and spermidine. In these studies, ubiquitin was incubated with methylglyoxal for 96 hours at 37°C. These studies revealed that structural changes do occur to ubiquitin over time. Further studies need to be carried out to examine what effect glucose modifications have on ubiquitin and how the microenvironment of ubiquitin affects the formation of AGEs. Other past studies have not addressed these issues, but the present study will address these questions along with using PM to study its influence on glycation in ubiquitin. Also, the three dimensional structure of ubiquitin has been determined, which will aide in the these studies (Vijay-Kumar, Bugg et al. 1987).

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials and Reagents

The following chemicals were purchased from Fisher Scientific: sodium carbonate, sodium phosphate monobasic, Tween 20.

The following chemicals were purchased from Sigma: sodium azide, casein, D(+)glucose, pyridoxamine dihydrochloride, anti-rabbit-IgG alkaline phosphatase conjugate, diethanolamine, bovine serum albumin (fatty acid free; low endotoxin), phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium.

Non-fat dry milk was purchased from Bio-Rad.

Ribonuclease A from bovine pancrease and lysozyme from hen egg whites were purchased from Worthington Biochemicals.

Human ubiquitin was a gift from Dr. Anthony Serianni (University of Notre Dame).

The following chemicals were purchased from Boston Biochem: ubiquitin-protein conjugation kit, ubiquitin aldehyde, anti-ubiquitin.

Methods

In Vitro Glycation of Proteins

Modification of RNase, lysozyme, BSA, and ubiquitin by glucose was carried out in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide to prevent bacterial growth. RNase, lysozyme, and BSA samples contained 8 mg/mL of protein, while ubiquitin samples contained 1 mg/mL of protein. The samples also contained various amounts of D-glucose (5, 30, or 100 mM) in the presence or absence of pyridoxamine (5 or 20 mM). Solutions were incubated in the dark at 37°C for forty days. Aliquots were removed at 0, 10, 20, and 40 days and stored at -20°C until analysis.

Determination of Enzymatic Activity

Ribonulclease A. The enzymatic activity of RNase was determined according to a previously published method (Kalnitsky, Hummel et al. 1959; Voziyan, Metz et al. 2002). Samples containing RNase were diluted to 3 μg/mL in 0.1 M sodium acetate, pH 5.0, and allowed to equilibrate at 37°C in a water bath for 5 minutes. 100 μL of the diluted RNase samples were mixed with 100 μL of 1% yeast RNA in the same buffer. After incubation at 37°C for 5 minutes, the reaction was stopped by the addition of 100 μL of an ice-cold solution of 0.8% lanthanum nitrate in 18% perchloric acid. The samples were then incubated on ice for 10 minutes to allow for complete precipitation of undigested RNA followed by centrifugation at 12,000 x *g* for 10 minutes. An aliquot of the supernatant (20 μL) was removed and diluted to 1 mL with distilled water. The

amount of digested RNA was determined by measuring the absorbance at 260nm. The relative activity was calculated by taking the zero day time point to be at 100% activity.

Lysozyme. The enzymatic activity of lysozyme was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* cells according to Shugar (Shugar 1952). Samples containing lysozyme were diluted to 17 μg/mL in distilled water and kept on ice. A solution of 0.3 mg/mL *Micrococcus lysodeikticus* cells in 0.1 M potassium phosphate buffer, pH 7.0, was made just prior to the assay. Then 100 μL of diluted lysozyme was mixed with 900 μL of *Micrococcus lysodeikticus* cells. The rate of lysis of the *Micrococcus lysodeikticus* cells was determined by measuring the change in absorbance at 450nm, which was measured every 15 seconds for two minutes (ΔA_{450}). The relative activity was calculated by taking the zero day time point to be at 100% activity.

Ubiquitin. Ubiquitin activity was determined using the assay kit from Boston Biochem according to the manufacturer's protocol. The following components were part of the reaction mixture: 3.3 μL energy solution, 13.3 μL (10 μg) conjugation fraction A, 13.3 μL (10 μg) conjugation fraction B, 44.0 μL (44 μg) ubiquitin from sample incubations, 3.4 μL (1.7 μg) ubiquitin aldehyde, and 1.6 μL sodium phosphate buffer. The reaction mixture was pre-incubated for 5 minutes at 37°C to allow for inhibition of ubiquitin C-terminal hydrolases followed by the addition 1.0 μL (100 ng) of the substrate (lysozyme) to initiate the reaction. The reaction mixture was incubated for 3 hours at 37°C. The reactions were stopped by adding concentrated (6X) SDS-PAGE sample buffer followed by SDS-PAGE on a 4-20% gradient gel. Samples were electrophoresed at a voltage of ~140V at room temperature until the bromophenol blue dye had reached the lower edge of the gel. For Western blot analysis, the samples were transferred to

Immobilon-P membrane at 80V at 4°C for 1 hour. The membrane was blocked in 5% non-fat dry milk in TBS for 0.5 hours at room temperature followed by incubation with anti-ubiquitin rabbit polyclonal antibody (1:1000 dilution) at room temperature overnight. The membrane was then washed 5 times for 15 minutes followed by incubation with an alkaline phosphatase-conjugated secondary antibody, goat anti-rabbit IgG (1:1000 dilution) for 1.5 hours at room temperature. The membrane was washed again and developed with alkaline phosphatase substrate solution until the desired intensity was achieved. The reaction was stopped by rinsing with water.

Determination of CML Formation by ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect AGE, N^{ε}-carboxymethyllysine (CML), formation on the model proteins studied. Antibodies R618 (Booth, Khalifah et al. 1996) raised against glycated RNase were used to detect CML modification on glycated lysozyme, BSA, and ubiquitin. Antibodies R1549 raised against glycated BSA were used to detect CML modification on glycated RNase. The following procedure was used for both antibodies. Glycated lysozyme, BSA, and ubiquitin were diluted to 1 ng/μL (for R618) or glycated RNase was diluted to 5 μg/mL in 0.05 M sodium carbonate buffer, pH 9.5-9.7. Diluted proteins (200 μL) were coated on a 96-well polystyrene plate overnight at room temperature. The wells were washed with a 0.15M NaCl and 0.05% Tween-20 solution. The wells were then blocked with 200 μL of 2% casein in sodium carbonate buffer for one hour at 37°C followed by extensive washing. Antibodies R618 (1:350 dilution) or antibodies R1549 (1:500 dilution) were added to the wells and incubated for one hour at 37°C followed by

washing. An alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:2,000 dilution) was added to the wells and the incubation was carried out for one hour at 37°C. After the extensive washing, the *p*-nitrophenylphosphate substrate in 1.0 M diethanolamine solution (200 µL/well) was added to the plates and the absorbance of the released *p*-nitrophenolate was detected at 405nm using a Spectra Max 190 microplate reader from Molecular Devices.

SDS-PAGE Analysis of Glycated Samples

Integrity and crosslinking of the glycated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were diluted in 2X sample buffer and incubated for 5 minutes in boiling water. The samples and standard molecular weight marker were then loaded onto a 4-20% gradient Tris-glycine SDS-PAGE gel. Samples were electrophoresed at ~140V at room temperature until the bromophenol blue dye had reached the lower edge of the gel. The proteins were visualized using Coomassie Brilliant Blue staining.

Mass Spectrometry Analysis of Gycated Samples

Lysozyme samples that had been incubated for 40 days at 37°C alone, with 100 mM glucose, or 100 mM glucose in the presence of 5 mM PM or 20 mM PM were selected for analysis by mass spectrometry. The samples were analyzed by the Proteomics Laboratory, which is part of the Mass Spectrometry Research Center at Vanderbilt University. The samples were prepared using the spin filter protocol for proteomic analysis (Manza, Stamer et al. 2005). The sample proteins were digested with

chymotrypsin. HPLC-MS (high performance liquid chromatography-mass spectrometry) analysis of the resulting peptides was performed using a ThermoFinnigan LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 1.4 instrument control. The peptides were separated on a packed capillary tip, 100 µm x 11 cm, with C_{18} resin (Jupiter C_{18}, 5 micron, 300 angstrom, Phenomonex, Torrance, CA) using an inline solid phase extraction column that was 100µm x 6cm and packed with the same C18 resin (using a frit generated with from liquid silicate Kasil 1). The flow from the HPLC pump was split prior to the injection valve. The flow rate during the solid phase extraction phase of the gradient was 1 µL/min, and during the separation phase it was 700 nL/min. Mobile phase A was 0.1% formic acid, while mobile phase B was acetonitrile with 0.1% formic acid. A 95 minute gradient was performed with a 15 minute washing period (100 % A for the first 10 minutes followed by a gradient to 98% A at 15 minutes) to allow for solid phase extraction and removal of any residual salts. After the initial washing period, a 60 minute gradient was performed in which the first 35 minutes was a slow, linear gradient from 98% A to 75 % A, followed by a faster gradient to 10 % A at 65 minutes and an isocratic phase at 10 % A at 75 minutes. MS/MS spectra of the peptides were obtained using data-dependent scanning, which consisted of one full MS spectrum (mass range of 400-2000 atomic mass units) followed by three MS/MS spectra.

Analysis of Mass Spectrometry Data

Proteomic analysis of the data generated from the HPLC-MS experiments was performed using Sequest (developed by John Yates and Jimmy Eng), which is an algorithm or program that identifies proteins by matching MS-MS data to database sequences (Liebler 2002). Essentially, Sequest works by recording the MS-MS scan from the instrument along with the m/z value of the precursor ion and the scan data. Parameters can then be defined to search for specificities relevant to the experiment. In this case, peptides were matched based on the chymotryptic digestion of the known lysozyme sequence. Searches were also performed for specific modifications, such as CML and HTRP. Then theoretical MS-MS spectra were generated from the specified peptides and compared with the actual MS-MS spectrum obtained from the instrument. A correlation score (Xcorr) was calculated based on the match between the actual and theoretical spectra. This value is determined by how well the b- and y-ions from the actual spectrum match the theoretical spectrum. Then a list of peptides was generated with all the corresponding information, such as the correlation score, charge state of the peptide (z), scan number, and MS-MS spectra. After confirming the identity of the peptide, the peak containing the peptide of interest was identified in the chromatogram and the area of the peak was determined. The program Xcalibur was utilized to view the HPLC chromatograms and MS spectra. The area of the peak containing the modified peptide was then normalized to a reference peptide, which did not contain any modifications and was present in all the samples.

CHAPTER III

RESULTS

Damage By Glucose Modification to Structure and Activity of Proteins and Effect of PM

RNase

*CML Formation***.** Previous studies have shown that CML is a dominant AGE that forms in glycation reactions, and PM has been found to inhibit glycation reactions at the Amadori intermediate. Therefore, ELISA experiments were conducted in order to determine the kinetics of CML formation on lysine residues of RNase at physiologically normal (5 mM) and diabetic (30 mM) glucose levels. No modification was observed at the 0 day time point, but CML formation did increase with time in samples treated with glucose, but not in control samples that were incubated without glucose. Only a small amount of CML is detected in samples treated with 30 mM glucose, while no CML formation is seen in samples treated with 5 mM glucose (Fig 6*A*). The presence of PM prevents CML formation in samples incubated with 30mM glucose. Supraphysiological glucose levels (100 mM) were also employed in order to obtain more robust signals. The biological process of glucose modification is a relatively slow process, so the use of supraphysiological levels of glucose allows for a faster reaction. Compared to the RNase samples incubated with 30 mM glucose, the samples that were incubated with 100 mM glucose showed a much more dramatic increase in CML formation over time (Fig 6*B*). Again, PM did prevent CML formation in samples treated with 100 mM glucose. Thus,

RNase treated with glucose showed a time and concentration dependent increase in CML formation, whereas RNase treated with glucose in the presence of PM resulted in no detectable CML formation.

Degradation and Cross-Linking of RNase. SDS-PAGE was carried out to determine what effects glycation had on the degradation and cross-linking of RNase. RNase incubated with 5 mM glucose resulted in a very small decrease in band intensity over the incubation time period (Fig 7*A*) as determined by gel automated digitizing software. The decrease in band intensity was more pronounced in samples treated with 30 mM glucose both in the presence and absence of 5 mM PM (Fig 7*B*), especially at 40 days in samples with and without PM. RNase samples treated with 100 mM glucose show an even more significant decrease in band intensity compared to the 30 mM samples (Fig 7*C*). PM also appears to protect this decrease in band intensity to some extent especially at 40 days (100 mM). The decrease in band intensity can be attributed to protein degradation as a result of glycation. Protein degradation is also apparent by the low molecular weight band observed under the main band (Fig 7*B* and *C*). No intermolecular cross-links were detected in any of the samples. Therefore, it appears that the degradation of RNase occurs in a glucose concentration dependent manner and that PM exhibits some degree of protection against degradation.

Enzymatic Activity. In order to determine if glucose modifications affect the function of RNase and what affect PM has on function, enzymatic activity assays were carried out. The control (RNase incubated alone) remains at ~90% activity over the course of the incubation time period. Samples containing RNase incubated with 5 mM glucose, 30 mM glucose, and 30 mM glucose in the presence of 5 mM PM showed a

gradual decline in enzymatic activity, with ~40% activity remaining on day 40 (Fig 8*A*). Since RNase incubated with 30 mM glucose in the presence and absence of 5 mM PM declined in activity levels at the same rate, PM had no effect on the protection of enzymatic function. Similar results were also found for samples treated with 100 mM glucose. RNase incubated with 100 mM glucose or 100 mM glucose in the presence of 5 mM PM showed a decline in activity levels, with ~30% activity remaining on day 40 (Fig 8*B*). Again, PM had no effect on enzymatic activity levels. Thus, RNase treated with glucose showed a time and concentration dependent decrease in enzymatic activity, and PM had no effect on the activity levels.

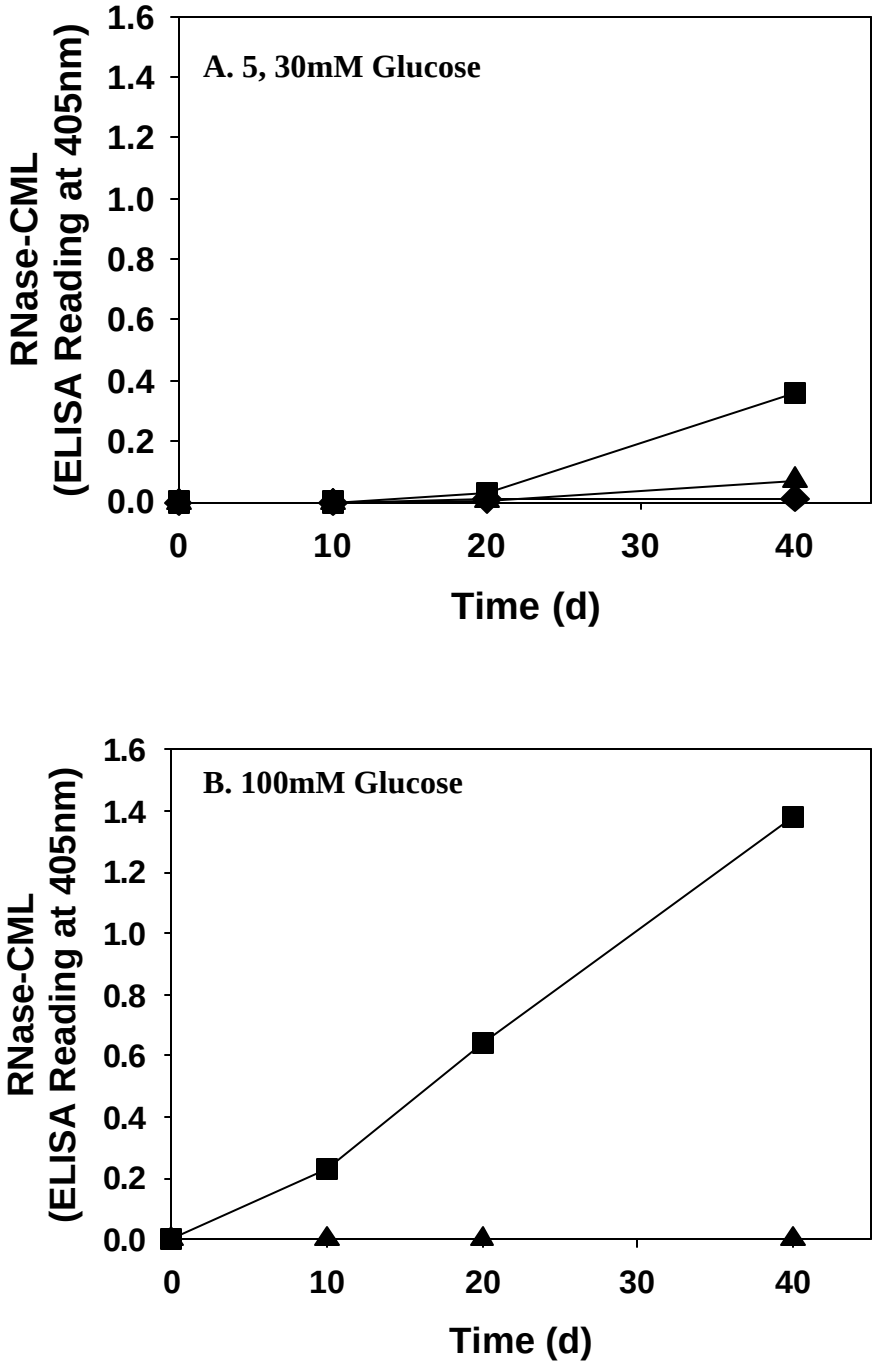

Figure 6. **Effect of PM on CML Formation on RNase**. Samples containing RNase (8 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. ELISA was used to determine the amount of CML formation. The protocol is described under "Methods." Each point represents an average of triplicate measurements.
5 mM glucose-*diamonds*, 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*

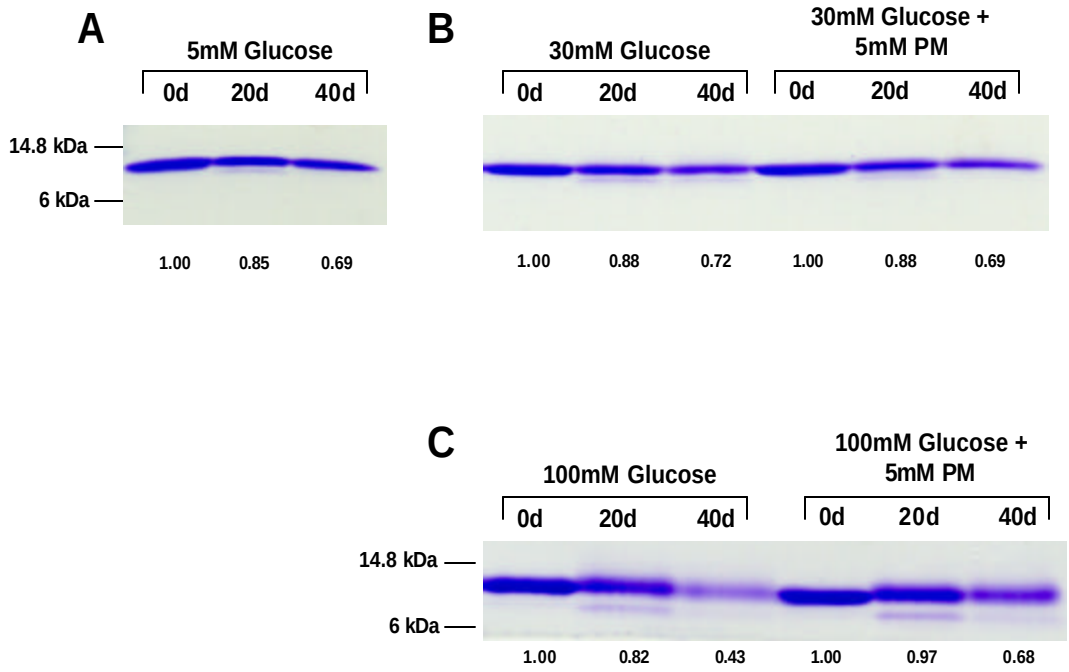

Figure 7. **SDS-PAGE of RNase Samples.** Samples containing RNase (8 mg/mL) were incubated with 5 mM glucose (*A*) or 30 mM glucose in the presence or absence of 5 mM PM (*B*) or 100 mM glucose in the presence or absence of 5 mM PM (*C*). The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. The samples were analyzed by 4-20% SDS-PAGE as described in the "Methods." The numbers underneath the gel represent band intensity as determined by UN-SCAN-IT Gel Automated Digitizing System.

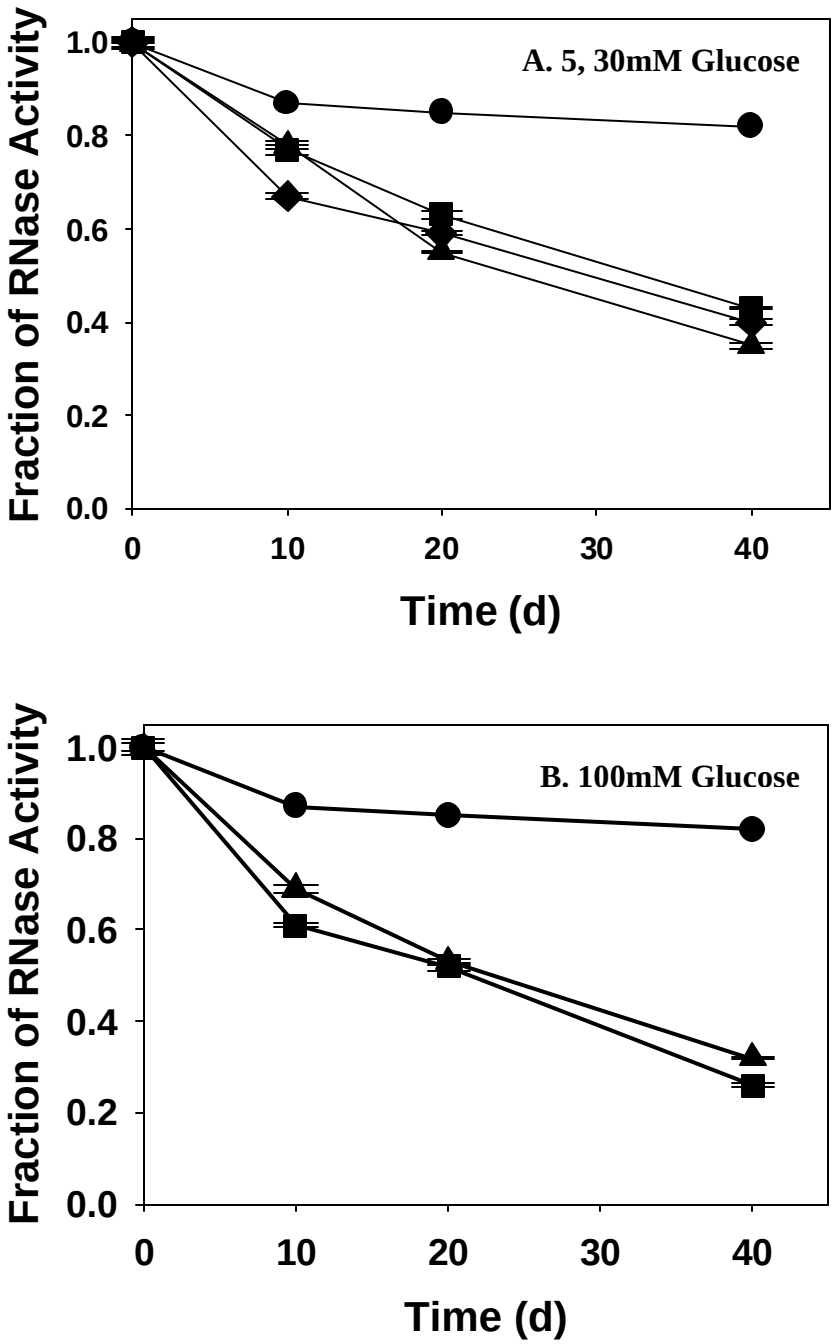

Figure 8. **Effect of PM on RNase Activity Levels.** Samples containing RNase (8 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. RNase activity levels were determined as described under "Methods." Each point represents an average of triplicate measurements. For the control, activity values from four different experiments were averaged together due to inconsistencies in the activity levels of different batches of RNase.
Control-*circles*, 5 mM glucose-*diamonds*, 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*

Lysozyme

*CML Formation***.** In addition to the model protein RNase, lysozyme was also utilized to study glycation reactions. ELISA experiments were conducted in order to determine the kinetics of CML formation on lysine residues of lysozyme. Only a small amount of CML is detected in samples treated with diabetic physiological levels of 30 mM glucose, while no CML formation is seen in samples treated with normal physiological levels of 5 mM glucose (Fig 9*A*). The presence of PM prevents CML formation in samples incubated with 30 mM glucose. Compared to the lysozyme samples incubated with 30mM glucose, the samples that were incubated with 100 mM glucose (supraphysiological levels) showed a much more dramatic increase in CML formation (Fig 9*B*). Both 5 and 20 mM PM were incubated with lysozyme to determine if 20 mM PM produced a more dramatic effect than 5 mM PM. However, in these experiments, both 5 mM and 20 mM PM prevented CML formation to the same extent in samples treated with 100 mM glucose. Thus, lysozyme treated with glucose showed a time and concentration dependent increase in CML formation, whereas lysozyme treated with glucose in the presence of PM resulted in no detectable CML formation.

Degradation and Cross-Linking of Lysozyme. SDS-PAGE analysis was carried out to determine what effects glycation had on the degradation and cross-linking of lysozyme. Lysozyme incubated with 5 mM glucose resulted in stable band intensity over the incubation time period (Fig 10*A*) as determined by gel automated digitizing software. Samples treated with 30 mM glucose in the absence of PM showed intermolecular cross-links of lysozyme forming over time. This is evident in the bands located at 25.9 kDa, which is consistent with the molecular weight for intermolecular cross-links of lysozyme.

These cross-links are not seen at 0 days, but do appear at 20 and 40 days. However, the presence of PM in samples incubated with 30 mM glucose resulted in decreased cross-linking formation (Fig 10*B*). Similar results as those obtained with the 30 mM glucose samples are also seen with lysozyme treated with 100 mM glucose (Fig 10*C*). These results indicate that PM is decreasing the cross-linking of lysozyme in glycated samples. Glucose modifications do not appear to cause protein degradation in lysozyme. Therefore, it appears that the cross-linking of lysozyme occurs in a glucose concentration dependent manner and that PM does exhibit a pronounced effect on the protein cross-linking in these samples.

Enzymatic Activity. In order to determine if glycation affects lysozyme functions and if PM ameliorates the effects of glycation, enzymatic activity assays were conducted. The control (lysozyme incubated alone) remains at 100% activity over the course of the incubation time period. Samples containing lysozyme incubated with 30 mM glucose showed a gradual decline in enzymatic activity, with ~30% activity remaining on day 40. Samples incubated with 5 mM glucose and samples incubated with 30 mM glucose in the presence of 5 mM PM showed a greater protection of enzymatic activity, with activity levels only decreasing to ~75% on day 40 (Fig 11*A*). Both 5 mM PM and 20 mM PM showed similar levels of activity protection in samples incubated with 100 mM glucose, with activity levels decreasing to ~55% on day 40 compared to levels of ~10% in samples treated with 100 mM glucose in the absence of PM (Fig 11*B*). The results of these experiments show that PM is protecting against the loss of enzymatic activity. Therefore, PM did have an effect on enzymatic activity levels of lysozyme, which is unlike the results seen with RNase.

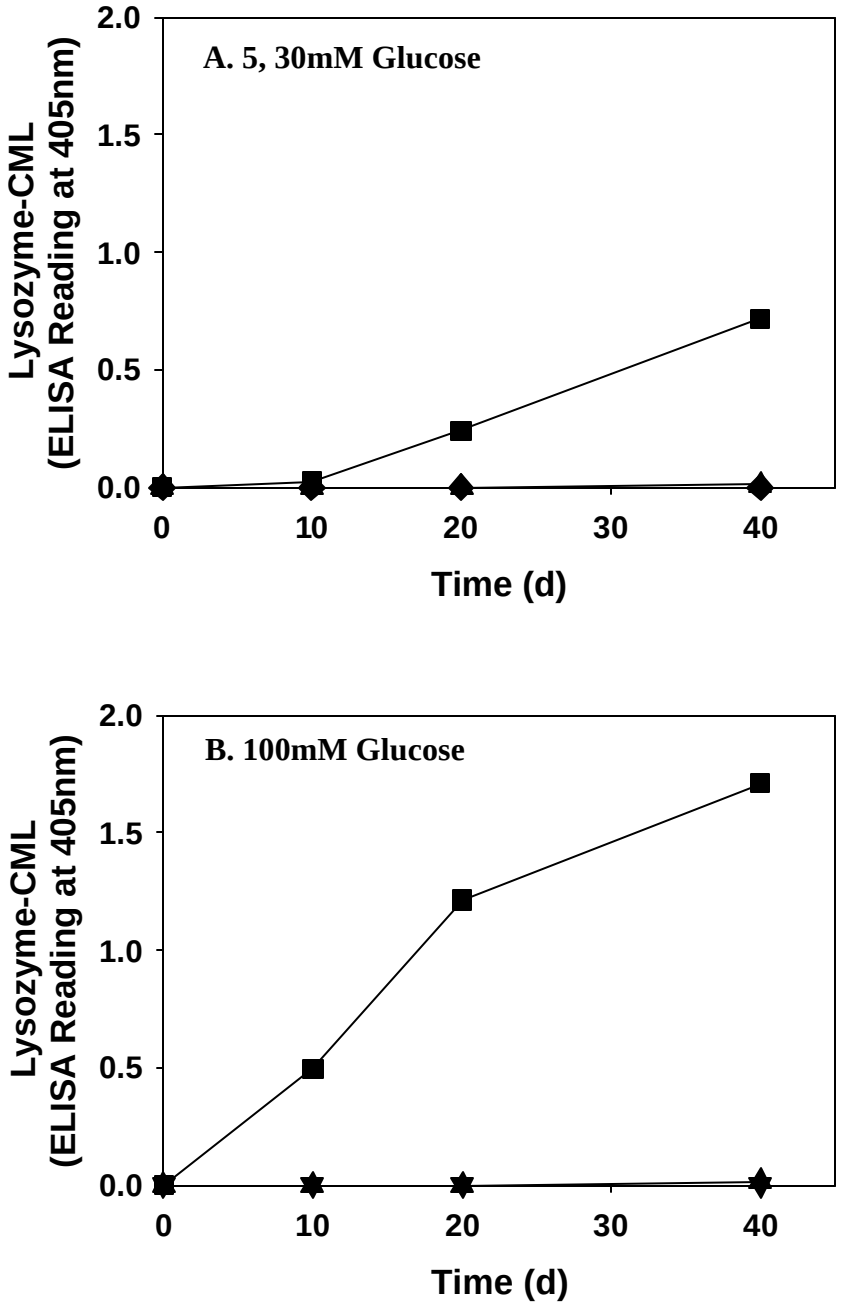

Figure 9. **Effect of PM on CML Formation on Lysozyme**. Samples containing lysozyme (8 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. ELISA was used to determine the amount of CML formation. The protocol is described under "Methods." Each point represents an average of triplicate measurements.
5 mM glucose-*diamonds,* 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*, 20 mM PM-*inverted triangles*

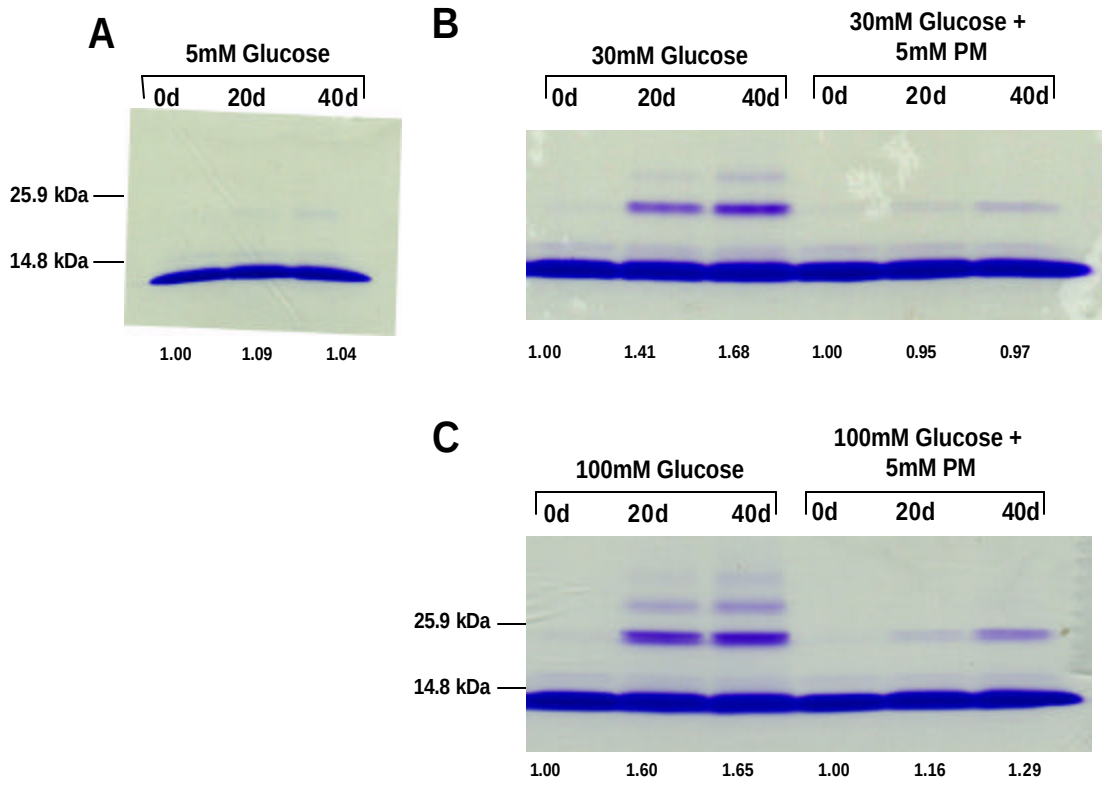

Figure 10. **SDS-PAGE of Lysozyme Samples.** Samples containing lysozyme (8 mg/mL) were incubated with 5 mM glucose (*A*) or 30 mM glucose in the presence or absence of 5 mM PM (*B*) or 100 mM glucose in the presence or absence of 5 mM PM (*C*). The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. The samples were analyzed by 4-20% SDS-PAGE as described in the "Methods." The numbers underneath the gel represent band intensity as determined by UN-SCAN-IT Gel Automated Digitizing System.

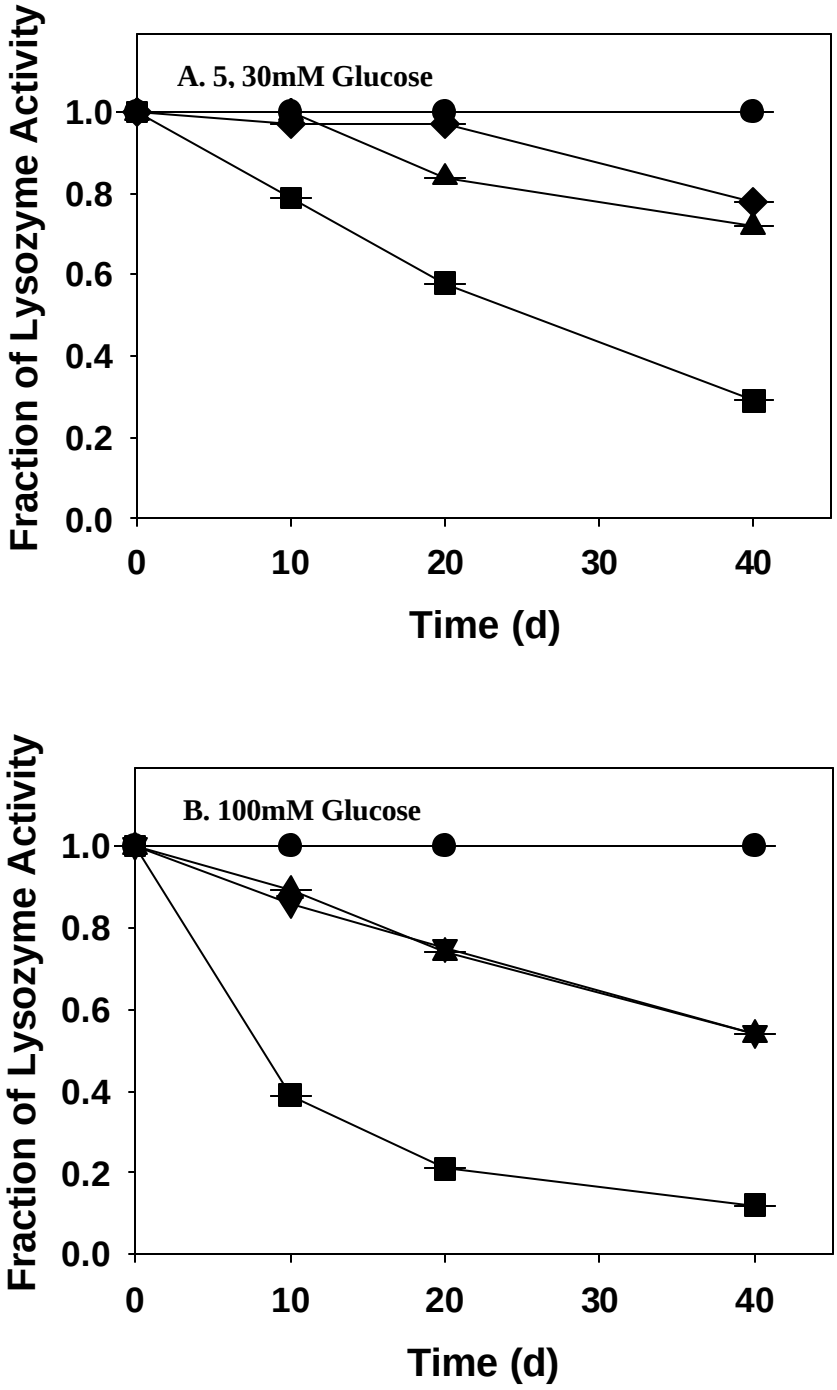

Figure 11. **Effect of PM on Lysozyme Activity Levels.** Samples containing lysozyme (8 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. Lysozyme activity levels were determined as described under "Methods."
5 mM glucose-*diamonds*, 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*, 20 mM PM-*inverted triangles*

BSA

*CML Formation***.** BSA is another model protein that was used in the glycation studies. ELISA was performed to determine the kinetics of CML formation on BSA. Only a small amount of CML is detected in samples treated with diabetic physiological levels of 30 mM glucose, while no CML formation is seen in samples treated with normal physiological levels of 5 mM glucose (Fig 12*A*). The presence of PM prevents CML formation in samples incubated with 30mM glucose. Compared to the BSA samples incubated with 30 mM glucose, the samples that were incubated with 100 mM glucose (supraphysiological levels) show a much more dramatic increase in CML formation (Fig 12*B*). Again, PM did prevent CML formation in samples treated with 100 mM glucose. Thus, BSA treated with glucose showed a time and concentration dependent increase in CML formation, whereas BSA treated with glucose in the presence of PM resulted in no detectable CML formation.

Degradation and Cross-Linking of BSA. SDS-PAGE analysis was carried out to determine what effects glycation had on the degradation and cross-linking of BSA. BSA incubated with 5 mM glucose resulted in stable band intensity over the incubation time period (Fig 13*A*) as determined by gel automated digitizing software. Samples treated with 30 mM glucose in the absence of PM showed some changes over time, which is evident in the smearing of the bands at 20 and 40 days. Slightly higher molecular weight bands are also present in addition to the band smearing. These higher molecular weight bands likely represent intramolecular cross-links of BSA, since the molecular weight is not consistent with the formation of multimers of BSA. However, the presence of PM in samples incubated with 30 mM glucose resulted in decreased band smearing (Fig 13*B*).

There is less smearing of the bands seen at 20 and 40 days. Similar results as those obtained with the 30 mM glucose samples are also seen with BSA treated with 100 mM glucose (Fig 13*C*). These results indicate that PM is decreasing the intramolecular cross-linking of BSA in glycated samples. Therefore, it appears that the intramolecular cross-linking of BSA occurs in a glucose concentration dependent manner and that PM does exhibit a distinct effect on the protein cross-linking in these samples.

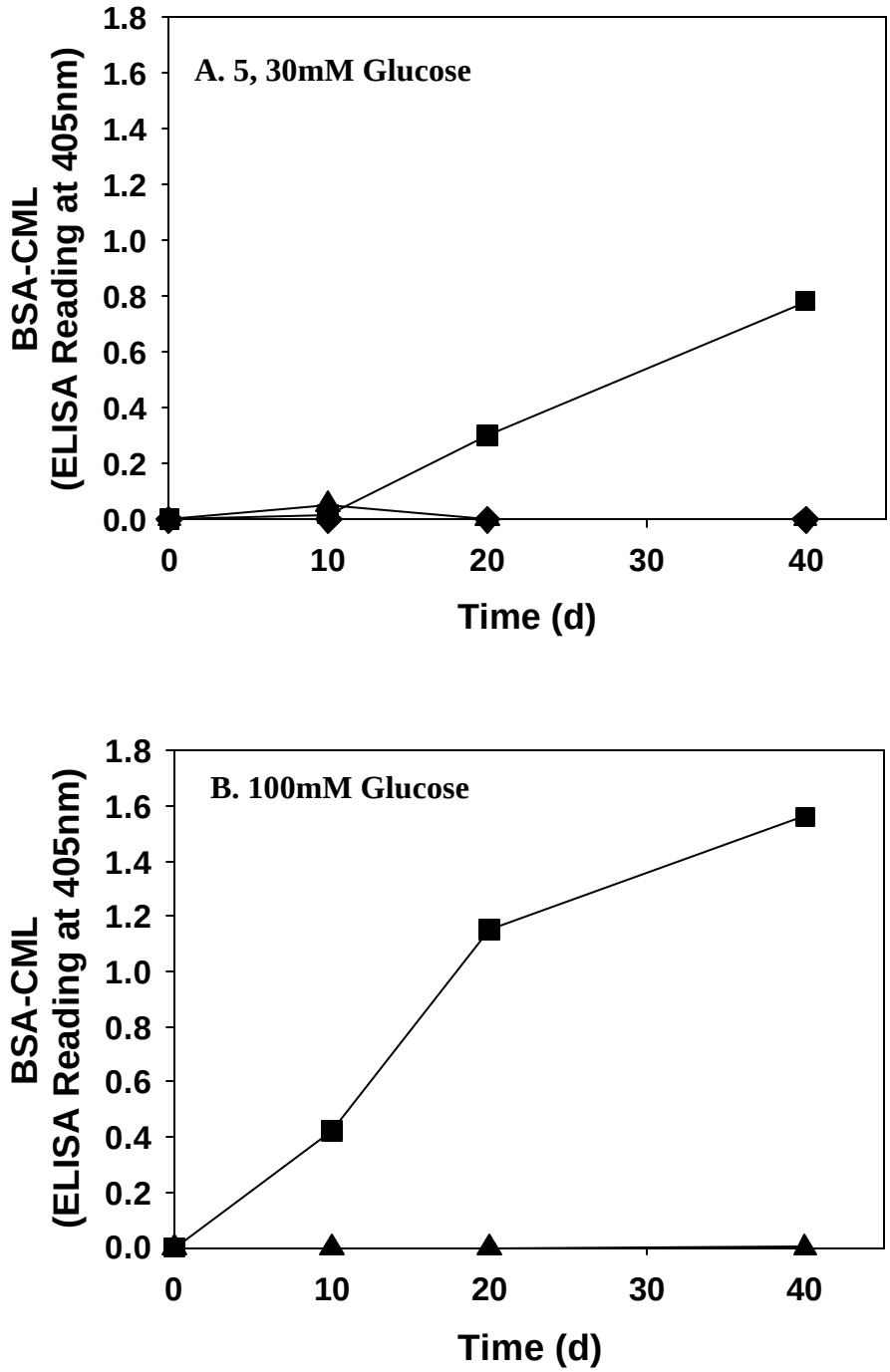

Figure 12. **Effect of PM on CML Formation on BSA**. Samples containing BSA (8 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. ELISA was used to determine the amount of CML formation. The protocol is described under "Methods." Each point represents an average of triplicate measurements.
5 mM glucose-*diamonds*, 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*

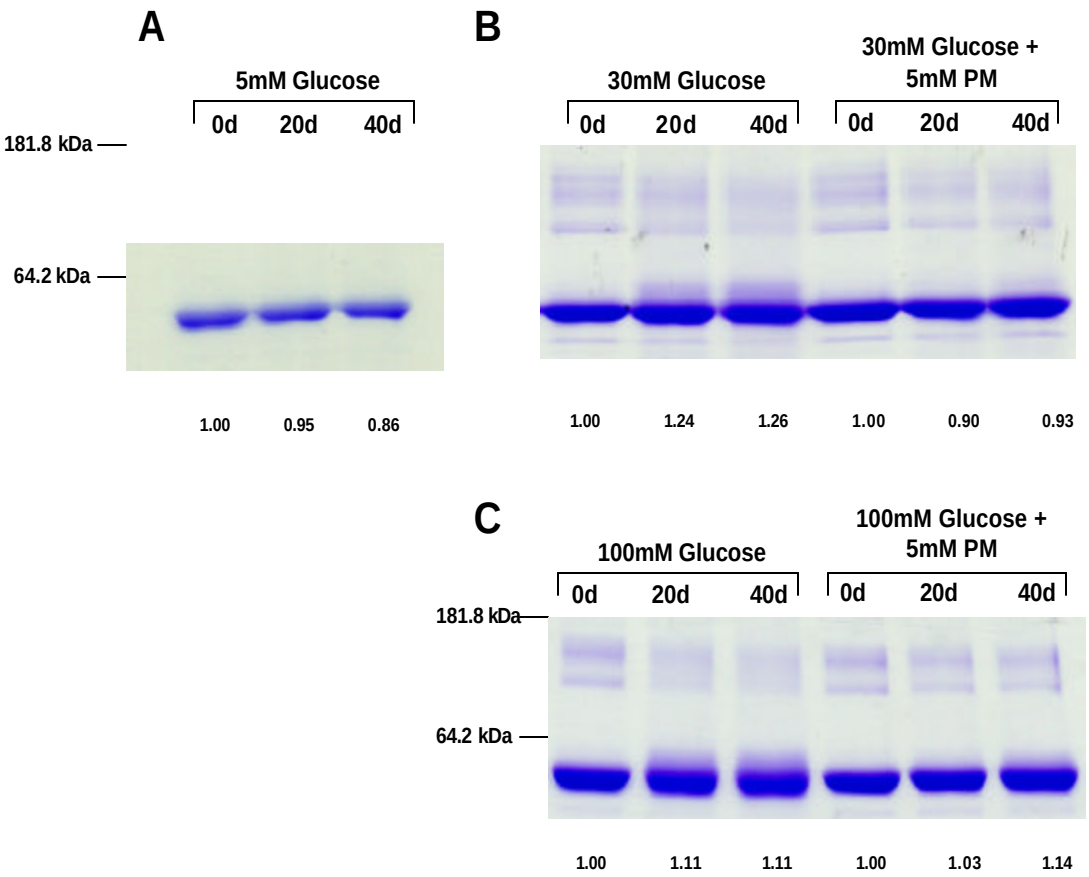

Figure 13. **SDS-PAGE of BSA Samples.** Samples containing BSA (8 mg/mL) were incubated with 5 mM glucose (*A*) or 30 mM glucose in the presence or absence of 5 mM PM (*B*) or 100 mM glucose in the presence or absence of 5 mM PM (*C*). The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. The samples were analyzed by 4-20% SDS-PAGE as described in the "Methods." The numbers underneath the gel represent band intensity as determined by UN-SCAN-IT Gel Automated Digitizing System.

Ubiquitin

*CML Formation***.** Ubiquitin was also used as a model protein of interest in the glycation studies. ELISA experiments were carried out to determine CML formation on ubiquitin. Only a small amount of CML is detected in samples treated with diabetic physiological levels of 30 mM glucose, while no CML formation is seen in samples treated with normal physiological levels of 5 mM glucose (Fig 14*A*). The presence of PM prevents CML formation in samples incubated with 30 mM glucose. Compared to the ubiquitin samples incubated with 30 mM glucose, the samples that were incubated with 100 mM glucose (supraphysiological levels) show a much more dramatic increase in CML formation (Fig 14*B*). Again, PM did prevent CML formation in samples treated with 100 mM glucose. Thus, ubiquitin treated with glucose showed a time and concentration dependent increase in CML formation, whereas ubiquitin treated with glucose in the presence of PM resulted in no detectable CML formation.

Degradation and Cross-Linking of Ubiquitin. SDS-PAGE analysis was carried out to determine what effects glycation had on the degradation and cross-linking of ubiquitin. Ubiquitin incubated with 5 mM glucose resulted in stable band intensity over the incubation time period (Fig 15*A*) as determined by gel automated digitizing software. Samples treated with 30 mM glucose in the absence of PM showed some degradation over time with more pronounced degradation occurring at 40 days. However, the presence of PM in samples incubated with 30 mM glucose resulted in less degradation of the protein (Fig 15*B*). Similar results as those obtained with the 30 mM glucose samples are also seen with ubiquitin treated with 100 mM glucose (Fig 15*C*). Only protein

degradation was observed in these samples; no cross-linking was apparent. These results indicate that PM is decreasing the degradation of ubiquitin in glycated samples.

Enzymatic Activity. Enzymatic activity assays were conducted to determine what affect glycation has on ubiquitin function and to observe if PM ameliorates the effects of glycation. Ubiquitin can conjugate with other ubiquitin molecules resulting in self-ubiquitiniation. As a result of ubiquitin conjugation, ubiquitin ladders are observed. Differences in the ubiquitin ladders are observed in these particular glycated samples and are dependent on glucose concentration and the presence of PM. The control reactions are seen in Figure 15*A*. In samples incubated with 5 mM glucose, there are less intense ubiquitin ladders observed at 40 days compared to 0 days (Fig 16*A*). In samples treated with 30 mM glucose, there is a time dependent decrease in the intensity of the ubiquitin ladder, but when PM is present, it prevents the decrease (Fig 16*B*). Similar results are seen in samples treated with 100 mM glucose with and without PM (Fig 16*D*). The decrease in the ubiquitin ladder does seem to be more prominent in samples that were incubated with 100 mM glucose compared to those with 30 mM glucose. However, in the presence of PM, there the ubiquitin ladder is intact. Therefore, PM is protecting the ubiquitin conjugation reactions in these samples.

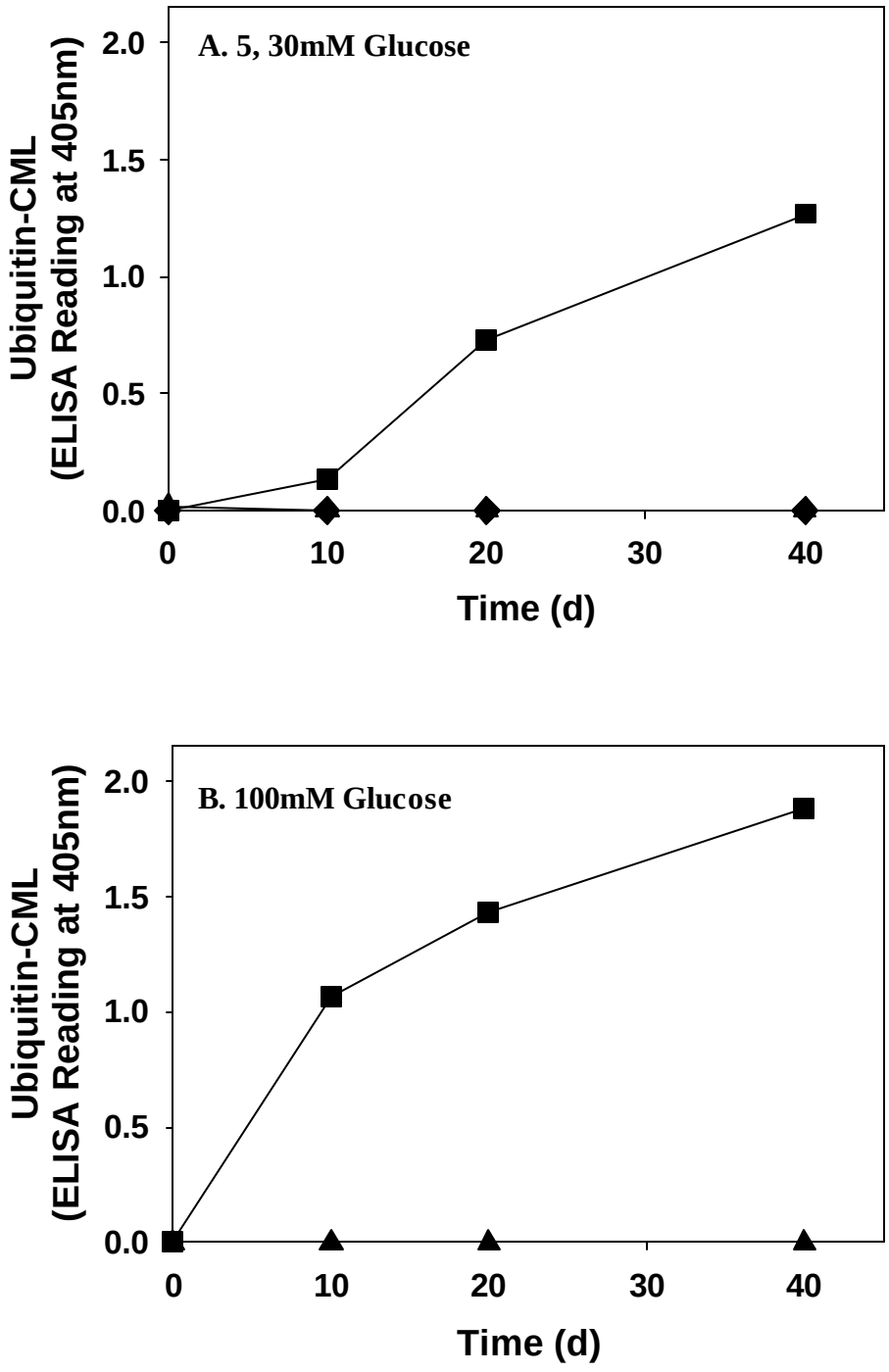

Figure 14. **Effect of PM on CML Formation on Ubiquitin**. Samples containing ubiquitin (1 mg/mL) were incubated with 5 mM glucose or 30 mM glucose (*A*) in the presence or absence of 5 mM PM or 100 mM glucose (*B*) in the presence or absence of 5 mM PM. The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. ELISA was used to determine the amount of CML formation. The protocol is described under "Methods." Each point represents an average of triplicate measurements.
5 mM glucose-*diamonds*, 30 mM and 100 mM glucose-*squares*, 5 mM PM-*triangles*

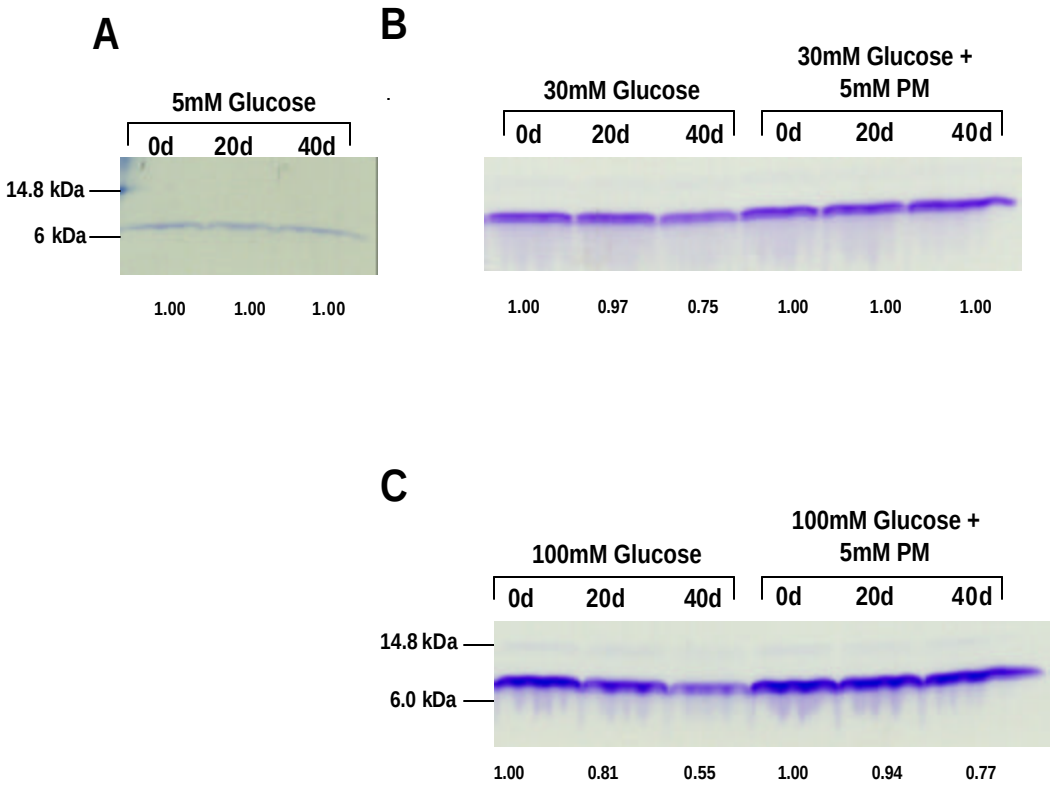

Figure 15. **SDS-PAGE of Ubiquitin Samples.** Samples containing ubiquitin (1 mg/mL) were incubated with 5 mM glucose (*A*) or 30 mM glucose in the presence or absence of 5 mM PM (*B*) or 100 mM glucose in the presence or absence of 5 mM PM (*C*). The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. The samples were analyzed by 4-20% SDS-PAGE as described in the "Methods." The numbers underneath the gel represent band intensity as determined by UN-SCAN-IT Gel Automated Digitizing System.

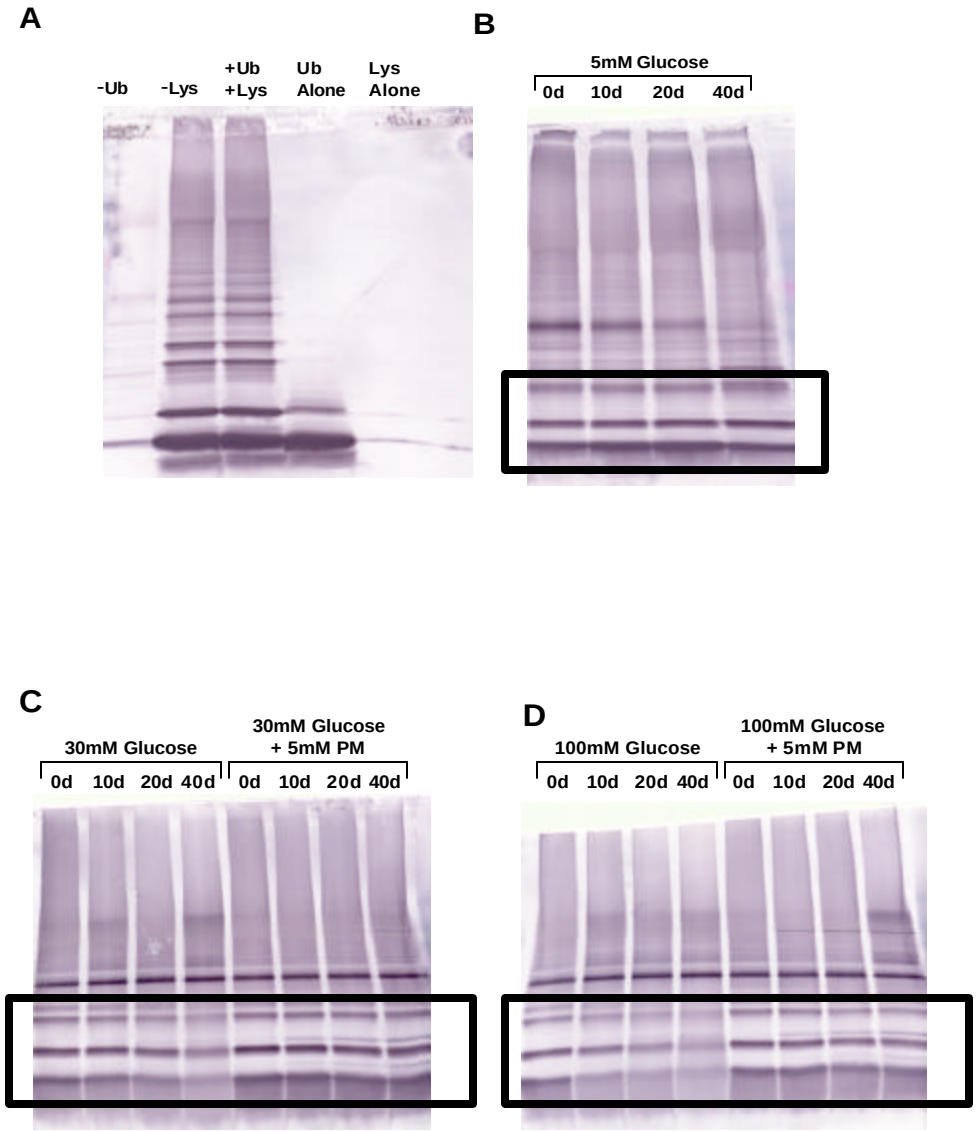

Figure 16. **Effect of PM on Ubiquitin Activity.** Samples containing ubiquitin (1 mg/mL) were incubated with 5 mM glucose (*B*) or 30 mM glucose in the presence or absence of 5 mM PM (*C*) or 100 mM glucose in the presence or absence of 5 mM PM (*D*). Control reactions contain the reaction mixture minus the listed component or only the individual component (*A*). The sample incubations were carried out at 37°C in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.03% sodium azide. Ubiquitin activity was determined as described under "Methods."

Detection of AGE Formation on Lysozmye by Mass Spectrometry

PM Inhibition of CML Formation

In order to probe more deeply into the differences seen in glycation patterns and enzymatic activity profiles, lysozyme was chosen as the model protein to study using mass spectrometry. While studies have been conducted to detect glucose modifications such as CML (Fig 17) on lysozyme, there are no studies using MS to look at the differences in modifications detected when PM is present. Lysozyme samples which had been incubated for 40 days with 100 mM glucose in the absence or presence of 5 mM and 20 mM PM were examined.

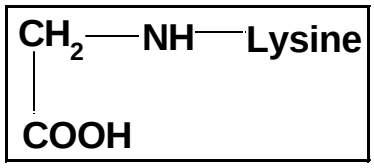

Figure 17. **Structure of CML.**

After MS analysis, the modifications of CML on lysine residues and HTRP on tryptophan residues were searched for using the program Sequest. Currently, no studies exist which examine the modification of tryptophan residues as a result of glycation reactions Table 1 shows the results found for peptides containing CML on K-96 and K-97, such as the following peptide found in the glucose sample: $AK^{CML}K^{CML}IVSDGNGMNAW$. All six lysine residues were analyzed, but detailed results are shown for only K-96 and K-97. Using the information obtained from Sequest, the elution profiles of the respective peptides were located in the chromatogram (Fig 18).

Then peptide identification was confirmed by the MS spectrum (Fig 19). Most of the corresponding b and y ions are detected in the spectrum and are consistent with mass shifts of 58 atomic mass units (molecular mass difference between lysine and CML) on K-96 and K-97. In order to determine the relative intensity of the modifications present in each sample, the area of the peak containing the peptide of interest was determined and normalized to a reference peptide found in all the samples, which was not subject to modification (Fig 18 and Table 1). All six lysine residues, K-1, 13, 33, 96, 97, and 116, were analyzed, and the highest CML relative intensity occurs on K-96 and K-97 found in the glucose treated lysozyme sample (Fig 20). The data for K-96 and K-97 also shows that the control contains very little CML, while lysozyme incubated with glucose in the presence of 5 mM PM shows less CML detection, and even less CML relative intensity levels are found in lysozyme incubated with glucose in the presence of 20 mM PM. Similar trends are also seen on K-33. This data is consistent with PM preventing the formation of CML. CML was also detected on other lysine residues, such as K-1, while PM treated samples exhibit relatively little or no CML. There was practically no CML detected on K-13 or K-116 in any of the samples. This can be explained by not all of the residues being accessible to modification due to protein structural constraints, which illustrates the effects of protein configuration and environment on glycation reactions.

Table 1. **MS Data on Peptides containing CML on K-96 and K-97.**

Sample	Residue	Peptide	Xcorr	Scan #	z	MH^+	MH^{2+}	MH^{3+}	Area	Relative Intensity
Control	K-96 & K-97	$AK^{CML}K^{CML}$IVSDGNGMNAWVAW	3.44	7460	2	1962.1	981.6	654.7	3843204	0.15
Glucose	K-96 & K-97	$AK^{CML}K^{CML}$IVSDGNGMNAW	3.68	5174	2	1605.8	803.4	535.9	175623050	6.55
Glucose + 5 mM PM	K-96 & K-97	$AK^{CML}K^{CML}$IVSDGNGMNAW	4.25	5114	2	1607.2	804.1	536.4	105884025	3.85
Glucose + 20 mM PM	K-96 & K-97	$AK^{CML}K^{CML}$IVSDGNGMNAW	4.28	5102	2	1608.3	804.7	536.8	62968232	1.82

The following defined parameters were obtained from Sequest. The Xcorr value is the cross correlation score, which is the normalized score that is a measure of how well the actual spectrum matches to the theoretical spectrum. The z value is the charge state of the peptide. MH^+ is the mass of the singly charged peptide, while MH^{2+} and MH^{3+} represent the mass of the doubly and triply charged peptides, respectively. The area values were obtained from the elution chromatogram. The relative intensity values were obtained by normalizing the area values to a matching reference peptide.

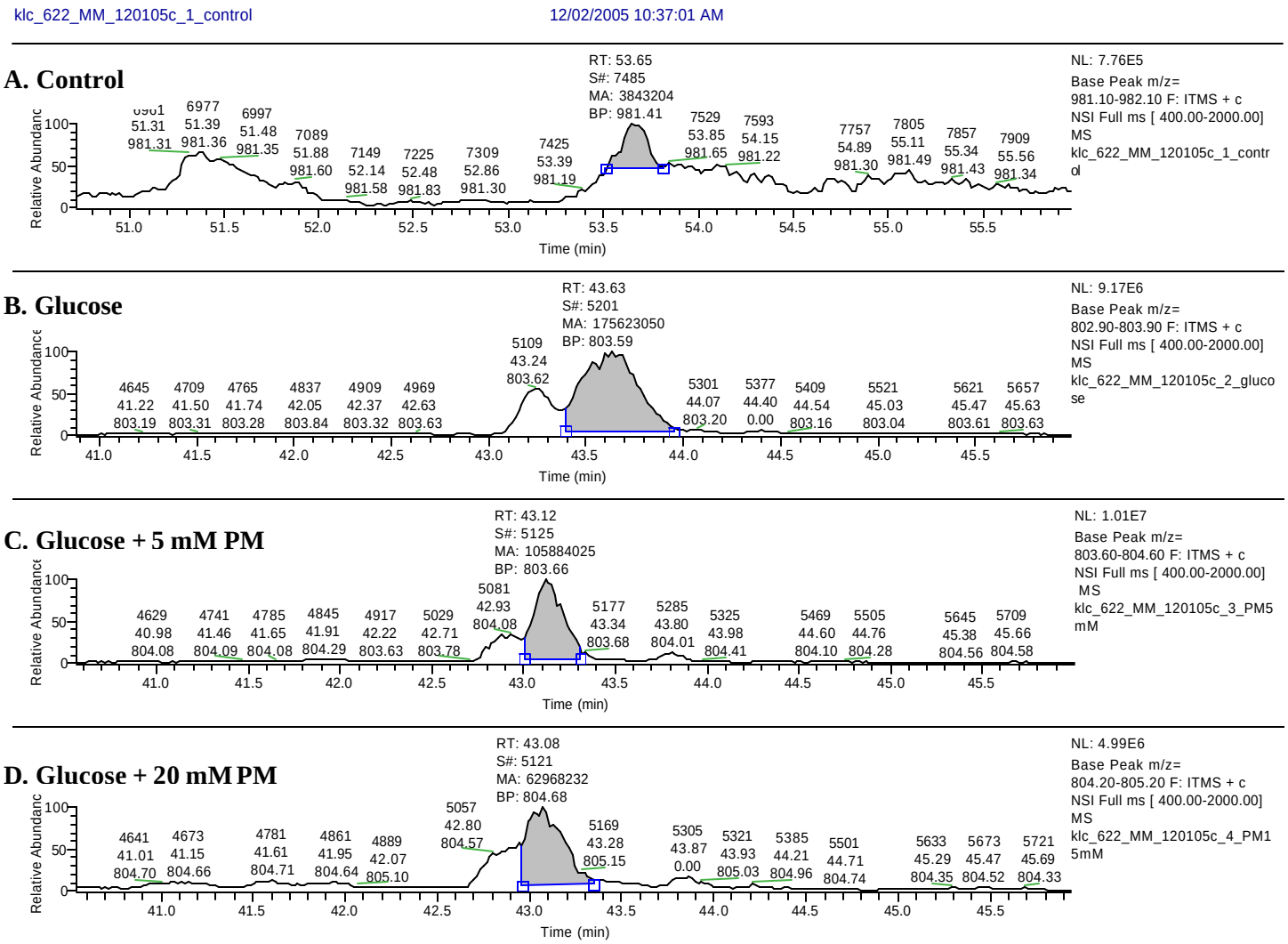

Figure 18. **HPLC Elution Profiles of Lysozyme Peptides containing CML on K-96 and K-97.**

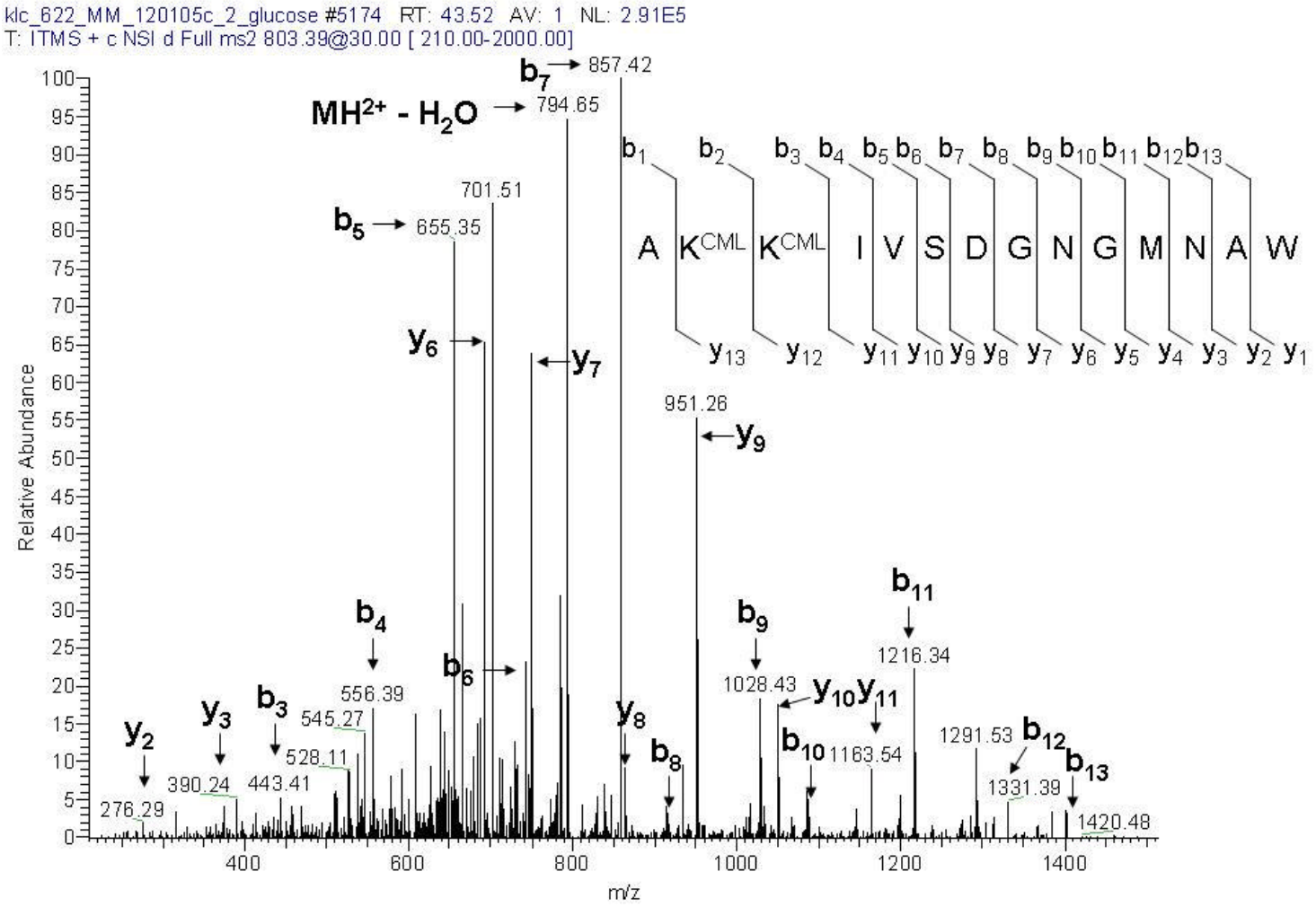

Figure 19. **MS Spectrum of Lysozyme Peptide containing CML on K-96 and K-97.**

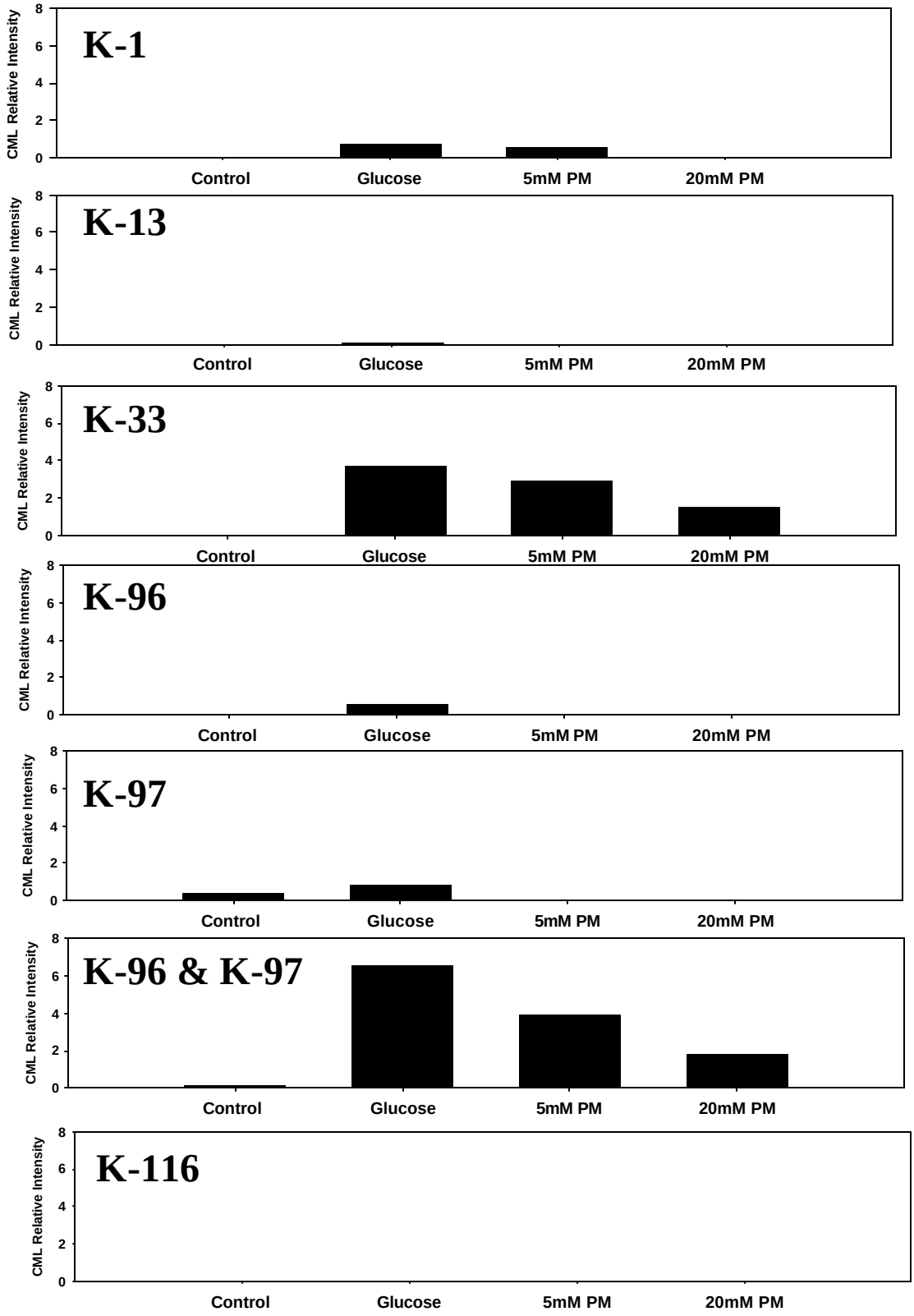

Figure 20. **CML Relative Intensity Chart.**

PM Inhibition of HTRP Formation

As mentioned above, modifications of HTRP (Fig 21) on tryptophan were also searched for using Sequest. It should be pointed out that glucose does not directly react with tryptophan residues and modifications like HTRP are not considered AGEs. However, HTRP may form as a result of glycation reactions, since ROS are generated from glucose autoxidation and from intermediates formed in the AGE formation pathway.

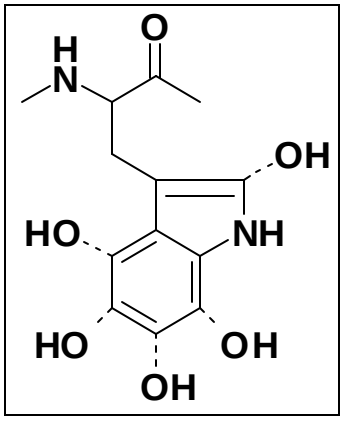

Figure 21. **Structure of HTRP.**

Indeed, the results from this study do show that HTRP forms in glycation reactions. Table 2 shows the results found for peptides containing HTRP on W-62 and W-63, such as the following peptide found in the glucose sample: GILQINSRW^{HTRP}W^{HTRP}. Again, all six tryptophan residues were analyzed, but detailed results are shown for only W-62 and W-63. Using the information obtained from Sequest, the elution profiles of the respective peptides were located in the chromatogram (Fig 22). Then peptide identification was confirmed by the MS spectrum (Fig 23). Most of the corresponding b and y ions are detected in the spectrum, and the y ions detected are consistent with mass

shifts of 16 atomic mass units (molecular weight of HTRP) on W-62 and W-63. The relative intensities of HTRP were determined in the same manner as those for CML, as explained above. All six tryptophan residues, W-28, 62, 63, 108, 111, and 123, were analyzed, and the highest HTRP relative intensity occurs on W-62 and W-63 found in the glucose treated lysozyme sample (Fig 24). The data for W-62 and W-63 also shows that the control contains very little HTRP, while lysozyme samples treated with glucose in the presence of PM (both 5 and 20 mM) contain much less HTRP than samples incubated with glucose alone. Similar results are seen with W-28, W-62, and W-108. This data is consistent with PM protecting against damage by ROS. Some data inconsistencies are seen, such as detection of HTRP on W-63 only in samples containing PM but not in samples treated only with glucose. This could be attributed to only detecting the most abundant peptides, which in this case, may be the peptides that contain HTRP on both W-62 and W-63. Perhaps, this occurs because the chymotryptic digestion of lysozyme resulted in preferential cleavage of peptides containing both W-62 and W-63, which in this case could be considered an artifact of the digestion of glycated lysozyme.

Table 2. **MS Data on Peptides containing HTRP on W-62 and W-63.**

Sample	Residue	Peptide	Xcorr	Scan #	z	MH^+	MH^{2+}	MH^{3+}	Area	Relative Intensity
Control	W-62 & W-63	GILQINSRW^{HTRP}W^{HTRP}	2.93	8103	2	1304.8	652.9	435.6	38580746	1.49
Glucose	W-62 & W-63	GILQINSRW^{HTRP}W^{HTRP}	2.86	8198	2	1305.1	653.1	435.7	564049595	21.05
Glucose + 5 mM PM	W-62 & W-63	GILQINSRW^{HTRP}W^{HTRP}	2.88	8174	2	1305.2	653.1	435.7	83601230	3.34
Glucose + 20 mM PM	W-62 & W-63	GILQINSRW^{HTRP}W^{HTRP}	2.15	7055	2	1304.9	653.0	435.6	271993810	7.88

The following defined parameters were obtained from Sequest. The Xcorr value is the cross correlation score, which is the normalized score that is a measure of how well the actual spectrum matches to the theoretical spectrum. The z value is the charge state of the peptide. MH^+ is the mass of the singly charged peptide, while MH^{2+} and MH^{3+} represent the mass of the doubly and triply charged peptides, respectively. The area values were obtained from the elution chromatogram. The relative intensity values were obtained by normalizing the area values to a matching reference peptide.

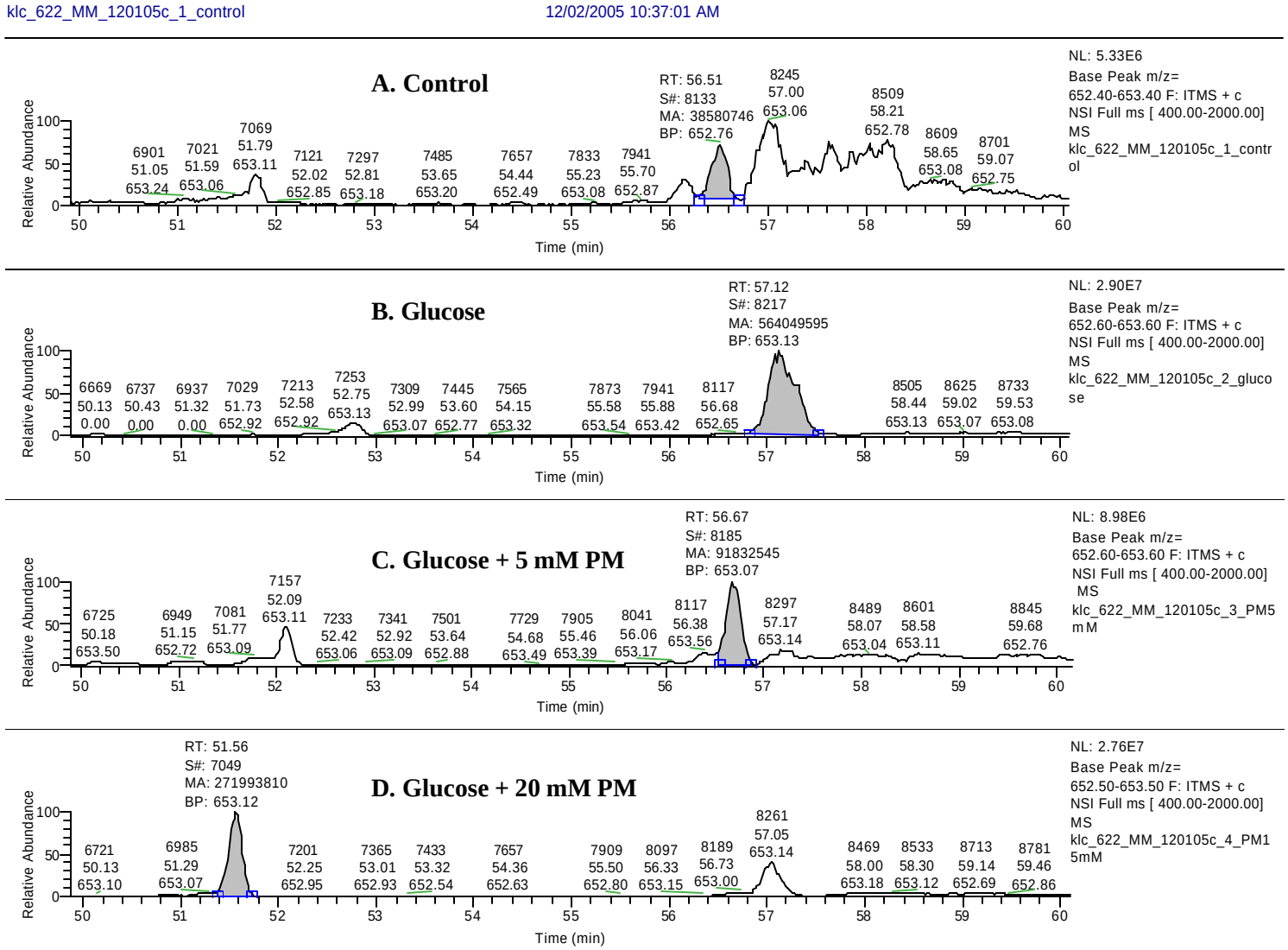

Figure 22. **HPLC Elution Profiles of Lysozyme Peptides containing HTRP on W-62 and W-63.**

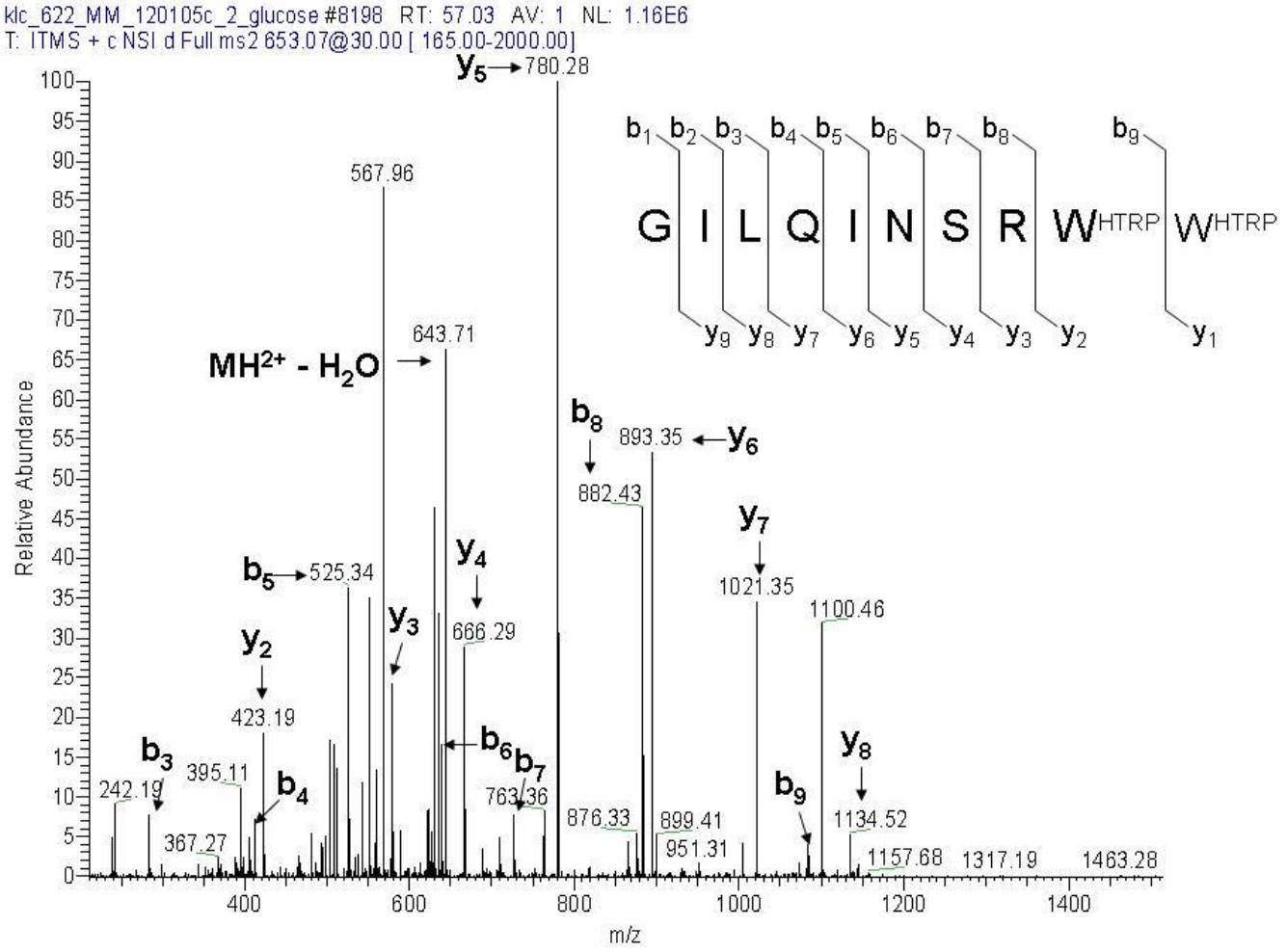

Figure 23. **MS Spectrum of Lysozyme Peptide containing HTRP on W-62 and W-63.**

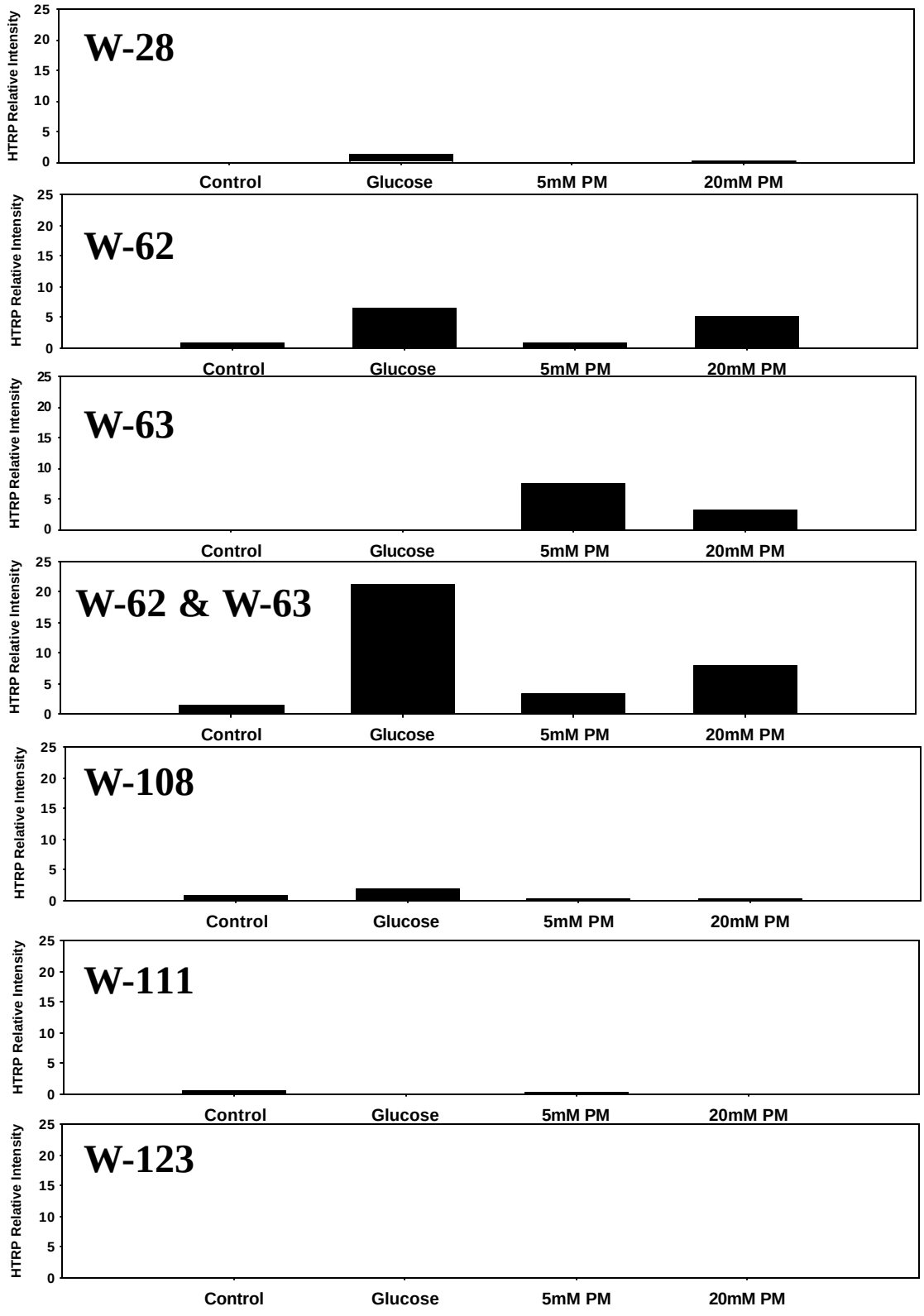

Figure 24. **HTRP Relative Intensity Chart.**

CHAPTER IV

DISCUSSION

AGEs have been implicated in many diseases, particularly diabetic complications. While PM has shown promising results against preventing the formation of AGEs and the complications that arise as a result of AGEs, a lack of knowledge still exists as to how PM functions as an AGE inhibitor. The purpose of this study was to examine how glucose modifications affect the function of model proteins and how PM inhibits these glycation reactions. The model proteins RNase, lysozyme, BSA, and ubiquitin were all studied in order to determine how different protein environments affect the formation of AGEs and efficacy of PM.

PM Inhibition of AGE Formation

The prominent AGE, CML, has been detected in diseases such as diabetic nephropathy. This study found that CML levels increase over time in all of the model proteins that were incubated with various concentrations of glucose ranging from 5 mM to 100 mM (Fig 6, 9, 12, and 14). The glucose levels employed in this study are of physiological relevance. These results are of particular importance since previous studies have not been conducted utilizing physiological glucose concentrations, which are the normal 5 mM glucose levels, the diabetic 30 mM glucose levels, and the supraphysiological 100 mM glucose levels. The higher concentrations of glucose invoke a greater increase in CML formation. However, PM was found to inhibit CML formation

in all the proteins. These results indicate that PM is functioning to block the conversion of the Amadori intermediate to CML in different protein environments.

PM Protection Against Protein Degradation and Cross-Linking

Electrophoresis experiments revealed differences in glycation patterns among the model proteins. After prolonged incubation with glucose, especially 100 mM glucose, RNase appears to undergo degradation (Fig 7). This correlates with previous studies stating that ROS, formed from either glucose autoxidation or oxidative degradation of the Amadori intermediate, can cause fragmentation of the protein backbone (Ookawara, Kawamura et al. 1992). However, PM protected RNase from degradation, suggesting that PM may be functioning to scavenge the ROS generated in these reactions.

Different glycation patterns were seen with lysozyme. After extended incubation times, such as those seen at 40 days, lysozyme, which was treated with 30 mM and 100 mM glucose, was cross-linked (Fig 10). This conclusion is made based on the fact that the molecular weight of lysozyme is approximately 14 kD and the bands seen above 14 kD correspond to multimers of lysozyme, such as the band at 28 kD which would indicate an intermolecular cross-linking of two lysozyme molecules. Also, the electrophoresis experiments were performed under reducing conditions, so only strong covalent interactions would be able to withstand such conditions. Previous studies do state that glycation reactions can result in the cross-linking of proteins, particularly through arginine residues, such as pentosidine and glucosepane. Cross-links can also form through lysine residues, such as MOLD and GOLD. Lysozyme does contain six lysine residues and eleven arginine residues. Therefore, it is reasonable to conclude that

the glucose modifications present on lysozyme are inducing the formation of cross-links. Conversely, PM protected against the formation of cross-links, as is evident when comparing lysozyme treated with 100 mM glucose in the presence of PM at 40 days to that of lysozyme treated in the absence of PM. While some cross-linking is still present in PM treated samples, it is dramatically reduced. Therefore, PM is protecting against cross-linking. PM protection against cross-linking has not been previously reported, so it could be another mechanism of PM action.

BSA also exhibits cross-linking when exposed to glycation reactions, though it is a different type of pattern than that observed for lysozyme. Electrophoresis experiments of BSA incubated with 30 mM and 100 mM glucose in the absence of PM showed possible intramolecular cross-linking of BSA over the incubation time, which is indicated by the smearing of the bands and the slightly higher molecular weight band seen at 20 and 40 days (Fig 13). This higher molecular weight band is not consistent with intermolecular cross-linking, or the formation of multimers of BSA. However, it is quite possible that intramolecular cross-links are formed within the BSA molecule itself as a result of glycation. PM protects against BSA cross-linking, which is evident is decreased band smearing and the less intense band of the higher molecular weight species.

Ubiquitin exhibits a different glycation pattern compared to lysozyme and BSA, but one that is similar to RNase. Under conditions that involve ubiquitin incubation with 30 mM and 100 mM glucose in the absence of PM, ubiquitin also appears to degrade over time, which is evident by the decrease in band intensity seen at 40 days (Fig 15). Again, PM may be protecting against ROS formation, because in the presence of PM, there is less degradation.

Though the model proteins do vary in their glycation patterns, PM does protect the integrity of RNase, lysozyme, BSA, and ubiquitin. This data supports other studies, which have concluded that PM is capable of several different mechanisms of protection from glycation (Voziyan and Hudson 2005). However, the data presented here points out that even in different protein environments, there is still PM protection from protein degradation and cross-linking. The fact that PM can be effective even in different protein environments could be attributed to the effectiveness seen in PM usage in animal experiments and clinical trials.

PM Protection of Protein Function

Another important aspect of this study was to determine how effective PM is in protecting protein function. Enzymatic activity assays revealed that glucose incubation with both RNase and lysozyme resulted in diminished enzyme function over time (Fig 8 and 11). Declines in enzymatic activity levels occurred over the incubation period in the presence of both 30 mM and 100 mM glucose, with more pronounced declines seem with 100 mM glucose. This indicates that glucose modifications do affect protein function, which can be indicative of why physiological complications arise in the presence of excess glucose levels. Though both RNase and lysozyme exhibit similar declines in enzymatic function in the presence of glucose, differences in activity levels were observed when the proteins were incubated with PM. In the presence of 5 and 20 mM PM, lysozyme maintains high levels of enzymatic activity, suggesting that PM is inhibiting against the loss of protein function (Fig 11). Different concentrations of PM were utilized in order to determine if more efficient results were achieved in the presence

of higher concentrations of PM. Since glucose is an aldehyde, it possesses the potential to react with PM, which means that PM could be functioning to scavenge the excess glucose. The glucose concentrations in these experiments were several folds higher than that of the PM concentrations. However, similar activity levels were observed in the presence of both 5 and 20 mM PM. Therefore, PM is protecting lysozyme function in these experiments, but not through glucose scavenging. The results seen also indicate that the Amadori intermediate is not as detrimental to protein function as AGEs, such as CML, since PM is blocking the formation of AGEs at the Amadori intermediate.

Though there is protection of lysozyme function in the presence of 5 mM PM, RNase activity levels decline to the same extent as those of RNase incubated with glucose alone (Fig 8). Therefore, RNase behaves differently in response to PM. PM protects against cross-linking, degradation, and CML formation, but not from the loss of enzymatic function, which suggests that in certain proteins blocking the conversion of the Amadori intermediate to AGEs may not be necessary to affect function. Perhaps, the initial glucose modifications present on RNase are enough to induce damage to protein functionality. Though these are model proteins, similar situations may be encountered *in vivo* as well. Some proteins may functionally benefit from PM blocking the formation of AGEs. However, in other proteins PM may be unable to prevent the initial damage caused by the glucose modifications.

Protection of ubiquitin function was also observed in the presence of PM (Fig 16). Ubiquitin ladders, or ubiquitin conjugation with itself, were observed in samples treated in the presence of 5 mM PM along with 30 or 100 mM glucose. However, ubiquitin incubated with glucose alone resulted in decreased band intensity along the ubiquitin

ladder. These results indicate that glucose modifications may affect the ability of ubiquitin to conjugate with other ubiquitin molecules. Ubiquitination occurs by ubiquitin binding to other ubiquitin molecules through lysine residues. Since glucose modifications target lysine residues, it is reasonable to conclude that glucose induced modifications present on lysine would hinder ubiquitination (Passmore and Barford 2004). However, PM seems to protect the ability of ubiquitin conjugation, which again suggests that blocking the formation of AGEs, even at intermediates along the pathway, protects protein function.

PM preserved the function of both lysozyme and ubiquitin in the presence of excess glucose. As mentioned above, it is currently understood that PM blocks the formation of AGEs by inhibiting the conversion of the Amadori intermediate. Therefore, in the cases of both lysozyme and ubiquitin, it seems that inhibiting the formation of AGEs was sufficient to maintain protein function. Again, protecting protein function may be one of the means of effectiveness seen with PM treatment in diabetic nephropathy. Since glycation occurs very slowly, it may be clinically beneficial that PM can inhibit AGE formation even in cases where excess glucose levels may have been present for an extended period of time.

PM Inhibition of CML Formation

Since differences were seen in enzymatic activity levels for RNase and lysozyme in the presence of PM, mass spectrometry experiments were performed to better study lysozyme and to assess those observed differences, along with determining the site-specific differences. ELISA experiments showed that lysozyme incubation with glucose

resulted in increased CML formation that occurred in a time and glucose concentration dependent manner, while PM inhibited the formation of CML (Fig 9). PM also preserved lysozyme function, while glucose modifications resulted in diminished enzyme activity (Fig 11). The purpose of the mass spectrometry experiments was to identify the sites of glucose modifications and to determine if PM did in fact prevent the formation of AGEs. Using mass spectrometry to study glycation has been employed in numerous previous studies. However, the use of mass spectrometry to study differences in glycation patterns induced on lysozyme due to PM has not been explored.

Lysozyme contains six lysine residues, which are potential sites for glucose modification. After searching for CML modifications present on lysozyme, which had been incubated with only glucose, it was determined, that CML was present on K-1, 33, 96, and 97 (Fig 20). The highest CML relative intensity was found to occur on peptides that contained CML on both K-96 and K-97. Not all lysine residues were subject to glucose modification. As mentioned earlier, studies involving peptide glycation determined that charged residues located near lysine residues increase the reactivity of lysines to glycation (Mennella, Visciano et al. 2005). Acidic and basic residues in lysozyme comprise 21% of the amino acid sequence. By examining the x-ray structure of lysozyme (Blake, Koenig et al. 1965), it was determined that charged residues, such as H-15 and D-101, are located near K-96 and K-97. Thus, the proximity of these charged residues to K-96 and K-97 might promote glycation at these sites. K-96 and K-97 are also located near the active site cleft, so CML formation at these sites might be linked to the loss of enzyme function (Fig 11). Residues located in the active site of lysozyme are D-52, D-101, and E-35, which are acidic residues. The formation of CML on K-96 and

K-97 changes the positive charge carried on these basic lysines to negative charges, thus changing the dynamics of the active site. The disturbance in these interactions could lead to the loss of protein function. Further examination of the x-ray structure also reveals that K-13 and K-116 are located close to one another and are not surrounded by as many charged residues as the other lysine residues. Consequently, these two residues show the least intense CML signals (Fig 20).

Notable differences in CML intensity levels were observed in the presence of PM. Less CML was observed when lysozyme was incubated with glucose in the presence of 5 mM PM and those levels were reduced even further in the presence of 20 mM PM (Fig 20). Less CML is detected on K-96 and K-97 in the presence of PM. In the absence of PM, these were the sites of the most intense CML signals. As mentioned earlier, by examining the x-ray structure, it was reasonable to conclude that CML formation at K-96 and K-97, which are located near the active site of the enzyme, could contribute to the loss of enzymatic activity. However, by PM blocking the conversion of the Amadori intermediate to CML, the enzyme function is protected (Fig 11). Thus, perhaps the Amadori intermediate is not as harmful as CML to enzyme function. While the Amadori intermediate is a more bulky constituent, it possesses a neutral charge, which is unlike the negatively charged CML. This charge difference could contribute to the protection seen in enzymatic activity. These mass spectrometry results clearly indicate that PM does prevent and or reduce the amount of CML formed due to glucose modifications of lysine residues. These results also support the findings obtained from assessing CML formation, glycation patterns, and enzymatic activity. While ELISA experiments found no CML formation in the presence of PM, mass spectrometry experiments do indicate

that there is CML present. It is reasonable to conclude that ELISA experiments may not have detected low levels of CML present on some residues due to the inaccessibility of some lysine residues to antibody binding. Also, according to the mass spectrometry results, the majority of CML formation occurs on certain residues, such as K-33, 96, and 97, so CML detected on these residues could have led to very strong signals, which overshadowed the CML present on other residues. The reduction of CML levels detected in the presence of PM by the mass spectrometry experiments could also account for the preservation of lysozyme function in the presence of PM. Again, the results obtained from the different biochemical analyses all suggest that PM does prevent against the formation of CML.

PM Protection Against the Oxidation of Tryptophan Residues

Mass spectrometry results revealed that PM, in addition to inhibiting CML formation on lysozyme, also protects against the oxidation of tryptophans (Fig 24). Again, the oxidation of tryptophan residues in response to glycation reactions has not been previously reported. Therefore, PM protection of tryptophans from oxidation due to glucose is novel. Lysozyme contains six tryptophan resides, of which W-28, 62, 63, and 108 were found to be oxidized. The highest HTRP relative intensity levels were found to occur on W-62 and W-63, while decreased levels of HTRP were found in the presence of PM (Fig 24). Examination of the x-ray structure of lysozyme reveals that W-62 and W-63 are located near the vicinity of K-97, which is the lysine residue, along with K-96, found to contain the highest CML relative intensity levels. Therefore, perhaps glycation at these lysine residues influenced the oxidation of W-63 and W-63. Again, oxidized

tryptophan residues can occur due to damage by ROS. In this particular case, ROS may have been generated due to the autoxidation of glucose or the oxidation of other intermediates in the glycation pathway. In this case, as in previous studies, PM is protecting against the damage caused by ROS. Thus, the mass spectrometry experiments have confirmed that PM is indeed protecting against the formation of oxidized tryptophans.

Final Conclusions

The purpose of this study was to gain insight into how glucose modifications that arise from physiologically relevant levels of glucose affect model proteins and how PM inhibits these reactions. Biochemical analyses on RNase, lysozyme, BSA, and ubiquitin have revealed that glucose modifications do induce the formation of CML, cause protein degradation and cross-linking, and hinder enzyme function. However, PM prevents CML formation, protects protein integrity, and preserves enzyme function. More detailed mass spectrometry experiments involving lysozyme revealed site-specific information on the formation of CML and HTRP modifications along with providing evidence that the use of PM prevents these modifications. The novelties obtained from this study are as follows: (1) knowledge of glucose modifications arising from physiological concentrations of glucose; (2) confirmation of PM protection of protein function; (3) evidence of PM protection of protein cross-linking; and (4) indication of tryptophan oxidation due to glucose and PM protection of tryptophan oxidation. The data gained from this study further advances the knowledge of the benefits of PM along with providing insight into the mechanism of PM action.

Future Work

Future plans are ongoing to further study lysozyme by mass spectrometry. The experiments presented here will be repeated several times in order to obtain statistically significant data. Also, there are other modifications that arise from glucose, which can be identified and quantified. Though beyond the scope of this work, similar studies involving mass spectrometry can also be extended to involve the other model proteins in this study, RNase, BSA, and ubiquitin, in order to gain further knowledge of PM mechanism and how glucose modifications are affected by protein environment.

REFERENCES

Ahmed, M. U., E. Brinkmann Frye, et al. (1997). "N-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins." Biochem J **324 (Pt 2)**: 565-70.

Ahmed, M. U., S. R. Thorpe, et al. (1986). "Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein." J Biol Chem **261**(11): 4889-94.

Basta, G., A. M. Schmidt, et al. (2004). "Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes." Cardiovasc Res **63**(4): 582-92.

Baynes, J. W. (2000). "From life to death--the struggle between chemistry and biology during aging: the Maillard reaction as an amplifier of genomic damage." Biogerontology **1**(3): 235-46.

Blake, C. C., D. F. Koenig, et al. (1965). "Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom resolution." Nature **206**(986): 757-61.

Booth, A. A., R. G. Khalifah, et al. (1996). "Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine." Biochem Biophys Res Commun **220**(1): 113-9.

Booth, A. A., R. G. Khalifah, et al. (1997). "In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways." J Biol Chem **272**(9): 5430-7.

Brock, J. W., D. J. Hinton, et al. (2003). "Proteomic analysis of the site specificity of glycation and carboxymethylation of ribonuclease." J Proteome Res **2**(5): 506-13.

Brown, B. E., R. T. Dean, et al. (2005). "Glycation of low-density lipoproteins by methylglyoxal and glycolaldehyde gives rise to the in vitro formation of lipid-laden cells." Diabetologia **48**(2): 361-9.

Brownlee, M. (1995). "Advanced protein glycosylation in diabetes and aging." Annu Rev Med **46**: 223-34.

Brownlee, M., A. Cerami, et al. (1988). "Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications." N Engl J Med **318**(20): 1315-21.

Brownlee, M., A. Cerami, et al. (1988). "Advanced products of nonenzymatic glycosylation and the pathogenesis of diabetic vascular disease." Diabetes Metab Rev **4**(5): 437-51.

Carlisle, C. H., R. A. Palmer, et al. (1974). "The structure of ribonuclease at 2-5 Angstrom resolution." J Mol Biol **85**(1): 1-18.

Ciechanover, A. (1998). "The ubiquitin-proteasome pathway: on protein death and cell life." Embo J **17**(24): 7151-60.

Degenhardt, T. P., N. L. Alderson, et al. (2002). "Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat." Kidney Int **61**(3): 939-50.

Degenhardt, T. P., S. R. Thorpe, et al. (1998). "Chemical modification of proteins by methylglyoxal." Cell Mol Biol (Noisy-le-grand) **44**(7): 1139-45.

Finley, E. L., J. Dillon, et al. (1998). "Identification of tryptophan oxidation products in bovine alpha-crystallin." Protein Sci **7**(11): 2391-7.

Hayase, F., R. H. Nagaraj, et al. (1989). "Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction in vivo." J Biol Chem **264**(7): 3758-64.

Heidland, A., K. Sebekova, et al. (2001). "Advanced glycation end products and the progressive course of renal disease." Am J Kidney Dis **38**(4 Suppl 1): S100-6.

Huang, B. X., H. Y. Kim, et al. (2004). "Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry." J Am Soc Mass Spectrom **15**(8): 1237-47.

Imanaga, Y., N. Sakata, et al. (2000). "In vivo and in vitro evidence for the glycoxidation of low density lipoprotein in human atherosclerotic plaques." Atherosclerosis **150**(2): 343-55.

Jain, A. K., G. Lim, et al. (2002). "Effect of high-glucose levels on protein oxidation in cultured lens cells, and in crystalline and albumin solution and its inhibition by vitamin B6 and N-acetylcysteine: its possible relevance to cataract formation in diabetes." Free Radic Biol Med **33**(12): 1615-21.

Jain, S. K. and G. Lim (2001). "Pyridoxine and pyridoxamine inhibits superoxide radicals and prevents lipid peroxidation, protein glycosylation, and (Na+ + K+)-ATPase activity reduction in high glucose-treated human erythrocytes." Free Radic Biol Med **30**(3): 232-7.

Kalnitsky, G., J. P. Hummel, et al. (1959). "[Some factors which affect the enzymatic digestion of ribonucleic acid.]." J Biol Chem **234**(6): 1512-6.

Khalifah, R. G., J. W. Baynes, et al. (1999). "Amadorins: novel post-Amadori inhibitors of advanced glycation reactions." Biochem Biophys Res Commun **257**(2): 251-8.

Kislinger, T., A. Humeny, et al. (2003). "Relative quantification of N(epsilon)-(Carboxymethyl)lysine, imidazolone A, and the Amadori product in glycated lysozyme by MALDI-TOF mass spectrometry." J Agric Food Chem **51**(1): 51-7.

Lapolla, A., D. Fedele, et al. (2001). "Advanced glycation end products: a highly complex set of biologically relevant compounds detected by mass spectrometry." J Mass Spectrom **36**(4): 370-8.

Li, J. M. and A. M. Shah (2003). "ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy." J Am Soc Nephrol **14**(8 Suppl 3): S221-6.

Liebler, D. C. (2002). Introduction to Proteomics. Totowa, NJ, Humana Press.

Manza, L. L., S. L. Stamer, et al. (2005). "Sample preparation and digestion for proteomic analyses using spin filters." Proteomics **5**(7): 1742-5.

Mennella, C., M. Visciano, et al. (2005). "Glycation of lysine-containing dipeptides." J Pept Sci.

Metz, T. O., N. L. Alderson, et al. (2003). "Pyridoxamine, an inhibitor of advanced glycation and lipoxidation reactions: a novel therapy for treatment of diabetic complications." Arch Biochem Biophys **419**(1): 41-9.

Odani, H., T. Shinzato, et al. (1998). "Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia." FEBS Lett **427**(3): 381-5.

Onorato, J. M., A. J. Jenkins, et al. (2000). "Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine." J Biol Chem **275**(28): 21177-84.

Ookawara, T., N. Kawamura, et al. (1992). "Site-specific and random fragmentation of Cu,Zn-superoxide dismutase by glycation reaction. Implication of reactive oxygen species." J Biol Chem **267**(26): 18505-10.

Passmore, L. A. and D. Barford (2004). "Getting into position: the catalytic mechanisms of protein ubiquitylation." Biochem J **379**(Pt 3): 513-25.

Raabe, H. M., J. H. Hopner, et al. (1998). "Biochemical and biophysical alterations of the 7S and NC1 domain of collagen IV from human diabetic kidneys." Diabetologia **41**(9): 1073-9.

Raabe, H. M., H. Molsen, et al. (1996). "Biochemical alterations in collagen IV induced by in vitro glycation." Biochem J **319 (Pt 3)**: 699-704.

Reddy, S., J. Bichler, et al. (1995). "N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins." Biochemistry **34**(34): 10872-8.

Sasaki, N., R. Fukatsu, et al. (1998). "Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases." Am J Pathol **153**(4): 1149-55.

Sell, D. R. and V. M. Monnier (1989). "Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process." J Biol Chem **264**(36): 21597-602.

Shugar, D. (1952). "The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme." Biochim Biophys Acta **8**(3): 302-9.

Stitt, A., T. A. Gardiner, et al. (2002). "The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes." Diabetes **51**(9): 2826-32.

Takatori, A., Y. Ishii, et al. (2004). "Amelioration of the beta-cell dysfunction in diabetic APA hamsters by antioxidants and AGE inhibitor treatments." Diabetes Metab Res Rev **20**(3): 211-8.

Thornalley, P. J. (2005). "Dicarbonyl intermediates in the maillard reaction." Ann N Y Acad Sci **1043**: 111-7.

Thorpe, S. R. and J. W. Baynes (1996). "Role of the Maillard reaction in diabetes mellitus and diseases of aging." Drugs Aging **9**(2): 69-77.

Thorpe, S. R. and J. W. Baynes (2003). "Maillard reaction products in tissue proteins: new products and new perspectives." Amino Acids **25**(3-4): 275-81.

Vijay-Kumar, S., C. E. Bugg, et al. (1987). "Structure of ubiquitin refined at 1.8 A resolution." J Mol Biol **194**(3): 531-44.

Voziyan, P. A. and B. G. Hudson (2005). "Pyridoxamine as a multifunctional pharmaceutical: targeting pathogenic glycation and oxidative damage." Cell Mol Life Sci **62**(15): 1671-81.

Voziyan, P. A., R. G. Khalifah, et al. (2003). "Modification of proteins in vitro by physiological levels of glucose: pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation end-products through binding of redox metal ions." J Biol Chem **278**(47): 46616-24.

Voziyan, P. A., T. O. Metz, et al. (2002). "A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation." J Biol Chem **277**(5): 3397-403.

Yeboah, F. K., I. Alli, et al. (2004). "Effect of limited solid-state glycation on the conformation of lysozyme by ESI-MSMS peptide mapping and molecular modeling." Bioconjug Chem **15**(1): 27-34.