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Glycation of Lysozyme

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GLYCATION OF LYSOZYME

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In partial fulfillment of
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Master of Science in Chemistry
by
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Approved by
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Dr. Bin Wang

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DEDICATION

I dedicate my thesis work to my family and friends, especially to my beloved mother, who taught me that even the largest task can be accomplished if it is done one step at a time, whose prayers and words of encouragement got me to this point, and to my deceased father, Mr. Talib, who had dreamt to live to see this moment. Likewise, I want to dedicate this work and to my siblings who have been a constant encouragement and support throughout the duration of my study. This work is also dedicated to my brother, Marwan, for his moral support that helped me to overcome all the difficulties that I have encountered during my study. Finally, I would like to dedicate this work to my relatives who have supported me in various ways even though they were far away from me.

ACKNOWLEDGEMENTS

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In addition, I would like to thank the Higher Committee for Education Development in Iraq (HCED), for sponsoring me and providing me with a financial support that covered my master program's expenses. Finally, I would like to thank the Marshall University Department of Chemistry for accepting me as a master student and allowing me to use its laboratories.

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ABSTRACT

For this research project, I propose a method for the analysis of glycated lysozyme using Matrix Associated Laser Desorption / Ionization-Time of Flight Mass spectrometry (MALDI-TOF MS). Glycation or "Maillard reaction" is a non-enzymatic reaction that occurs between amino acid residues, usually primary and secondary amines, of proteins and the carbonyl group of reducing sugars to yield Schiff bases. The glycation mechanism occurs between the carbonyl group of the reducing sugars and a primary amine by nucleophilic addition, resulting in the formation of a Schiff base. The Schiff base can then rearrange into the more stable Amadori product which is the first level of protein glycation. By processes such as oxidation, dehydration, and condensation, the Amadori product rearranges in time to form a heterogeneous group of compounds referred to advanced glycation end products (AGEs). Carnosine, a dipeptide consisting of β-alanine and histidine, has been shown to have anti-glycating characteristics that can hinder the damaging impact of aldo- and keto-sugars through oxidation, and it also defends against such age-stimulating processes as glycation, oxidation, and protein cross-linking. Lysozyme is an enzyme capable of destroying the bacterial cell wall, and it acts as protection from bacterial infection. The goal of this project was to determine the initial glycation sites on chicken and human lysozyme *in vitro*, determine how carnosine affects the initial glycation of lysozyme and the formation of AGE structures, and investigate how glycation affects the enzymatic activity of the enzyme. Lysozyme was incubated with D-glucose and D-galactose for three days at 37°C in the presence and absence of carnosine and analyzed by MALDI-TOF mass spectrometry. Lysozyme was primarily found to condense with one sugar molecule, and the preferred amino acid site of glycation for chicken lysozyme was identified as Lysine-115 which is not located near the catalytic cleft of the enzyme. In fact, the enzymatic activity of chicken

lysozyme did not change significantly when it was glycated using my glycation conditions. When both chicken and human lysozyme was incubated with sugars in the presence of carnosine, I found that carnosine did not inhibit the initial glycation reaction between lysozyme and D-galactose or D-glucose. The final experiment was to determine the effect of carnosine on the formation of AGE structures on both chicken and human lysozyme after the initial glycation reaction had occurred. The results of these experiments indicated that carnosine can decelerate or hinder the formation of AGE structures that can be obtained after the initial glycation of the protein.

INTRODUCTION

For this research project, I propose a method for the analysis of glycated lysozyme using Matrix Associated Laser Desorption / Ionization-Time of Flight Mass spectrometry (MALDI-TOF MS). The goal of this project is to determine the initial glycation sites on chicken and human lysozyme, determine how carnosine affects the initial glycation of lysozyme and the formation of AGE structures, and investigate how glycation affects the enzymatic activity of the enzyme.

Lysozyme

Lysozyme is a protein that was discovered and characterized by Alexander Fleming in 1922. It was the first enzyme whose tertiary protein structure was obtained by X-ray crystallography.[1] The name (lysozyme) was very suitable for this protein, because the first part *lyso-* refers to the ability to lyse bacteria and the second part *-zyme* because of its enzymatic activity. Lysozyme obtained from the egg white of hen eggs is a small enzyme protein that possesses a molecular weight about 14600 Da. and consists of 129 amino acids. From X-ray crystallography data, it was determined that lysozyme has about six disulfide bridges that provide stability to the protein structure.[1] Naturally occurring lysozyme can be found in animal tissues, organs, serum, tears, mucus, and saliva. Lysozyme also possess huge commercial potential, such as use as a food preservative, because of its ability to lyse bacteria and the fact that it is stable through a wide temperature and pH range.[2]

Lysozyme is an enzyme capable of destroying the bacterial cell wall, and it acts as protection from bacterial infection.[3] Lysozyme hydrolyzes the β (1-4) glycosidic linkage from N-acetylmuramic acid (NAM or MurNAc) to N-acetylglucosamine (NAG or GlcNAc) in particular polysaccharides as shown in Figure 1 below.[3] The linking oxygen that connects NAM and NAG

came from a hydroxyl group located on NAG. Lysozyme cleaves this bond which called the β (1-4) NAM-NAG glycosidic bond, because it originated from C-1 of NAM and the oxygen from the OH- group from the C-4 of NAG. This peptidoglycan consists of alternating chains of -NAG-NAM- units. The activity of the lysozyme enzyme can be measured in an activity assay by monitoring the lysis of the gram-positive bacterium *Micrococcus lysodeikticus* substrate by UV-Vis absorption spectrometry.

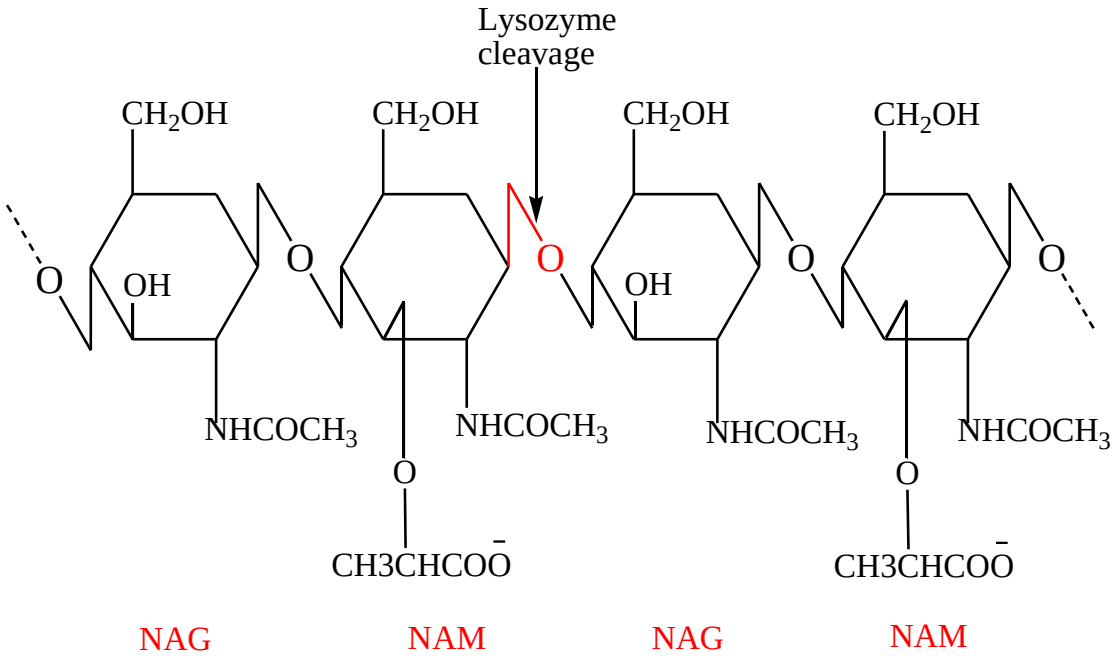

Figure 1: Diagram of the lysozyme cleavage site on a peptidoglycan. The lysozyme enzyme cleaves after a β (1-4) linkage in the alternating NAG-NAM polysaccharide component of bacterial cell walls.[4]

Hen Egg White Lysozyme

Hen egg white (HEW) possesses lysozyme by 3.5% of the total protein in the Hen Egg, and lysozyme serves as protection against bacteria and a source of protein for the chick.[5] Hen egg white lysozyme is one of the most significant types of lysozyme, which has been studied in a detailed manner.[3] It is a small protein with a single polypeptide chain that consists of 129 amino acid subunits bonded together on the inside by four disulfide bonds between the residues: Cys6-Cys12, Cys30-Cys115, Cys64-Cys80 and Cys76-Cys94. Its molecular weight is about 14,307 Da.[6] The conformation of lysozyme is nearly elliptical with dimensions 30 X 30 X 45 A˚, according to its shape by X-rays that have been clarified by David Philips in 1965 (Figure 2).[3]

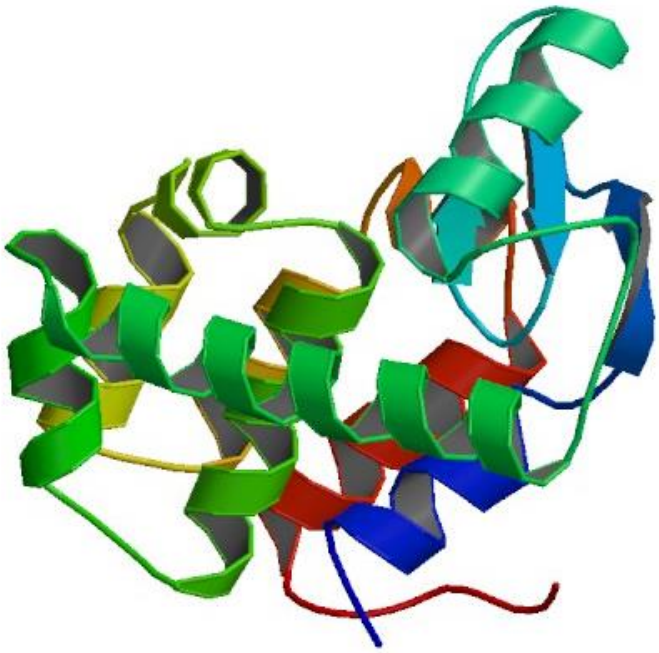

Figure 2: Tertiary structure of hen egg white lysozyme.[7]

Human Lysozyme:

Human lysozyme contains about 130 amino acid subunits and is found in secretions (such as milk, tears, spit, mucus, and perspiration), as well as in leukocytes and kidneys. Human lysozyme is able to hydrolyze the β (1-4) glycosidic linkage from N-acetylmuramic acid (NAM or MurNAc) to N-acetylglucosamine (NAG or GlcNAc) that take place in the cell wall of microorganisms.[8] Human milk possesses lysozyme that acts as a protection for the newborn because of its antimicrobial activity.[9] The amount of lysozyme that can be found in the milk of dairy animals (such bovine and caprine) are about 1600 to 3000 times lower than the amount of human milk; therefore, the bottle-fed babies are 3 times more likely to have diarrheal disease. An adult human milk has roughly 400 μg /mL lysozyme, while bovine and caprine milk has on average 0.130 μg /mL and 0.250 μg /mL of lysozyme.[9]

Glycation of protein:

Glycation or "Maillard reaction" is a non-enzymatic reaction that occurs between amino acid residues, usually primary and secondary amines, of proteins and the carbonyl group of reducing sugars to yield Schiff bases. In food chemistry, the reducing sugar, such as D-glucose, fructose, lactose, and their derivatives, are considered the main source of the carbonyl group. The N-terminal amino group and the ε-amino group of lysine residues are considered the main source of amines.[10]

The glycation mechanism occurs between the carbonyl group of the reducing sugars and a primary amine by nucleophilic addition, resulting in the formation of a Schiff base. The Schiff base can then rearrange into the more stable Amadori product which is the first level of protein glycation. By processes such as oxidation, dehydration, and condensation, the Amadori product rearranges in time to form a heterogeneous group of compounds referred to Advanced Glycation

End Products (AGEs).[11] The most common type of AGE structure formed on lysine residues is carboxymethyllysine (CML).[12] The reaction between the reducing sugar D-glucose and a lysine residue is shown in Figure 3.

The initial Schiff base formed upon glycation is a fully reversible product, and the sugar molecules can be hydrolyzed back off of the protein. Once the structure rearranges to the more stable Amadori product, the sugar residue is permanently attached to the protein. Over time, the sugar molecule attached to the protein can undergo oxidation, dehydration, and condensation reactions to form the AGE structures and even form cross-linked structures between amino acid residues within the same protein or between proteins which alters the structure and function of these proteins.

Protein glycation is influenced by many factors in vivo, including the nature and concentration of the carbonyl substrates, the availability and reactivity of the amino groups on proteins, and the chemical or enzymatic degradation of glycation products and their renal clearance.[13] The post-translational modification of proteins by sugars has been demonstrated in diabetes and classical galactosemia. The excess D-glucose in the bloodstream of patients suffering from diabetes results in the glycation of serum proteins, especially hemoglobin and albumin. In fact, glycated proteins may be the main pathogenic mediators of diabetic complications.[14] In patients with galactosemia, high levels of blood D-galactose can promote the galactation of serum proteins.[15]

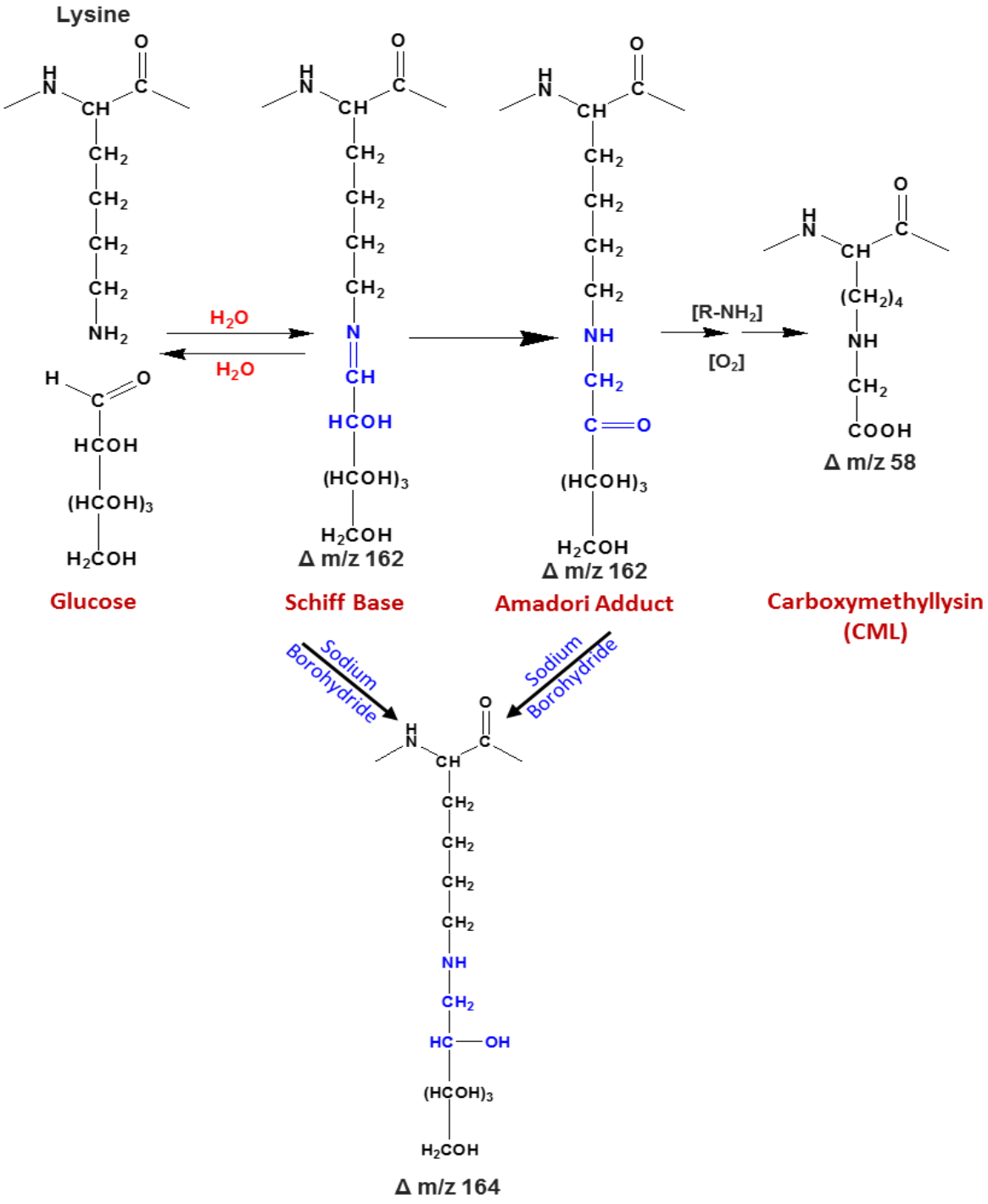

Figure 3: Reaction scheme for the glycation of lysine residues with D-glucose.[16]

Carnosine:

The dipeptide carnosine is a small molecule made up of two amino acids: β-alanyl-L-histidine (Figure 4). It was discovered over 100 years ago in Russia from beef extract; therefore, its name came from carno, carnis: meat.[17] It is one of the most common dipeptides in mammals. It has the ability to retard cellular senescence and renews cultured human fibroblasts; therefore, it has been implicated as an anti-aging agent.[18] It also plays an important role in the prevention of protein degradation. Carnosine is found in long-lived tissue such as the brain, kidney and muscle in a highly concentrated amount (up to 20 mM); however, its biological function is still under investigation. A comparison between carnosine concentrations in different animal species show that increased carnosine amounts correlates with increased life expectancy.[19] Carnosine has been shown to have anti-glycating characteristics that can hinder the damaging impact of aldo- and keto-sugars through oxidation, and it also defends against such age-stimulating processes as glycation, oxidation, and protein cross-linking.[18] Studies have shown that it can oppose the glycation of proteins and act to lessen some diabetic complications. None of the studies on the anti-glycating effect of carnosine have any direct evidence pertaining to its anti-glycating effect or mechanism of action. One of my aims for this work is to determine how carnosine affects the initial glycation of lysozyme and the formation of (advanced glycation endproduct) AGE structures.

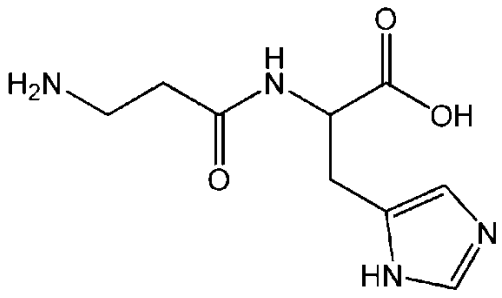

Figure 4: Carnosine structure: Beta-Alanyl-L-Histidine.[20]

MALDI-TOF MS:

MALDI-TOF MS (Matrix Associated Laser Desorption Ionization-Time of Flight Mass Spectrometry) is one of the numerous analytical technique that can be utilized for the biochemical analysis of peptides, proteins, polysaccharides, fatty acids, metabolites, and DNA molecules.[21] In the late 1980s, Hillenkamp, Karas, and Tanaka at the University of Munster in Germany developed the use of MALDI-TOF MS in the analysis of large nonvolatile molecules, such as DNA, proteins, and peptides.[19] The advantages of this technique are the accuracy, the speed, and the ease of use which allow researchers to measure the molecular weights of these molecules using a small amount of a sample. MALDI-TOF mass spectrometry has been successfully used for the analysis of glycated peptides and proteins[22, 23] making it an ideal choice for the analysis of the glycated lysozyme generated in this research project.

The MALDI-TOF Components:

MALDI is an ionization process which can generate gas-phase ions of intact proteins and peptides while the TOF is a sensitive mass analyzer used to separate the gas-phase ions by m/z value based upon flight times through a drift free region.[24] Every mass spectrometer possesses four main components: an ionization source which creates gas-phase ions, a mass analyzer which separates the ions by their m/z ratio, an ion detector, and a vacuum system. The ionization source is responsible for producing the gas phase ions of the molecules which can then be separated by the mass analyzer. Only ions, either positive or negative, will be affected by the mass analyzer.

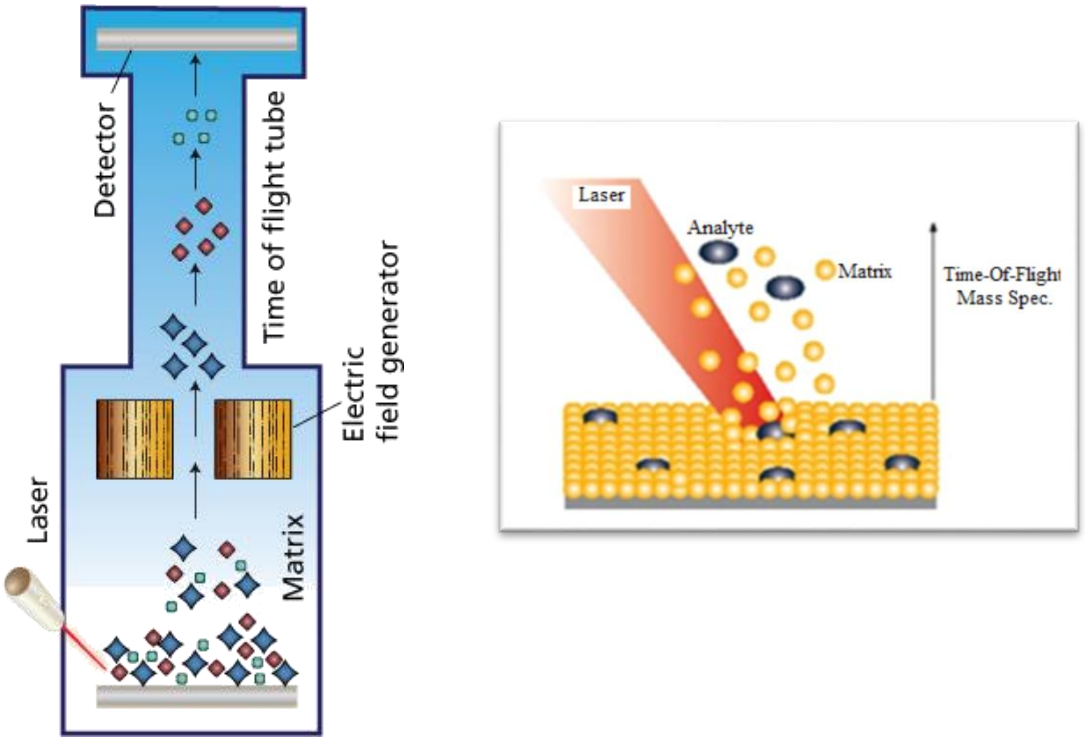

Figure 5: The MALDI-TOF mass spectrometer components.[25]

A good ionization leads to a successful analysis of the target molecules. The resulting ions could be either singly or multiple charged depending upon the ionization source used to create the ions. The ions that were produced by the ionizer will enter into the analyzer and get separated based upon their m/z ratio. There are different types of mass analyzers that differ from one another depending on the principle of the separation. The ions must not be allowed to react or collide with other gaseous molecules within the system which would interfere with their separation; therefore, the mass analyzer acts in a vacuum. The separation process in the mass analyzer and the ionization process occur very fast, thus, an efficient detection must be applied in order to obtain an accurate analysis. The detector, usually an electron multiplier tube, will detect the amount of ions striking its surface and convert this signal into an electrical signal (current). Finally, the data system will convert the current signals into a mass spectrum.

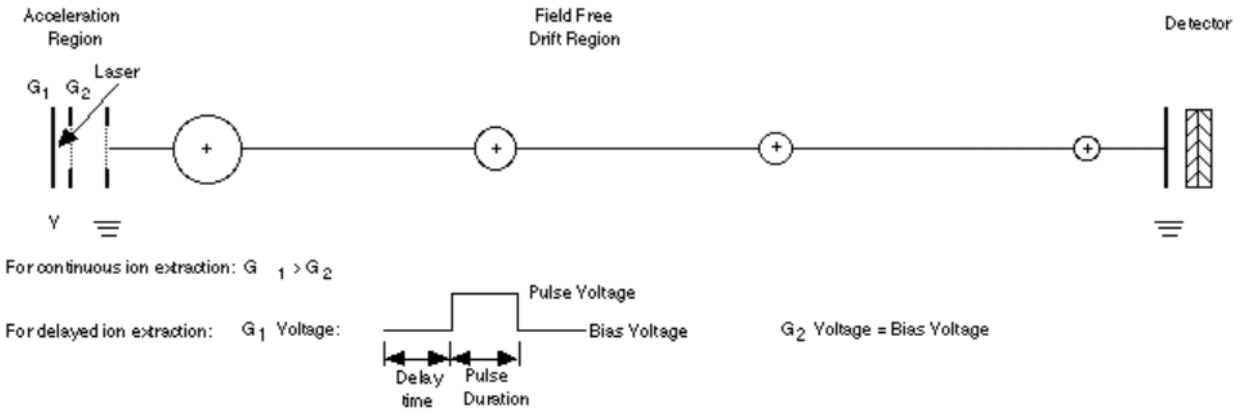

Figure 6: Basic components of a linear MALDI-TOF mass spectrometer.[26]

A linear MALDI-TOF mass spectrometer has a simple instrumental design (Figure 6). For MALDI-TOF analysis, the sample is uniformly mixed with excess matrix which absorbs the photons from the laser and converts it to heat energy (Figure 5). Some of the matrix heats rapidly and is vaporized along with the sample creating gas phase molecules. The matrix molecules then collide with the sample molecules donating a proton to the sample molecule upon collision creating $(M+H)^+$ sample gas phase ions. Generally only +1 ions are created for peptide samples, and both +1 and +2 ions are created for protein samples. The TOF mass analyzer separates ions based upon their m/z ratio. This mass analyzer is based on the rule that when a temporally and spatially well specific group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field (K.E. = [mv2]/2 = zeEs where K.E. = kinetic energy; m = the mass of the ion; v = velocity of the ion; z = number of charges; e = the charge on an electron in coulombs; E = electric field gradient; and s = the distance of the ion source region) and let to drift in area of constant electric field, then they are going to pass this area in a time which depends upon their m/z ratios.[26] Consequently, the time of ion flight differs according to the

mass-to-charge ratio (m/z) value of the ion. Each time the laser pulses, a packet of ions is generated and sent down the flight tube for detection.

Previous Experiments:

A few research labs have previously studied the glycation of lysozyme. Researchers at McGill University used electrospray ionization mass spectrometry (ESI-MS to study the glycation of lysozyme by both D-glucose and D-fructose under dry heating conditions.[27] They employed harsh glycation conditions which lead to the production of highly glycated chicken lysozyme which formed adducts containing up to 5 sugar residues. This study showed that lysozyme could be glycated in the presence of D-glucose; however, no actual sites of glycation were identified. In addition, the authors could not be certain which amino acid residues (Arg, Lys, His, Cys) were being glycated using these particular glycation conditions.

In 2010, Yukiko Maekawa and coworkers studied the glycation reaction of chicken lysozyme incubated with D-glucose at 37°C for 3, 9, 24, and 72 hours.[28] They analyzed the glycated lysozyme by MALDI-TOF mass spectrometry and found that the glycation reaction progressed within several hours under physiological conditions at 37°C. The resultant mass spectra can be seen in Figure 7 below. They also determined that lysozyme could take up to 10 D-glucose residues after 10 weeks of reaction.

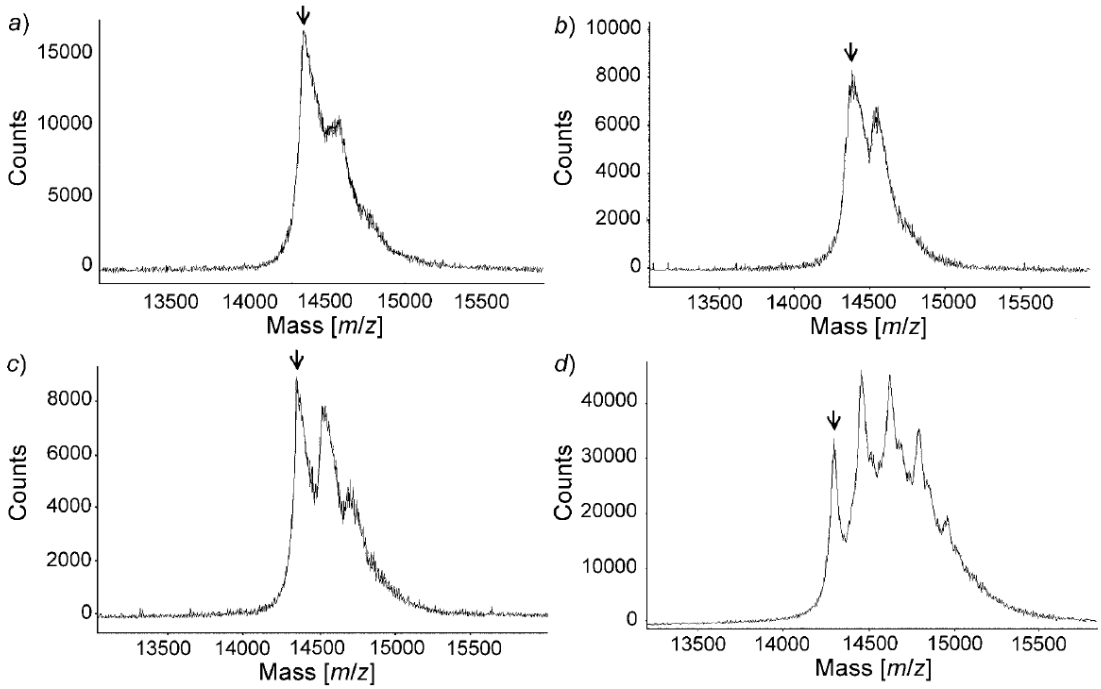

Figure 7: Reaction time-dependent MS spectra of glycated lysozyme a) 3 h, b) 9 h, c) 24 h, and d) 72 h after starting the glycation reaction. The arrow shows native lysozyme (m/z 14331).[28]

Researchers at the University of Wroclaw combined isotopic labelling and LCMS to identify glycated peptides produced from the tryptic digest of glycated lysozyme.[29] They used harsh glycation conditions of heating the lysozyme in the presence of excess D-glucose at 50°C for 14 days. Under these conditions, they identified glycated peptides which contained modified lysine residues from all six lysine residues present in chicken lysozyme. In addition, they identified several AGE structures located on arginine residues within the protein. When the researchers changed their glycation conditions to more resemble physiological conditions (incubation of protein in huge excess of D-glucose at 37°C for 40 days), they identified early glycation products on lysine residues 33, 96, and 97 within the amino acid sequence of chicken

lysozyme and AGE structures on several arginine residues. D-glucose was the only sugar used for these experiments.

Eric Dongliang Ruan and coworkers studied the glycation of lysozyme with D-glucose incubated at 50°C for up to 30 days.[30] They used a sugar to protein ratio of 1:6 for their experiments. Using both MALDI-TOF and ESI-MS, they were able to identify four lysine residues (1, 13, 97, and 116) that were glycated after 30 days of reaction. The MALDI-TOF spectra generated for the digested glycated lysozyme is shown in Figure 8 below.

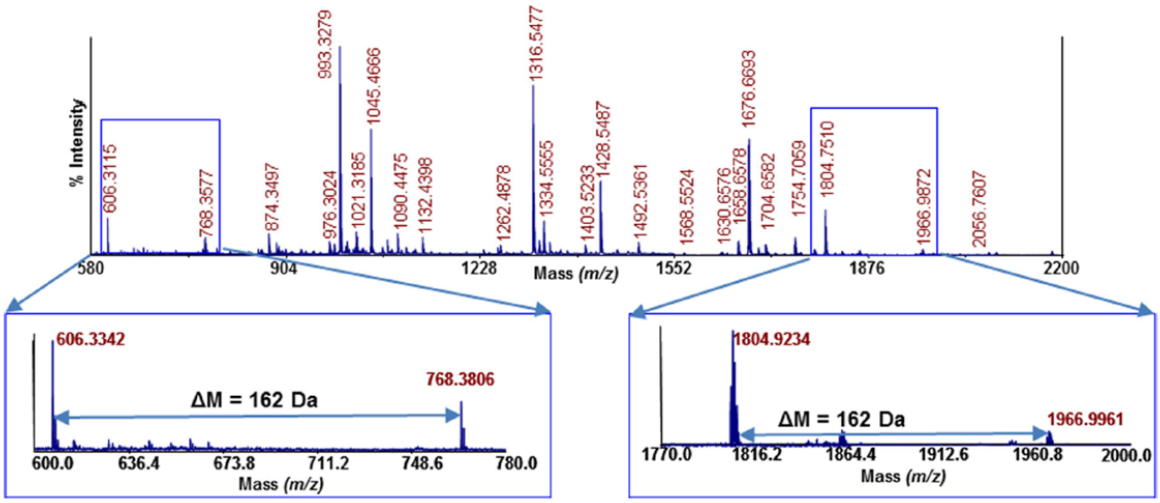

Figure 8: MALDI-TOF-MS spectra of digested glucosylated lysozyme (50°C for 30 days) with two potential glycated peptides (mass shift of 162 Da), comparison to original lysozyme (data not shown).[30]

Previous students in Frost's lab have studied the effect of carnosine on protein glycation. Derek Collins determined the effect of carnosine on the initial glycation of cytochrome *c* with D-galactose by monitoring the mass shifts that occurred when the protein was glycated (+162 Da/D-galactose addition). Protein samples incubated with D-galactose in the presence and absence of carnosine were analyzed daily for eleven days by MALDI-TOF mass spectrometry.

The resultant mass spectra can be seen in Figure 9. The glycation pattern when carnosine is added to the incubation mixture remains similar to when it is absent, indicating that carnosine does not inhibit the initial glycation reaction between cytochrome c and D-galactose.[31] Since carnosine did not inhibit the initial attachment of a sugar molecule to the cytochrome c protein, Miss Emma Gardner studied the effect of carnosine on the formation of the advanced glycation endproducts that occurs over time after the sugar is added to the protein. She determined that carnosine inhibited the formation of AGE structures on glycated cytochrome *c* (Figure 10). In particular, glycated Lys-13 was not converted to the AGE structure CML (carboxy methyl lysine) when carnosine was added to the incubation mixture. Lys-13 is located right next to the heme group in cytochrome *c*, so she speculated that the heme group could have participated in the conversion of the glycated lysine residue into the AGE structure and the carnosine could have been associating with the heme group preventing it from participating in the conversion.[16]

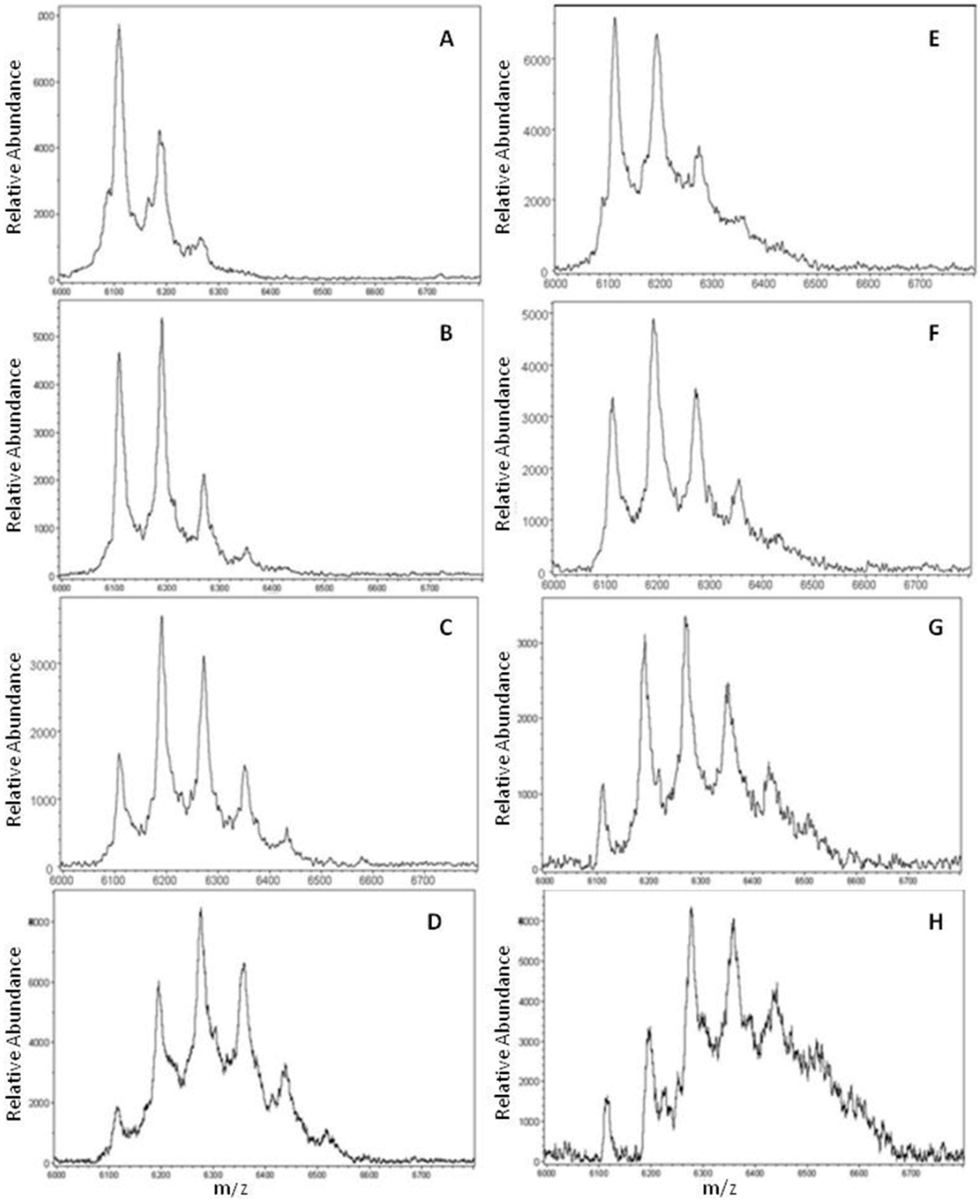

Figure 9: MALDI-TOF mass spectra of the +2 ion of bovine Cyt c incubated at 37°C in the presence of D-galactose for 2 days (A), 4 days (B), 7 days (C), and 11 days (D). Bovine cytochrome *c* incubated at 37°C in the presence of D-galactose and carnosine for 2 days (E), 4 days (F), 7 days (G), and 11 days (H).[31]

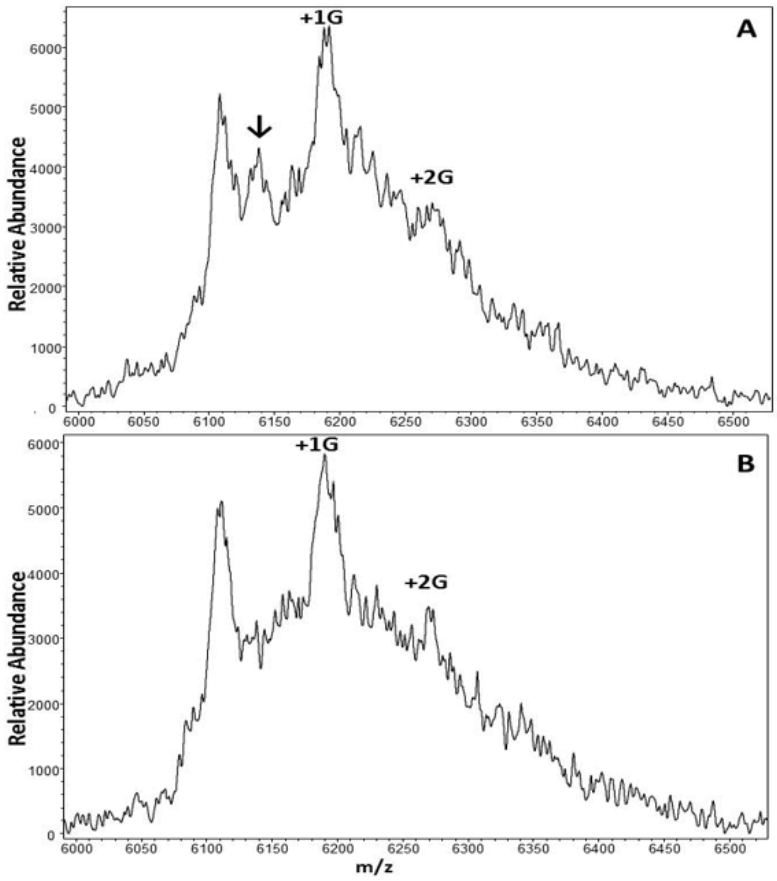

Figure 10: MALDI-TOF mass spectra of the +2 ion of glycated cytochrome *c* incubated at 37°C in the absence of carnosine (A) and in the presence of carnosine (B) for 42 days.[16]

For this research project, I propose studying the glycation of lysozyme with D-glucose and D-galactose under physiological conditions using short reaction times to identify physiologically relevant initial sites of glycation. In addition, I want to correlate these sites of glycation with the enzymatic activity of lysozyme to determine whether the initial glycation of lysozyme would have any effect on its reactivity.

Plasma lysozyme mainly comes from the disintegration of lysozyme containing cells of the blood (neutrophils and monocytes).[32] Plasma lysozyme was found to have a half-life of only 75 minutes in a rat using ^{125}I-labeled rat lysozyme, and the kidneys were found to take up

considerable amounts of lysozyme and retain the lysozyme for up to 4 days.[33] Thus, physiologically relevant glycation of lysozyme would occur in a shorter time frame than used in the previous experiments described above. Therefore, I propose glycating lysozyme at 37°C for two to three days and determining the most reactive amino acid sites towards glycation.

I also want to study the effect of carnosine on the initial glycation of lysozyme and the formation of AGE products. Previous experiments described above indicated that carnosine did not inhibit the initial glycation reaction but did inhibit the formation of AGE structures once glycation had occurred. These experiments were performed using the protein cytochrome *c* which contains a covalently attached heme group. It was speculated that the heme group may have been involved with the formation of the AGE structures that was inhibited by carnosine. Therefore, I wanted to repeat these experiments with a small protein that did not contain a heme group to see if the results would be the same as those with cytochrome *c*. Lysozyme is an ideal small protein for this study.

EXPERIMENTAL SECTION

Glycation of Chicken Lysozyme:

Briefly, 3 mg of lysozyme from chicken egg white (Sigma L4919) was dissolved into 300 μL of phosphate buffered saline (PBS). This solution was divided into three equal volumes and placed into three new microcentrifuge tubes. The total volume of the first tube was increased to 500 μL with PBS and labelled as control. For the second tube, 35 mg of D-galactose (Acros) was added, and the volume was diluted with PBS to a final volume of 500 μL. For the third tube, 35 mg of D-glucose (Sigma G7528) was added, and the volume was diluted to a final volume of 500 μL with PBS. All three solutions were vigorously vortexed until all the solid had been dissolved into the solution. The solutions were then incubated for two days at 37°C in a sand bath. The entire procedure was repeated using an incubation period of three days at 37°C. After incubation, an Ultracel YM-33 kDa cutoff filter was used to remove excess sugar from the sample prior to further analysis.

Glycation of Human Lysozyme:

The same procedure used for chicken lysozyme above was followed for the glycation of human lysozyme (recombinant protein, expressed in rice, Sigma L1667). The human recombinant protein has significantly higher bioactivity than the chicken lysozyme.

Effect of Carnosine on initial glycation of Lysozyme:

Briefly, 3 mg of chicken lysozyme (from chicken egg white) was dissolved into 300 mL of phosphate buffered saline (PBS). This solution was divided into three equal volumes and placed into three new microcentrifuge tubes. The total volume of the first tube was increased to 500 μL with PBS and labelled as control. For the second tube, 35 mg of D-galactose was added, and the volume was diluted with PBS to a final volume of 500 μL. For the third tube, 35 mg of

D-galactose and 50 mg of carnosine was added resulting in roughly a 2:1 molar ratio of carnosine to D-galactose, and the volume was diluted to a final volume of 500 μL with PBS. All three solutions were vigorously vortexed until all the solid had been dissolved into the solution. The solutions were then incubated for up to 11 days at 37°C in a sand bath. Aliquots of intact proteins were removed from the incubation mixtures each day during the 11 day incubation period and analyzed by MALDI-TOF mass spectrometry. The entire procedure was repeated using D-glucose as the reducing sugar.

The same procedure above was also used to study the effect of carnosine on the initial glycation of human lysozyme. The only difference in the procedure was that initially 3 mg of human recombinant protein was dissolved into 300 μL of PBS then split into three sample fractions.

Determining the Effect of Carnosine on AGE Structure Formation:

Glycated chicken lysozyme was prepared by incubating 2 mg of lysozyme with 70 mg of D-galactose in 500 μL of PBS, pH 7.4 for 3 days at 37°C. After incubation, an Ultracel YM-33 kDa cutoff filter was used to remove excess D-galactose from the sample prior to further analysis. The protein sample obtained after filtering was diluted to a volume of 500 μL with PBS and 250 μL of sample was transferred to a new microcentrifuge tube. Carnosine was added to one sample at a final concentration of 10 mM, and both samples were diluted to a total volume of 500 μL with PBS and allowed to incubate at 37°C for up to 42 days. A control sample of unglycated lysozyme was also incubated at 37°C for 42 days along with the glycated protein samples. Aliquots of intact proteins were periodically removed from each sample for analysis by mass spectrometry. The entire procedure was repeated using the human recombinant lysozyme protein. D-galactose was the only reducing sugar analyzed for these experiments.

Sodium Borohydride Reduction:

The Schiff base structure is not very stable making it difficult to analyze by MALDI-TOF mass spectrometry. To increase the stability of these structures, I reduced the Schiff base structure and even the Amadori structure to alcohols using sodium borohydride ($NaBH_4$). A newly prepared 100 mM solution of sodium borohydride (3.8 μg of $NaBH_4$ dissolved in 1.0 mL of 0.004N NaOH) was immediately added to each of the lysozyme protein samples in a 1:1 volume ratio. The solutions were incubated for 1 hour at room temperature, and the reactions were terminated by the addition of 1N HCl (1:12.5 vol/vol). After the addition of HCl, the mixtures were allowed to sit at room temperature for at least 5 min to make sure that the reaction was completely terminated. The pH of the samples was then adjusted to a pH of about 8 using 100 mM Tris buffer, pH 8.5.

Analysis of Intact Protein by MALDI-TOF Mass Spectrometry:

Protein samples were purified using C4 ZipTips (Millipore) and analyzed on a Bruker Autoflex MALDI-TOF mass spectrometer with instrument settings optimized for intact protein analysis. Prior to analysis, the protein preparations (0.7 μL) were each mixed with a 50% aqueous acetonitrile solution (0.7 μL) of saturated α-cyano-4-hydroxy-cinnamic acid containing 0.05% TFA as matrix, spotted onto a stainless steel sample plate and allowed to air dry. The mass spectra were recorded in positive ion mode with a 60 nanosecond delay in the m/z range from 4000 to 20,000. Typically, 2000 spectra were accumulated with 50 laser shots for each sample spot analyzed. Analysis of data was performed using FlexAnalysis and ClinProTools (Bruker Daltonics Inc. Billerica, MA).

Analysis of Tryptic Digests of Proteins by Mass Spectrometry:

The sodium borohydride reduced lysozyme protein samples adjusted to pH 8 were digested with trypsin (1:100 w/w) overnight at room temperature. Trypsin would lyse the amide bond of the protein at the C-terminal side of arginine and lysine residues; however, trypsin would be unable to lyse a lysine residue with a sugar attached to it. The resultant tryptic peptides produced were purified with C18 ZipTips (Millipore) and analyzed on a Bruker Autoflex MALDI-TOF mass spectrometer (Billerica, MA) to produce a peptide fingerprint mass spectrum which allowed us to determine candidate glycated peptides for further analysis. Prior to mass spectral analysis, the peptide preparations (0.7 μL) were mixed with a 50% aqueous acetonitrile solution (0.7 μl) of saturated α-cyano-4-hydroxy-cinnamic acid (CHCA), spotted onto a stainless steel sample MALDI plate sample and allowed to air dry. The mass spectra were recorded in positive ion mode with a 60 nsec delay in the m/z range from 500 to 3500. Roughly 2000 spectra were accumulated with 50 laser shots for each sample spot analyzed. The resulting peptide mass fingerprint spectra obtained was used to identify glycated peptides by comparing the experimental peptide molecular weights with the molecular weights of peptides obtained from a theoretical digest of lysozyme with trypsin.

The tryptic peptides were also fractionated on 10μm spherical C_{18} resins (YMC, Inc. Milford, MA) and eluted directly into the electrospray source of a ThermoFinnigan LCQ mass spectrometer (ThermoScientific, Waltham, MA) equipped with a quadrupole ion trap mass analyzer. The loading of peptides onto the columns was performed using a helium bomb. Once applied to the columns, the peptides were first washed with 0.1% acetic acid for 3 minutes and were then collectively eluted from the columns with 50% acetonitrile in 0.1% acetic acid at a flow rate of 2 μL/min using the helium bomb. The column eluates directed into the electrospray

source were analyzed by MS and tandem mass spectrometry (CID fragmentation with He) favoring isolation and fragmentation of doubly charged ions. CID mass spectra were generated for potentially glycated peptide identified by MALDI-TOF mass spectrometry to confirm the sites of glycation in the lysozyme protein.

The ESI/MS system was operated with the Xcalibur software (version 2.0, ThermoFinnigan), with the same software used also for data analysis. The mass spectrometer was set to function in the positive ion mode with parameters optimized during direct infusion of peptide standards with solvent. Nitrogen was used as the sheath gas (setting at 60), and ultrapure helium was used as the collision gas. The ion spray voltage was set as 4.5 kV and the capillary temperature was 210°C.

Lysozyme Enzymatic Assay:

For this experiment, chicken lysozyme was used to determine the effect of glycation on the enzymatic activity of lysozyme. According to the manufacturer, this assay procedure could not be used for the recombinant human lysozyme protein.

The lysozyme assay that I performed used *Micrococcus lysodeikticus* bacterial cells as a substrate for the lysozyme. The enzymatic rate determination (A450, Light path = 1 cm) depends on the following reaction:

$$\text{Micrococcus lysodeikticus Cells (Intact)} \xrightarrow{\text{Lysozyme}} \text{Micrococcus lysodeikticus Cells (Lysed)}$$

According to the assay procedure obtained on Sigma's website (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-lysozyme.html), one unit of lysozyme should produce a change in absorbance at a wavelength of 450 nm of 0.001 per minute at pH 6.24 and 25°C using a suspension of *Micrococcus lysodeikticus* as substrate in a 2.6 ml reaction mixture. Therefore, by monitoring the change of

absorbance over time at this wavelength, I was able to determine the rate of the enzymatic reaction for unglycated and glycated lysozyme protein samples. The absorbance was measured on a Cary 50 Scan UV-Visible spectrophotometer.

For each enzymatic assay performed, fresh suspensions of *Micrococcus lysodeikticus* were prepared by dissolving the cells in 66 mM Potassium Phosphate Buffer, pH 6.24, to create a 0.015% (w/v) *Micrococcus lysodeikticus* Cell Suspension. The absorbance of the solution was then checked to be sure that the starting absorbance values were between 0.7 to 0.9. For the analysis, 100 μL of the lysozyme solutions were added to 2.5 mL of the substrate suspension in a cuvette tube. The solution was immediately mixed by inversion, the cuvette was placed in the Cary UV-Visible spectrophotometer, and the absorbance values were recorded over a period of five minutes. The start time after mixing was kept constant for all samples assayed. The spectrophotometer was run in kinetics mode, and the rate of the reaction over the first 2 minutes was obtained using the instrument software assuming first order reaction kinetics.

Five solutions were assayed for this experiment. The lysozyme solutions were glycated using the same procedure as described in a previous section, except all concentration were doubled to obtain good assay results. Briefly, 12 mg of chicken lysozyme was dissolved into 600 μL of phosphate buffered saline (PBS). This solution was divided into three equal volumes and placed into three new microcentrifuge tubes. The total volume of the first tube was increased to 1.0 mL with PBS and labelled as control. For the second tube, 140 mg of D-galactose was added, and the volume was diluted with PBS to a final volume of 1.0 mL. For the third tube, 140 mg of D-glucose was added, and the volume was diluted to a final volume of 1.0 mL with PBS. All three solutions were vigorously vortexed until all the solid had been dissolved into the solution. The solutions were then incubated at 37°C in a sand bath for up to 33 days. In addition, two more

control samples of 140 mg of D-galactose in 1.0 mL of PBS and 140 mg of D-glucose in 1.0 mL of PBS were incubated at 37°C for up to 33 days. Sample aliquots of all five sample solutions were removed at multiple time points to perform enzymatic assay experiments.

RESULTS AND DISCUSSION

1- Analysis of Glycated Lysozyme

A. Chicken Lysozyme

Chicken lysozyme was incubated with D-glucose and D-galactose for 2 or 3 days at 37°C. The excess sugar was then removed using a 3 KDa cutoff filter, and the protein was purified and concentrated using C18 ZipTips and analyzed by MALDI-TOF mass spectrometry. The basic experimental protocol is shown in Figure 11 below. Figure 12 shows the MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated with sugar at 37°C for 2 days. Initial protein glycation results in the formation of Schiff base and Amadori structures which would result in an increase of 162 Da to the protein for every sugar molecule attached. Since I am looking at the +2 ion of lysozyme, I should see a shift of 81 Da for every sugar molecules attached to lysozyme. There is a small amount of glycated protein seen in the spectra. The spectrum of unmodified chicken lysozyme (Figure 12A) showed a prominent single peak centered at m/z= 7,153. Under my conditions of incubation, which is biological conditions, I can see that the protein had begun to add some D-galactose molecules (Figure 12B) and D-glucose molecules (Figure 12C), but the glycated protein signal is weak. Therefore, I decided to incubate the proteins with the sugars for 3 days to see if this would result in a higher production of glycated protein. Figure 13 shows the results of MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days with D-galactose and D-glucose. Again, very little signal from glycated lysozyme was observed in the spectra (Figure 13B and 13C).

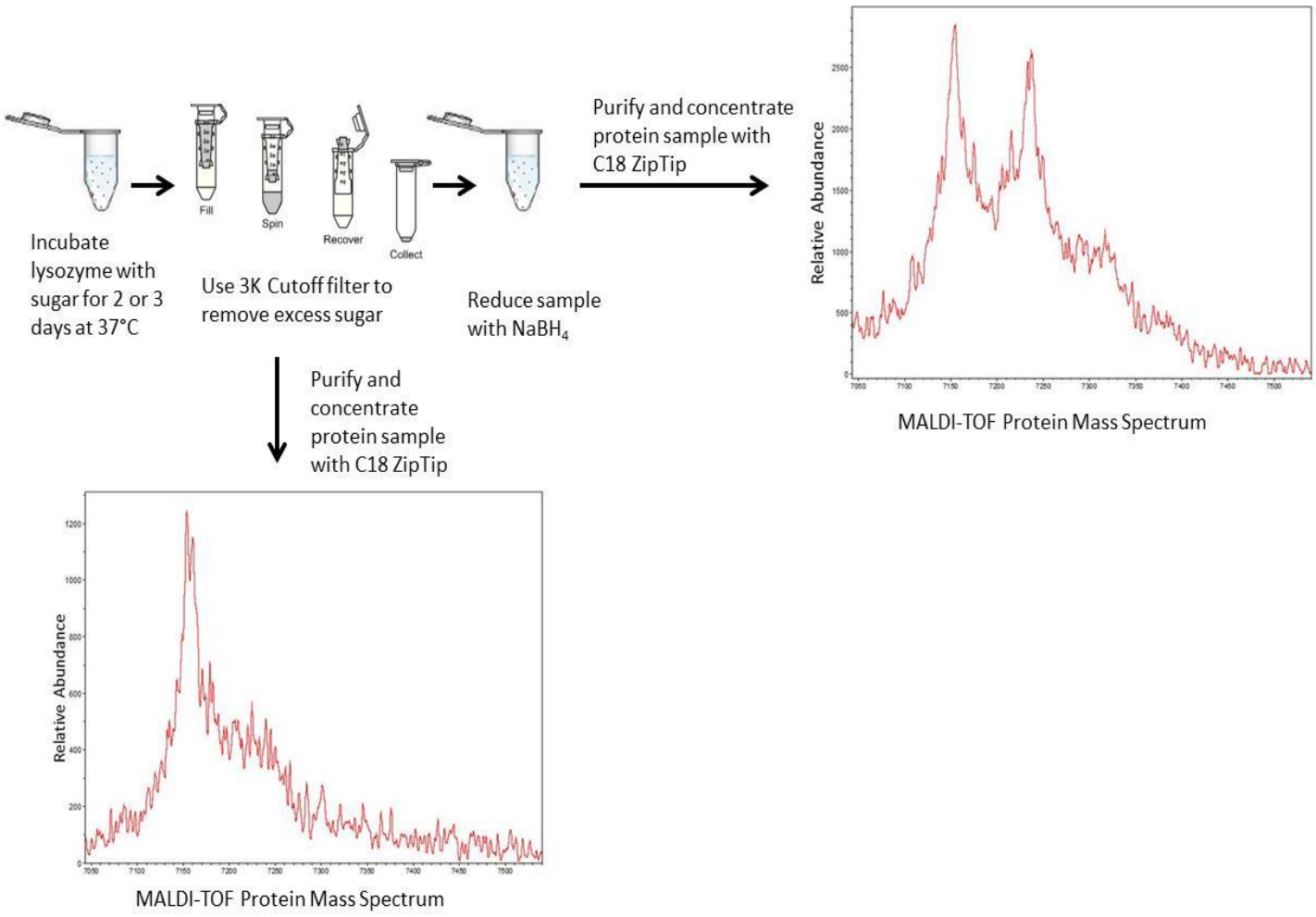

Figure 11: The experimental protocol for MALDI-TOF analysis of glycated lysozyme.

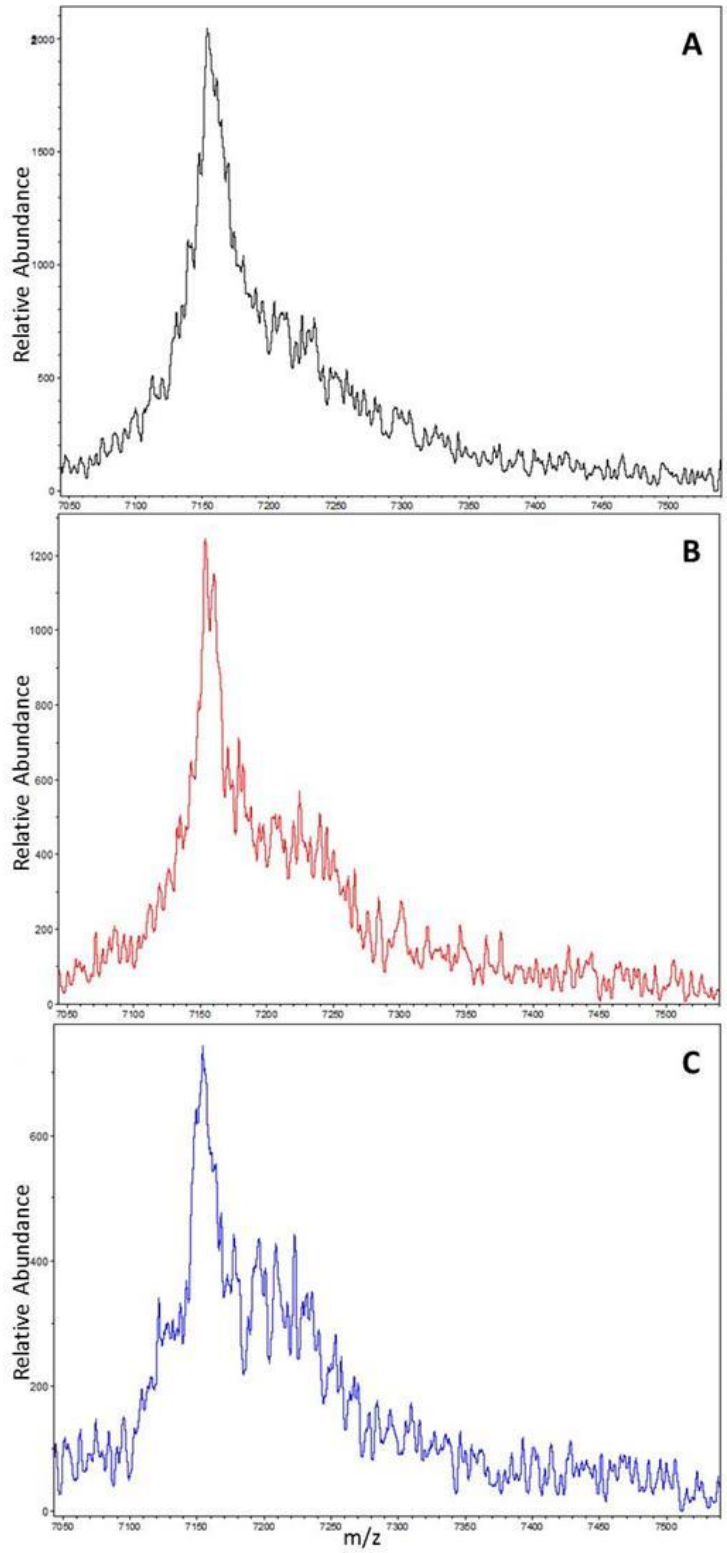

Figure 12: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 2 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C).

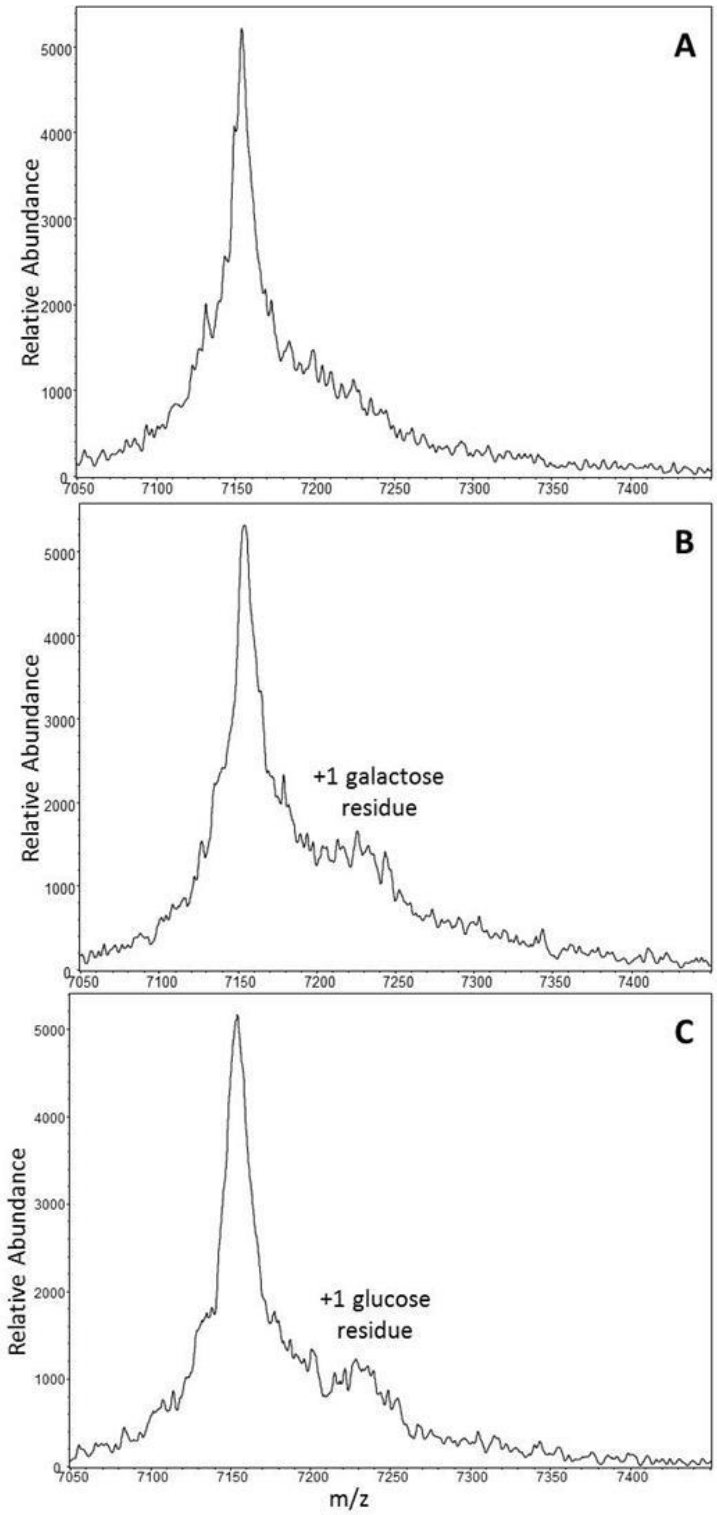

Figure 13: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C).

Schiff base structures are unstable, especially under the ionization conditions employed for MALDI-TOF mass spectrometry, making it difficult to analyze glycated proteins. Thus, sodium borohydride was utilized to reduce the samples in order to obtain a more stable glycation product to facilitate the analysis of glycated proteins. The Schiff base group is reactive making the structure less stable and could be causing the sugar to come off the protein during the MALDI desorption and/or ionization process. Reducing the structure with sodium borohydride ($NaBH_4$) would decrease the reactivity of this group making the molecule more stable (Figure 14). Likewise reducing the reactive carbonyl group of the Amadori structure to an alcohol would create a more stable structure which would hold up better during the MALDI desorption/ionization process. Every molecule of reduced sugar added to the protein would result in a protein mass shift of 164 Da. Since I am looking at the +2 ion of lysozyme, I should observe a mass shift of 82 Da for every sugar molecule attached to the protein.

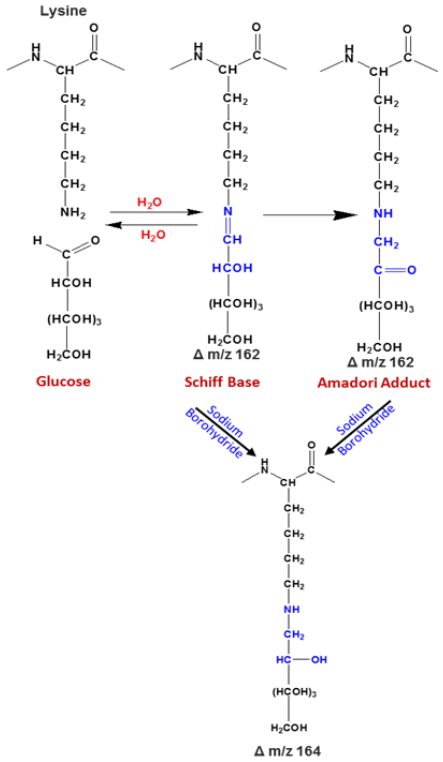

Figure 14: Reduction of glycated lysine structures with sodium borohydride.

Figure 15 shows the results of MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 2 days with sugar and reduced with sodium borohydride prior to mass spectral analysis. The mass spectrum for lysozyme incubated with D-galactose (Figure 15B) showed that lysozyme could be glycated with one D-galactose molecule under my incubation conditions. In fact, there is a small peak in the spectrum indicating the presence of protein modified with two D-galactose molecules. Likewise, when lysozyme was incubated with D-glucose, the protein was glycated with one sugar molecule, and a second molecule of sugar was beginning to attach to the protein. When the incubation period was extended to three days, the MALDI-TOF spectra of the resulting glycated proteins did not show much difference in the amount of glycation (Figure 16).

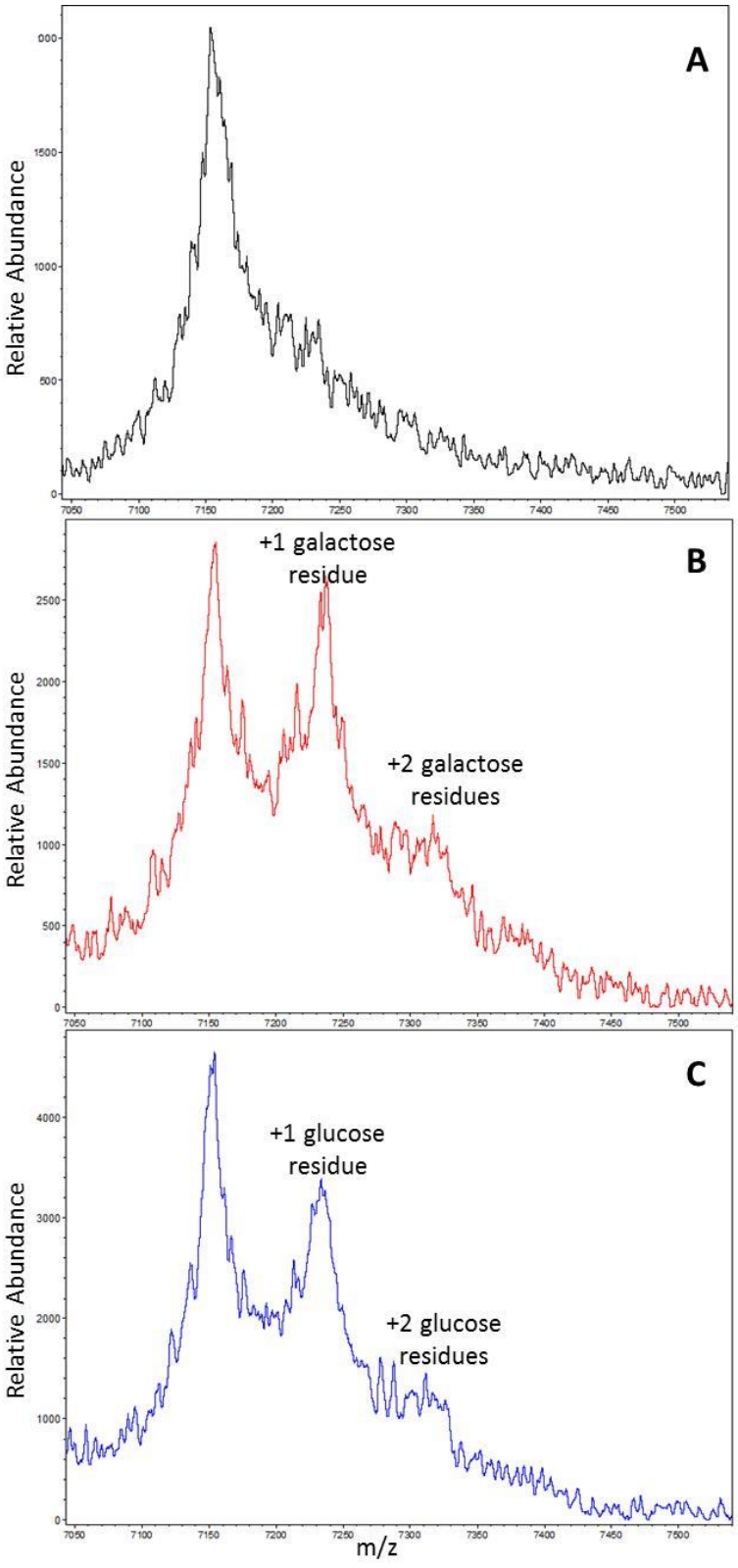

Figure 15: Reduced MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 2 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). All samples were reduced with $NaBH_4$ prior to analysis.

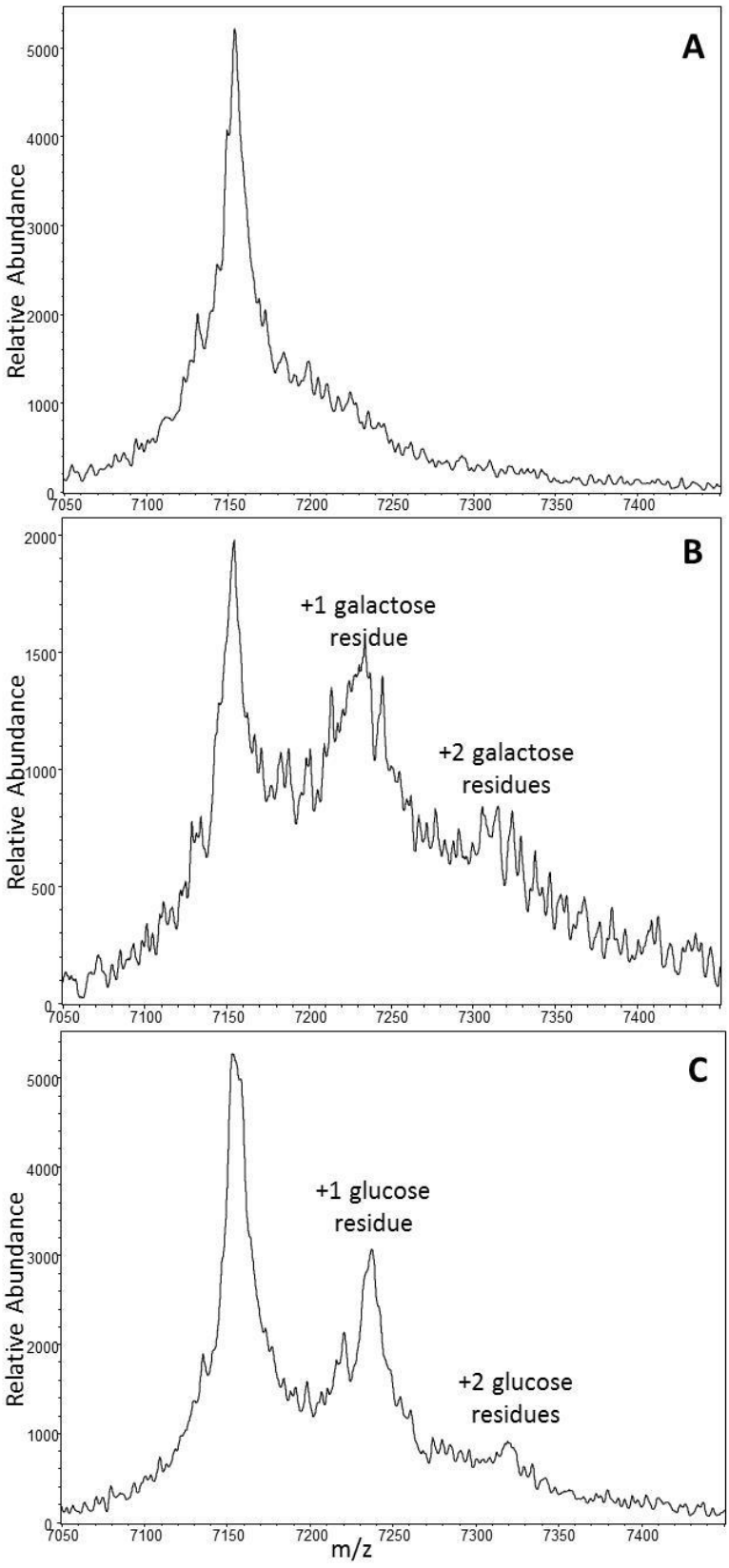

Figure 16: Reduced MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). All samples were reduced with $NaBH_4$ prior to analysis.

B. Human Lysozyme:

The glycation of human lysozyme was analyzed in the same manner as chicken lysozyme (Figure 11). Human lysozyme was incubated with D-galactose and D-glucose for 3 days at 37°C. I only used this glycation condition, because these were the conditions that produced enough glycated chicken lysozyme without overly glycating the protein. When the protein samples were reduced with sodium borohydride, the mass spectra revealed that roughly one sugar molecule was attached to the protein (Figure 17). The unmodified protein (Figure 17A) showed a prominent peak at about m/z 7340. Upon glycation with D-galactose (Figure 17B) and D-glucose (Figure 17C), the spectra contained protein peaks at about 82 Da higher than the unmodified protein indicating the addition of a sugar residue. In addition, the spectra revealed that a second molecule of D-galactose was just beginning to attach to the protein (Figure 17B).

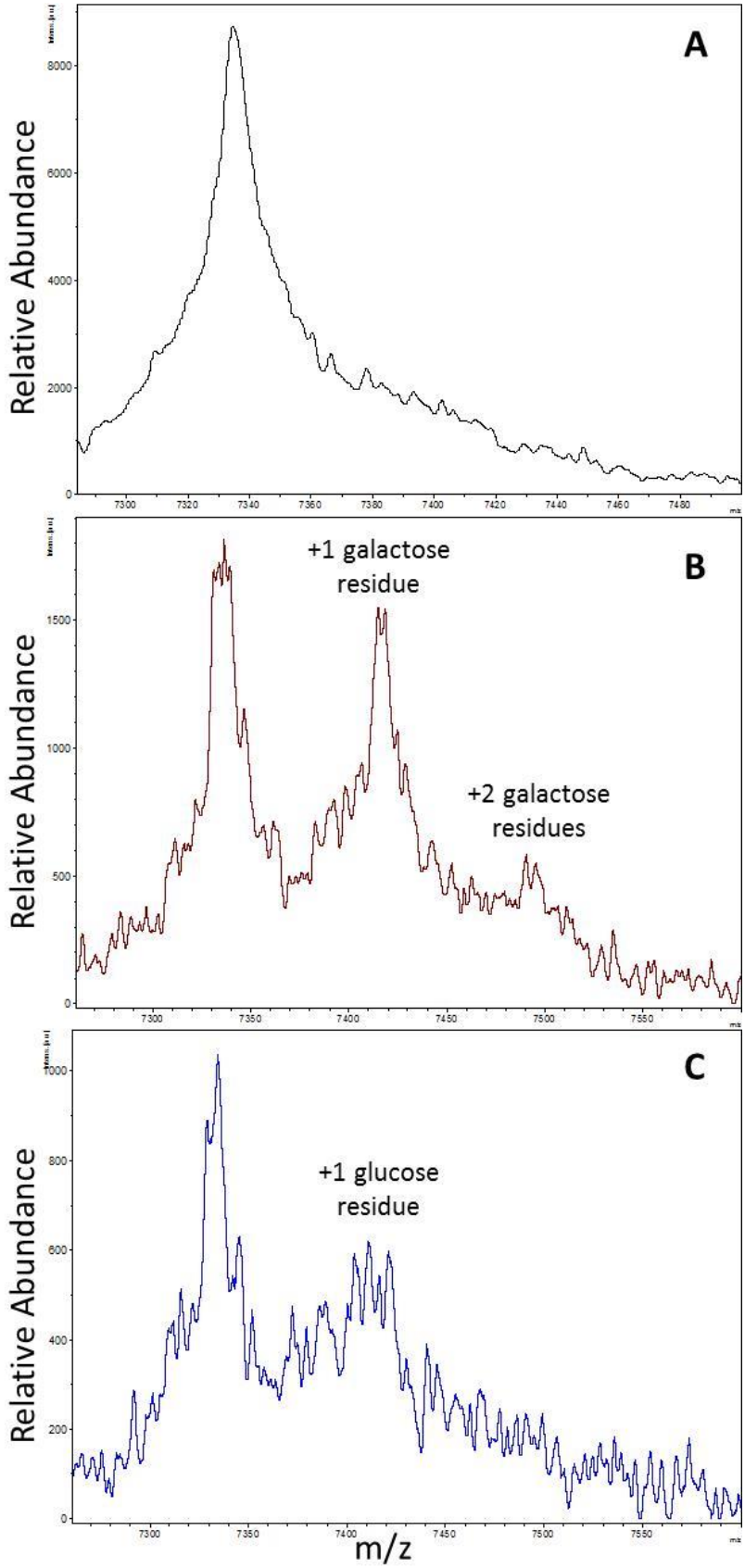

Figure 17: Reduced MALDI-TOF mass spectra of the +2 ion of human lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). All samples were reduced with $NaBH_4$ prior to analysis.

2- Effect of Carnosine on the Initial Glycation Reaction:

A. Chicken Lysozyme:

In order to study the effect of carnosine on the initial glycation reaction, I incubated samples of chicken lysozyme with D-galactose and D-glucose for 3 days at 37°C in the presence and absence of carnosine. The same basic protocol shown in Figure 11 was followed for these experiments. I was able to determine the extent of glycation for lysozyme in the presence of carnosine by monitoring the mass shifts that occur when the protein was glycated (+82 Da/D-galactose addition). The MALDI-TOF mass spectra for lysozyme incubated with D-galactose and D-glucose are shown in Figures 18 and 19, respectively. The signal for the glycated protein (+81 Da) is weak, so I again reduced the protein samples with sodium borohydride. The MALDI-TOF mass spectra for the reduced protein samples are shown in Figures 20 and 21. The signal for the glycated protein is much better after reduction showing that the protein could take up to 2 D-galactose molecules, $\Delta m/z$ 82 Da/sugar residue (Figure 20B), and up to 2 D-glucose molecules (Figure 21B). The glycation pattern when carnosine is added to the incubation mixture (Figure 20C and 21C) remains similar to when it is absent, indicating that carnosine does not inhibit the initial glycation reaction between lysozyme and D-galactose or D-glucose. In fact, carnosine may be aiding in the glycation of the protein and the formation of AGE structures. A previous student in Dr. Frost's lab conducted this same experiment with cytochrome *c* and carnosine and observed similar results (described in introduction).

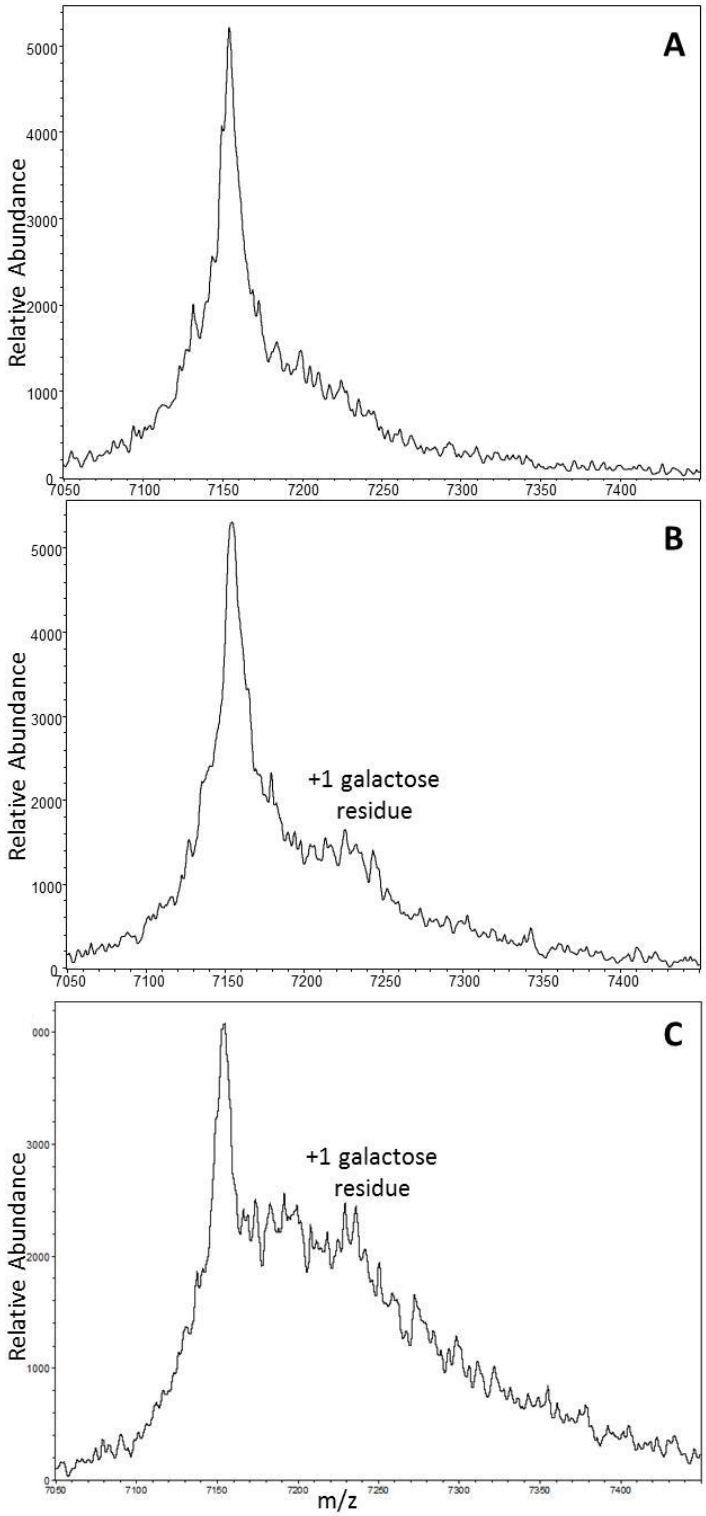

Figure 18: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-galactose and carnosine (C).

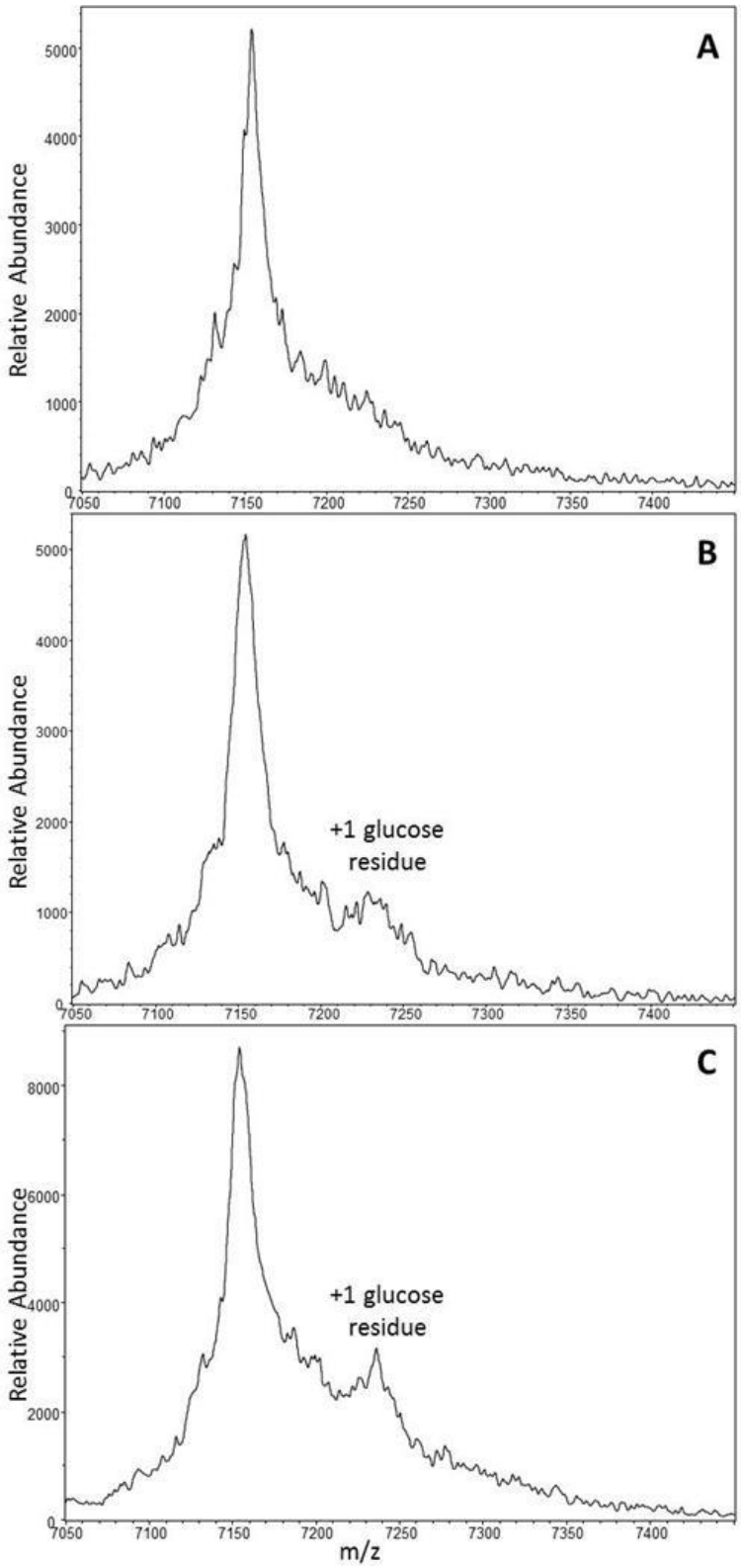

Figure 19: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-glucose (B), and in the presence of D-glucose and carnosine (C).

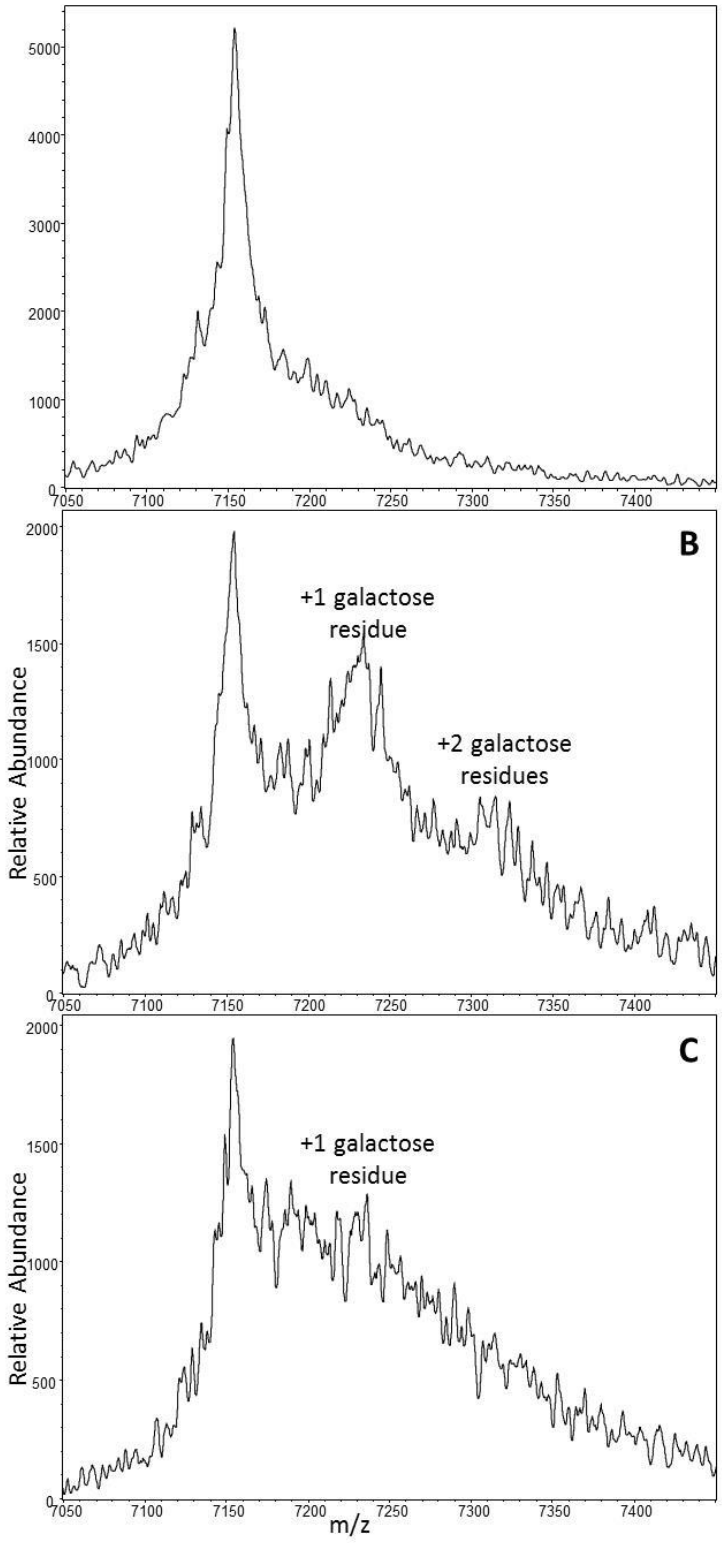

Figure 20: Reduced MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-galactose and carnosine (C). All samples were reduced with $NaBH_4$ prior to analysis.

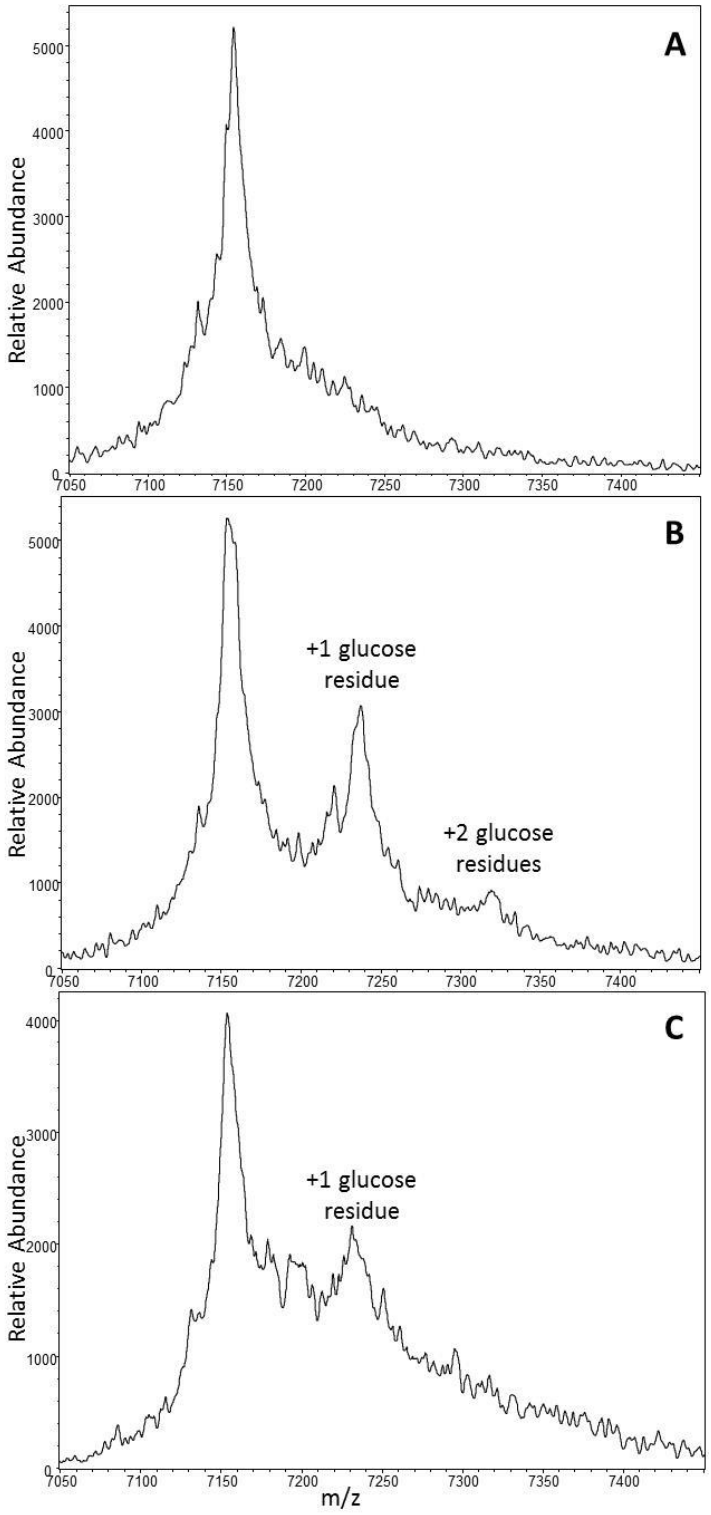

Figure 21: Reduced MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-glucose (B), and in the presence of D-glucose and carnosine (C). All samples were reduced with $NaBH_4$ prior to analysis.

B. Human Lysozyme:

I also studied the effect of carnosine on the initial glycation of human lysozyme. I incubated human lysozyme with D-galactose and D-glucose for 3 days at 37°C in the presence and absence of carnosine. The same basic protocol shown in Figure 11 was followed for these experiments. All samples were reduced with sodium borohydride prior to mass spectral analysis. The MALDI-TOF mass spectra for lysozyme incubated with D-galactose (Figure 22) and D-glucose (Figure 23) are shown below. The glycation pattern when carnosine is added to the incubation mixture (Figure 22C and 23C) remains similar to when it is absent (Figure 22B and 23B), indicating that carnosine does not inhibit the initial glycation reaction between lysozyme and D-galactose or D-glucose. In fact, the addition of carnosine to the incubation mixture may actually be promoting the formation of AGE structures. These results are the same as those obtained for both chicken lysozyme and cytochrome *c*.

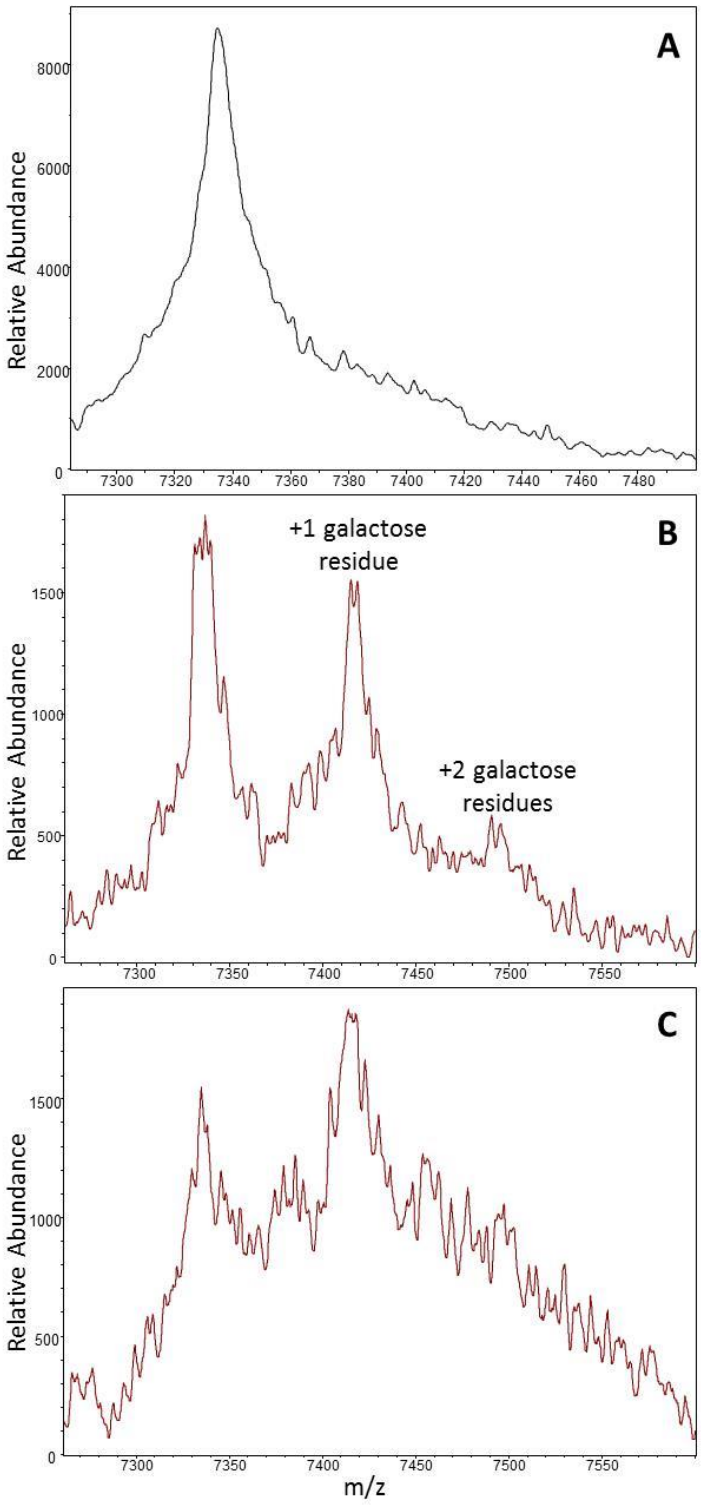

Figure 22: Reduced MALDI-TOF mass spectra of the +2 ion of human lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-galactose (B), and in the presence of D-galactose and carnosine (C). All samples were reduced with $NaBH_4$ prior to analysis.

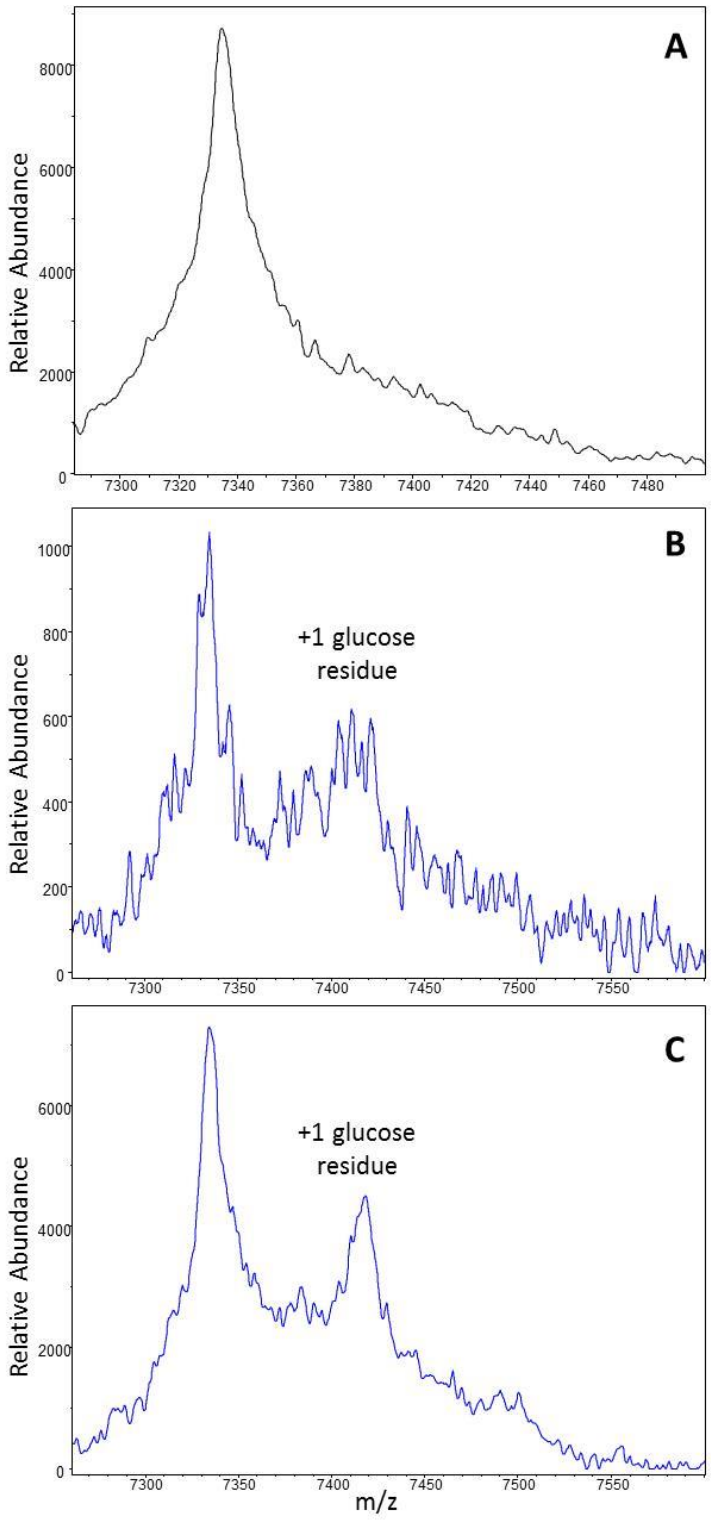

Figure 23: Reduced MALDI-TOF mass spectra of the +2 ion of human lysozyme incubated at 37°C for 3 days in the absence of sugar (A), and in the presence of D-glucose (B), and in the presence of D-glucose and carnosine (C). All samples were reduced with $NaBH_4$ prior to analysis.

3- Effect of Carnosine on Advanced Glycation Endproduct Formation:

A. Chicken Lysozyme:

In order to study the effect of carnosine on the formation of AGE structures, I initially incubated samples of chicken lysozyme without any sugar and with D-galactose for 3 days at 37°C. After incubation was complete, all excess sugar was removed using an Ultracel YM-33 kDa cutoff filter, and the remaining glycated protein solution was split into two equal portions. Carnosine was added to one sample to a final concentration of 10 mM, and both samples were diluted to 500 μL total volume with PBS. The unglycated protein and the glycated protein samples (with and without carnosine) were then incubated at 37°C for 42 days. Aliquots of protein were removed periodically and analyzed by MALDI-TOF mass spectrometry. The basic experimental protocol is shown in Figure 24. The resultant MALDI-TOF mass spectra for the protein samples after 28 days of incubation (Figure 25) and 42 days (Figure 26) are shown below. The arrows in the figures indicate the presence of AGE structures. The glycated chicken lysozyme that was not incubated with carnosine (Figure 26B) has a more prominent peak in the spectra at about m/z 7181 (indicated by the arrow) than when the glycated protein was incubated in the presence of carnosine. This peak had a mass that was about 58 Da (29 Da for the +2 ion) greater than the mass of the unmodified protein that would indicate the formation of an AGE structure. A known AGE structure that is formed between sugar and lysine residues is the carboxymethyllysine structure shown in Figure 3. This modification would result in the addition of 58 Da to the molecular weight of the protein. The addition of carnosine to the mixture (Figure 26C) decreases the formation of the AGE peak. In fact, the amount of glycation in general decreases when carnosine is added. Based on these results, this experiment suggests that carnosine can decelerate or hinder the formation of AGE structures that can be obtained after the

initial glycation of the protein. Recall that the formation of a Schiff base structure is an equilibrium reaction. Carnosine may actually serve to help shift this equilibrium back to the unmodified protein thus reversing the initial glycation of the lysozyme protein.

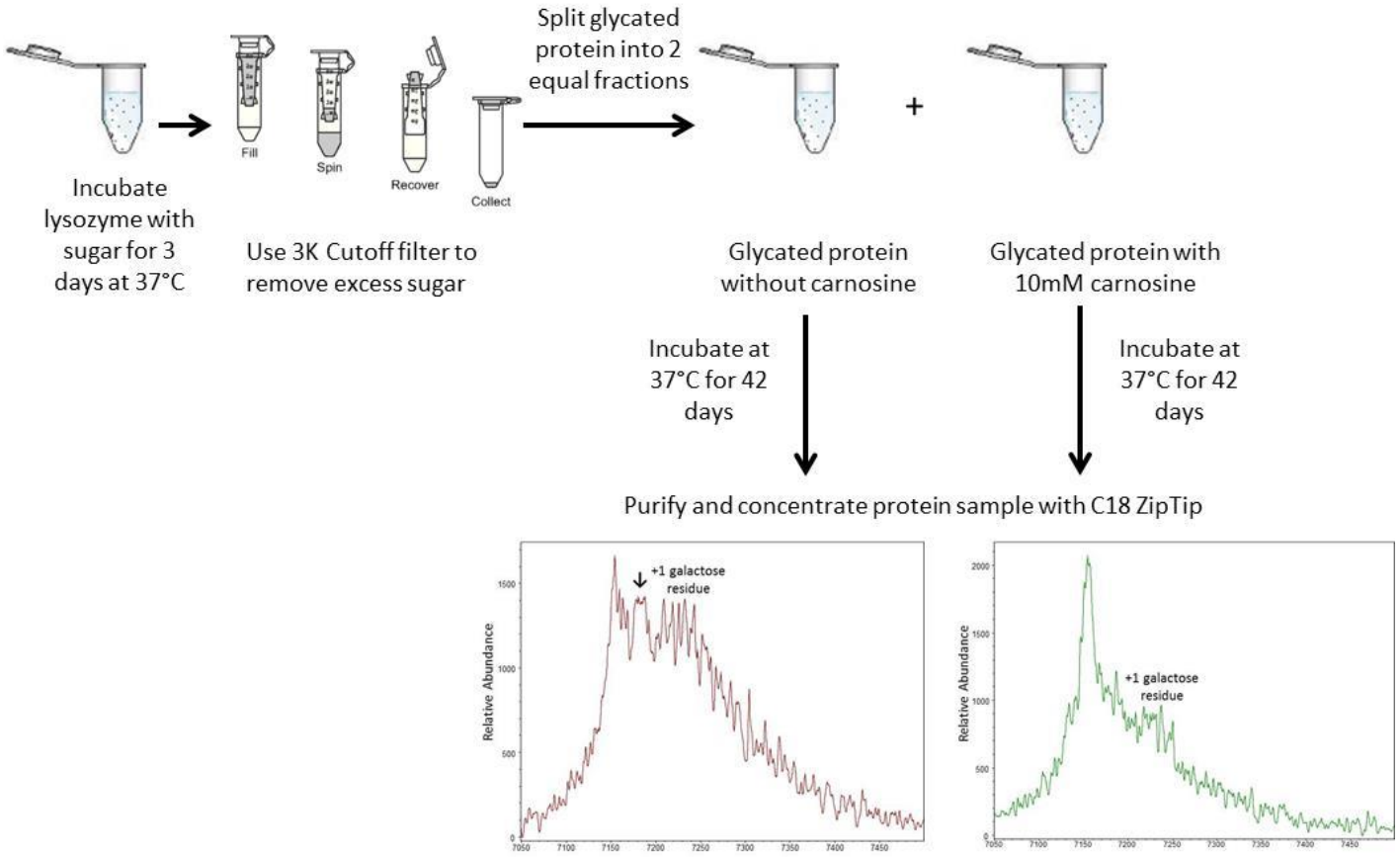

Figure 24: Experimental protocol for the study of the effect of carnosine on AGE structure formation.

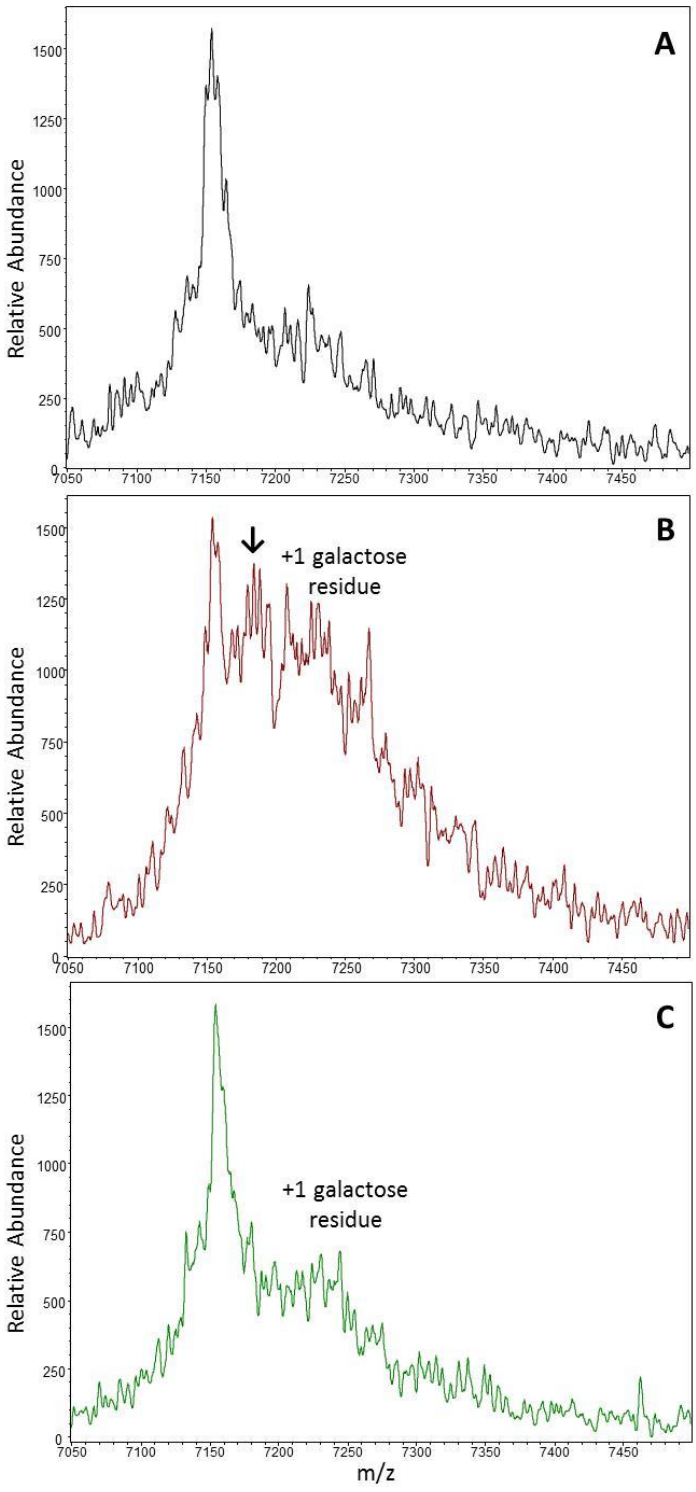

Figure 25: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 28 days. Nonglycated lysozyme (A) and glycated lysozyme incubated in the absence of carnosine (B) and in the presence of carnosine (C). The arrow indicates the presence of AGE structures.

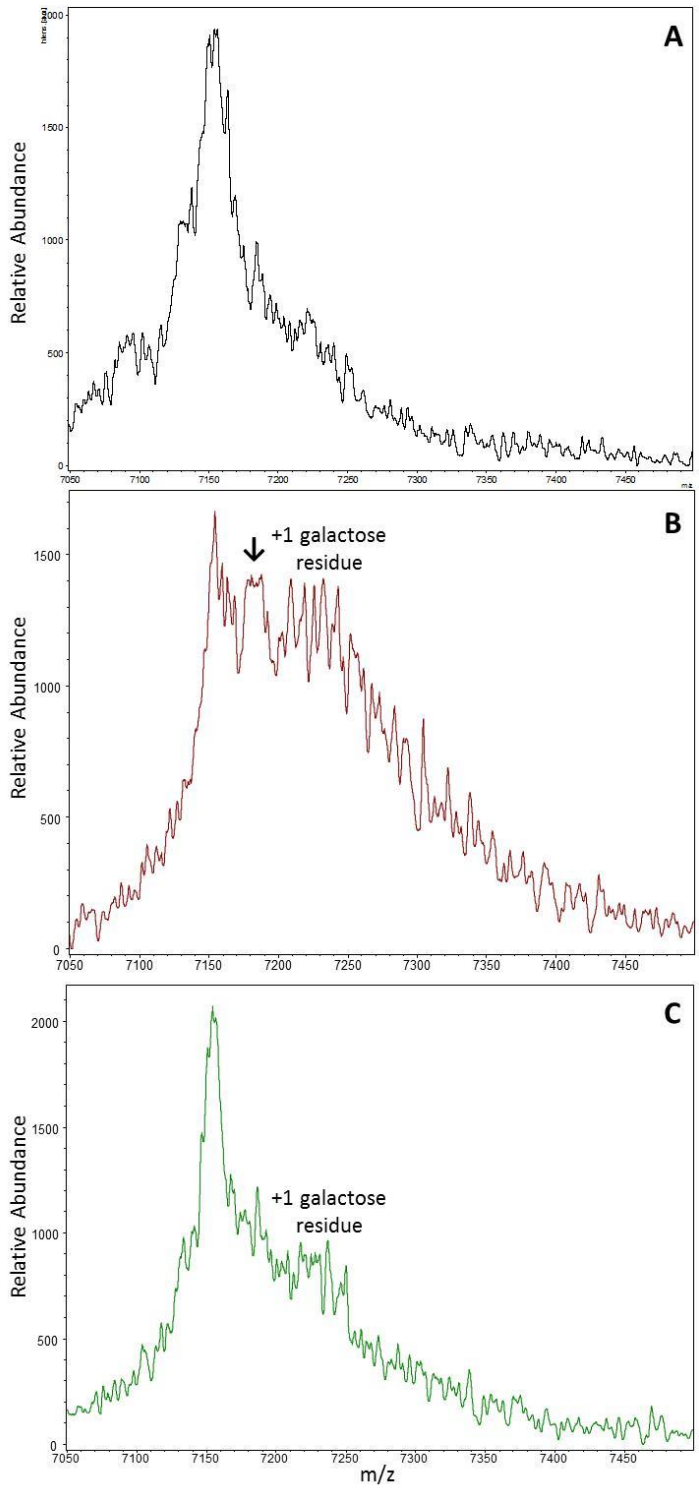

Figure 26: MALDI-TOF mass spectra of the +2 ion of chicken lysozyme incubated at 37°C for 42 days. Nonglycated lysozyme (A) and glycated lysozyme incubated in the absence of carnosine (B) and in the presence of carnosine (C). The arrow indicates the presence of AGE structures.

B. Human

The experiment to study the effect of carnosine on the formation of AGE structures was repeated using human lysozyme. The same experimental protocol outlined in Figure 24 was used for this experiment, except the glycated human lysozyme was only incubated for 8 days in the presence and absence of carnosine instead of 42 days because the intact protein began to degrade. The resultant MALDI-TOF mass spectra for the protein samples after 8 days of incubation are shown in Figure 27. Again, the presence of AGE structures (indicated by an arrow on Figure 27B) can be seen in the glycated protein sample that did not contain carnosine. This modified peak is much decreased in the glycated protein sample that contained carnosine (Figure 27C). Again, these results support the conclusion that carnosine can decelerate or hinder the formation of AGE structures that can be obtained after the initial glycation of the protein.

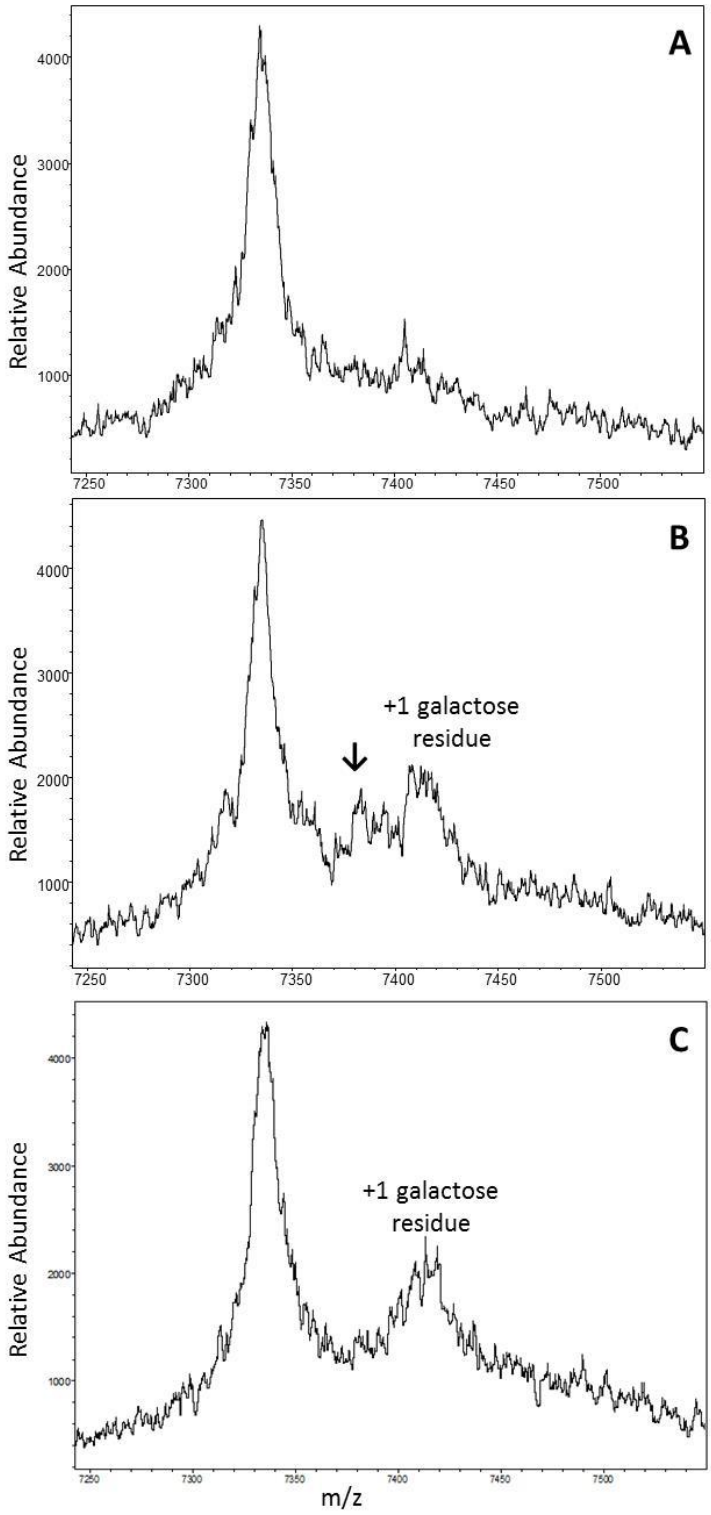

Figure 27: MALDI-TOF mass spectra of the +2 ion of human lysozyme incubated at 37°C for 8 days. Nonglycated lysozyme (A) and glycated lysozyme incubated in the absence of carnosine (B) and in the presence of carnosine (C). The arrow indicates the presence of AGE structures.

4- Identification of Glycation Sites:

A. Chicken Lysozyme:

When lysozyme was incubated with D-galactose for 3 days at 37°C, the protein added one molecule of sugar and began to add a second molecule (Figure 16B). My next goal was to determine the amino acid sites of glycation of lysozyme with D-galactose and D-glucose. Previous experiments conducted in the Frost lab identified glycation sites on only lysine residues of albumin and cytochrome *c* when these proteins were incubated with D-galactose and D-glucose using the same incubation conditions employed for my experiments.[26] Therefore, lysine residues are the most likely sites for glycation on lysozyme as well. The amino acid sequence for chicken lysozyme is shown in Figure 28 with all six lysine residues highlighted.

```
1 MRSLLILVLC FLPLAALGKV FGRCELAAAM KRHGLDNYRG YSLGNWVCAA KFESNFNTQA
61 TNRNTDGSTD YGILQINSRW WCNDGRTPGS RNLCNIPCSA LLSSDITASV NCAKKIVSDG
121 NGMNAWVAWR NRCKGTDVQA WIRGCRL
```

Figure 28: Amino acid sequence of chicken lysozyme with highlighted lysine residues.

To determine the amino acid sites of glycation, the non-glycated and lysozyme glycated with D-galactose and D-glucose samples reduced with $NaBH_4$ were separately reacted with trypsin, and the tryptic digest from each was analyzed by MALDI-TOF mass spectrometry. The resultant tryptic peptide mass fingerprint spectra for lysozyme condensed with D-galactose (Figure 29B) and D-glucose (Figure 29C) are shown in Figure 29. The experimental molecular weights obtained for the tryptic peptides were then compared with the molecular weights for a theoretical lysozyme tryptic digest to identify putative glycated peptides. Two peptides were identified which had mass shifts of +164 Da greater than the theoretical peptides (indicated with arrows in Figure 29).

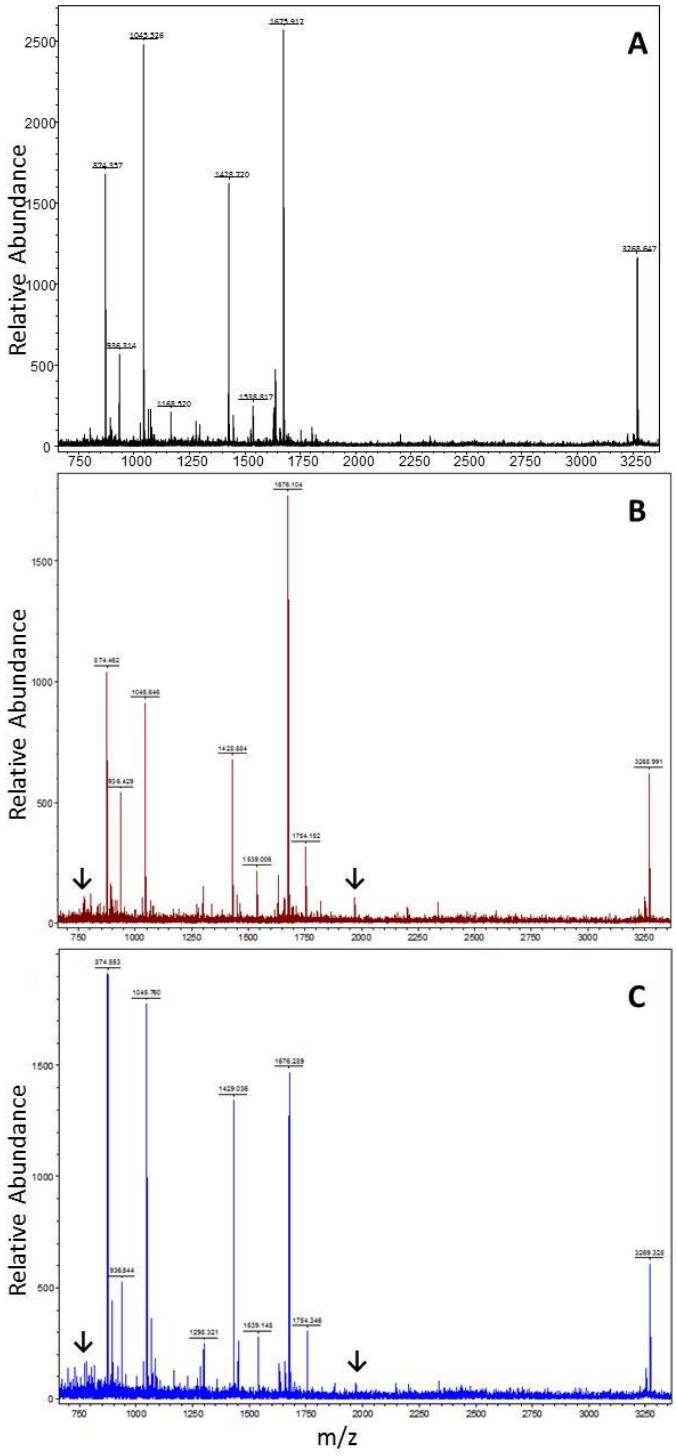

Figure 29: Reduced MALDI-TOF tryptic peptide mass fingerprint spectra of chicken lysozyme incubated at 37°C for 3 days in the absence of sugar and (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). All protein samples were reduced with $NaBH_3$ prior to digest and mass spectral analysis. The arrows designate glycated peptides.

One peptide with m/z 1804 in the unmodified, control lysozyme peptide mass fingerprint spectrum (Figure 30A) was found to exhibit a mass shift. This peptide was found to condense with one reduced D-galactose molecule (+164 Da) yielding a peak with m/z 1968 (Figure 30B), and the same peptide was also found to condense with one reduced D-glucose molecule (+164 Da) yielding a peak with m/z 1968 (Figure 30C). Based on the primary sequence of chicken lysozyme, the site of glycation was localized around peptide (aa 115-131) having an amino acid sequence consisting of KIVSDGNGMNAWVAWR. In order to confirm that this was a glycated peptide and identify the site of glycation, I obtained a tandem ESI-MS/MS CID mass spectrum for this peptide. Figure 31 shows the CID fragmentation for the +2 ion corresponding to the reduced glycated peptide at m/z 984. Analysis of the resulting y and b fragment ions produced upon the collision induced dissociation of the peptide confirmed that the lysine residue at position 115 was derivatized with a sugar residue. The b fragment ions present in the spectrum demonstrated mass shifts of +164 Da corresponding to reduced glycated peptide fragments which localized the glycation site to Lys 115. Another peptide with m/z 606 in the unmodified, control lysozyme peptide mass fingerprint spectrum was found to exhibit a mass shift. This peptide was also found to condense with one D-galactose molecule (+164 Da) yielding a peak at m/z 770 (Figure 32B), and the same peptide was found to condense with one D-glucose molecule (+164 Da) yielding a peak with m/z 770 (Figure 32C). The amino acid sequence of this peptide (aa 19-23) was KVFGR. The sugar molecule should be attached at the lysine residue at position 19; however, I was unable to obtain a good, clean CID tandem mass spectrum for this peptide to confirm the site of glycation. This was because the concentration of this peptide was very low within the complex tryptic peptide mixture and there were interfering peptides with m/z values too close to this peptide. Based upon all of these observations, the lysine residue at position 115

seems to be the preferred site of glycation under my experimental conditions. In order for a lysine residue to react with a sugar molecule, the lysine must be in its basic amine form with a lone pair on the nitrogen residue which acts as a nucleophile for the reaction. The presence of ^{114}K beside the ^{115}K residue could enhance the nucleophilicity of the epsilon amino group of ^{115}K, encouraging it to form a Schiff base with sugars. Also, the presence of an acidic amino acid nearby (^{119}D) could increase the likelihood of the sugar undergoing an Amadori rearrangement. The 115K residue is located on the surface of the folded protein in contact with the surroundings making it accessible to the sugar residues (Figure 33). The catalytic active site of the lysozyme enzyme consists of a glutamic acid residue at amino acid position 53 and an aspartic acid residue at amino acid position 70. The ^{115}K residue is not located near the catalytic cleft (Figure 33); therefore, I would not expect the glycation of this residue to interfere with the activity of the enzyme.

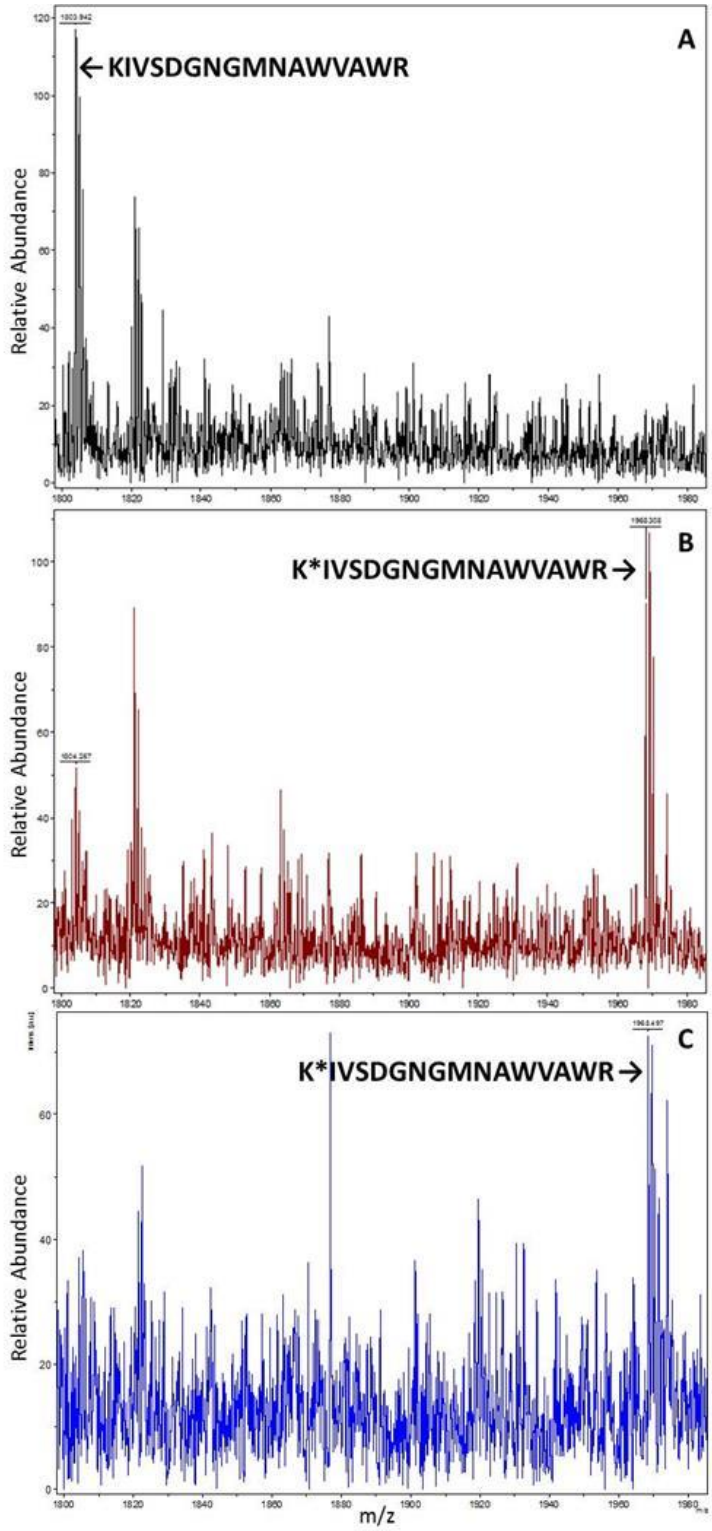

Figure 30: Reduced MALDI-TOF tryptic peptide mass fingerprint spectra of the unmodified and glycated peptide formed when chicken lysozyme was incubated at 37°C for 3 days in the absence of sugar and (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). K* represents a glycated lysine residue (∆m/z 164 Da).

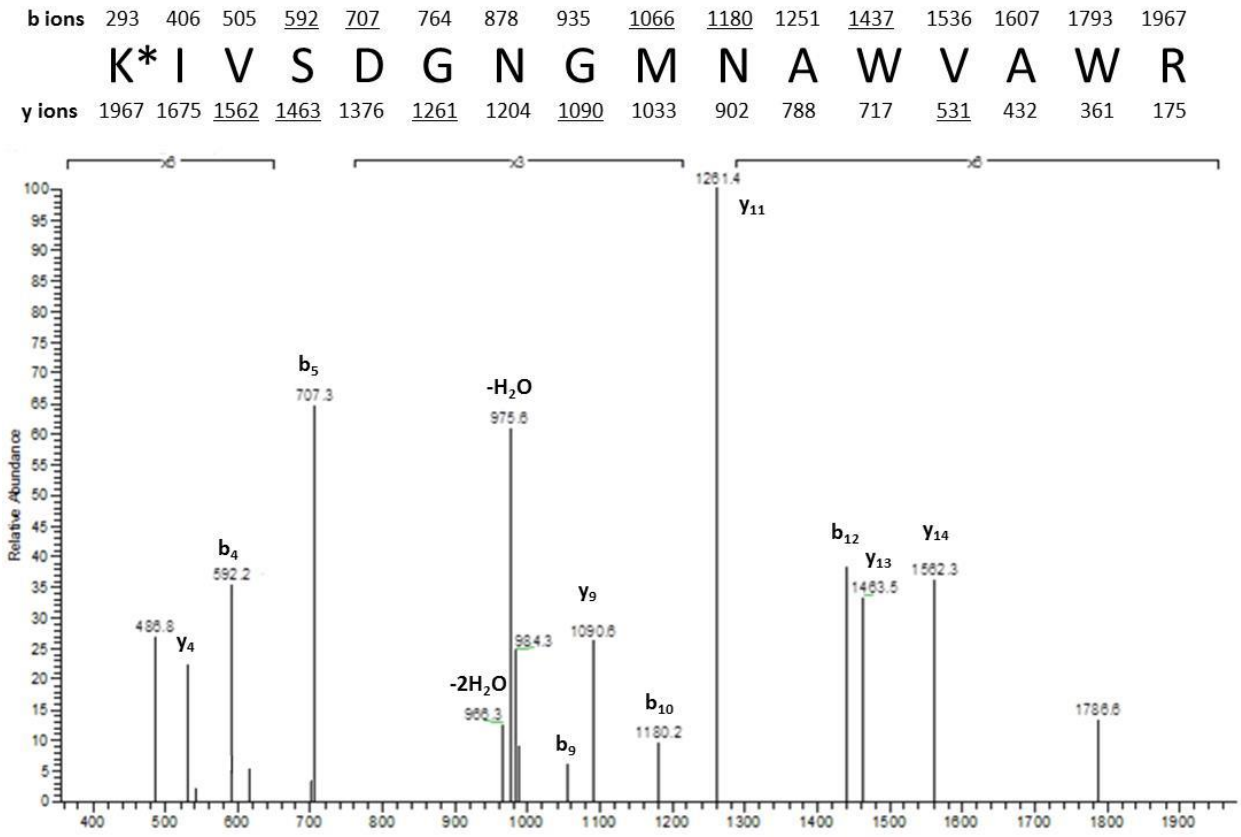

Figure 31: CID mass spectrum recorded on the $(M+2H)^{+2}$ ion at m/z 984. The sequence of the peptide is shown with the predicted y and b fragments. Ions observed in the spectrum are underlined. K* represents a galactated lysine residue of 292 Da (i,e., ^{115}K). The water loss series at m/z 975 and m/z 966 are characteristic of glycated peptides.

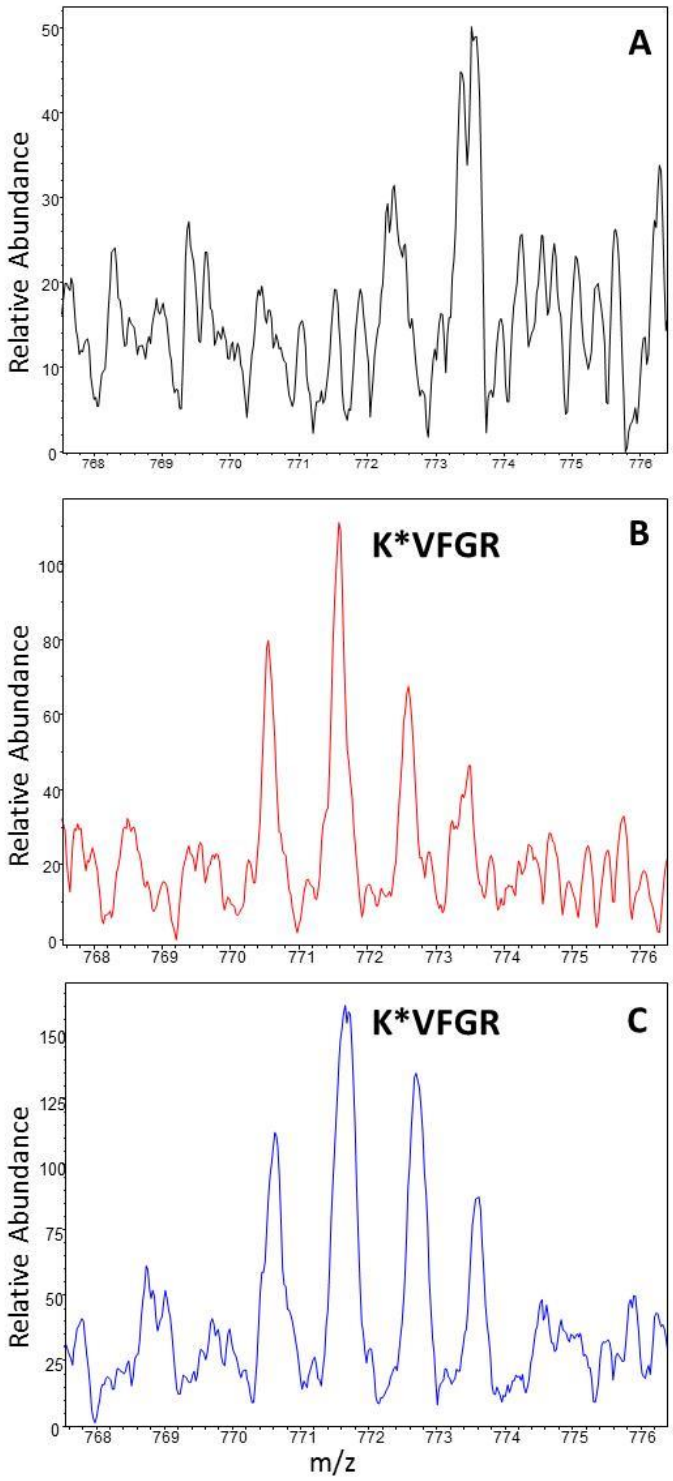

Figure 32: Reduced MALDI-TOF tryptic peptide mass fingerprint spectra of the unmodified and glycated peptide at m/z 770 formed when chicken lysozyme was incubated at 37°C for 3 days in the absence of sugar and (A), and in the presence of D-galactose (B), and in the presence of D-glucose (C). K* represents a glycated lysine residue (Δm/z 164 Da).

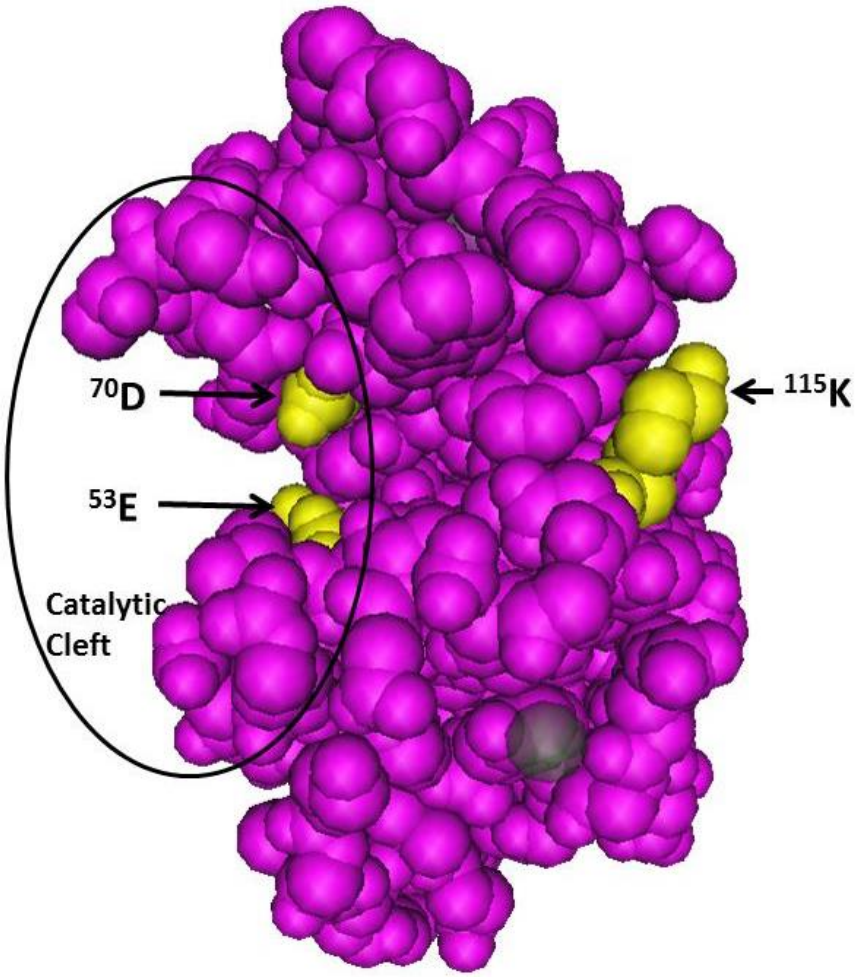

Figure 33: 3-D structure of chicken lysozyme. The ^{115}K residue is show in yellow. The ^{70}D and the ^{53}E residue which are the catalytic active sites for the enzyme are shown in yellow. Adapted from the high-resolution three-dimensional structure available at the Protein Data Bank PBD ID 4RW2.[34]

B. Human

The amino acid sequence for human lysozyme is shown in Figure 34 with all six lysine residues highlighted. To determine the amino acid sites of glycation, the non-glycated and lysozyme glycated with D-galactose and D-glucose samples reduced with $NaBH_4$ were separately reacted with trypsin, and the tryptic digest from each was analyzed by MALDI-TOF

mass spectrometry. Unfortunately, I repeated this experiment several times, but I was never able to get the trypsin to digest the human lysozyme protein. Each time I attempted to obtain a peptide mass fingerprint spectrum I was only able to obtain intact protein mass spectra. I thought that there may have been a component in the recombinant protein solution that was interfering with the trypsin digest, so I separated the glycated, reduced protein by SDS-PAGE prior to the digestion with trypsin. The resultant tryptic peptide mass fingerprint spectra for lysozyme condensed with D-galactose is shown in Figure 35. There were only two prominent peptides in the spectra, both of which are unmodified peptides. Even after separation, trypsin was not digesting this protein efficiently enough to be able to identify glycated peptides. I tried prolonging the digestion period for up to three days, but no better mass spectra were obtained. In the future, I could try digesting the glycated protein with different proteolytic enzymes. This may not result in the generation of peptides either, because trypsin is generally regarded as the best enzyme for protein digestion experiments. Perhaps I may have some luck switching to the chemical cyanogen bromide which cleaves proteins after methionine residues.

1 MKALIVLGLA LLSVTVQGKV FERCELARTL KRLGMDGYRG ISLANWMCLA KWESGYNTRA
61 TNYNAGDRST DYGIFQINSR YWCNDGKTPG AVNACHLSCS ALLQDNIADA AACAKRVVRD
121 PQGVRAWAAW RNRCQDRDVR QYVQGCGV

Figure 34: Amino acid sequence of human lysozyme with highlighted lysine residues.

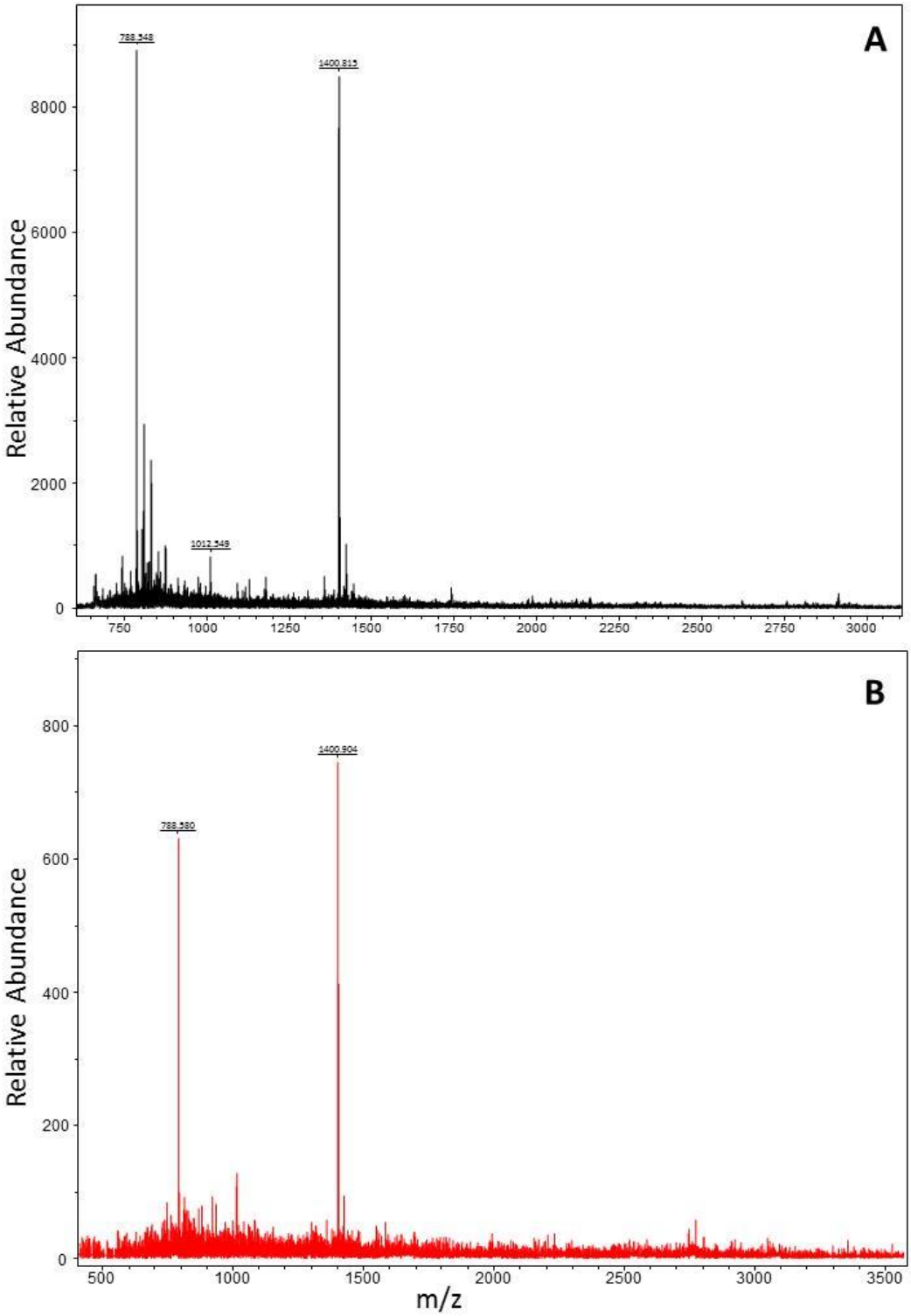

Figure 35: Reduced MALDI-TOF tryptic peptide mass fingerprint spectra of human lysozyme incubated at 37°C for 3 days in the absence of sugar and (A) and in the presence of D-galactose (B). All protein samples were reduced with $NaBH_3$ prior to digest and mass spectral analysis.

5- Effect of Glycation on Enzymatic Activity

A. Chicken

I determined the effect of protein glycation on the enzymatic activity of the protein by measuring the enzyme's lytic activity against *Micrococcus lysodeikticus* spectrophotometrically. This procedure could only be used for chicken lysozyme, because it would not work for the recombinant human lysozyme protein (as stated by Sigma-Aldridge). The absorbance value measured at a wavelength of 450 nm should decrease over time as lysozyme lysis the bacteria cells. For the first experiment, I simply assayed solutions of unmodified lysozyme, D-galactose, and D-glucose in buffer solution (Figure 36). The kinetic profile of absorbance shown in Figure 36A shows that the procedure was successful for measuring enzymatic activity. The kinetic profiles shown in Figures 36B and 36C indicate that solutions of D-galactose and D-glucose on their own were not able to lyse the bacterial cells. Next, I incubated chicken lysozyme with both D-galactose and D-glucose at 37°C for up to 33 days. Aliquots of the solutions were removed at different time points for enzymatic assay analysis. Figure 37 shows the kinetic profile for unmodified lysozyme (Figure 37A) and lysozyme glycated with D-galactose (Figure 37B) and D-glucose (Figure 37C) after three days of incubation. The kinetic profiles for these samples are very similar, and the rate constants do not differ significantly, indicating that protein glycation did not affect the enzymatic activity of the protein. I know from previous experiments that there would still be a large amount of unmodified protein even after three days of incubation; therefore, I decided to allow the incubation to proceed for longer amounts of time to obtain even more glycated protein in solution. Figure 38 shows the kinetic profile of the enzyme after 14 days of incubation, and Figure 39 shows the kinetic profile after 33 days of incubation. All of the profiles remain

similar in these figures, and again the rate constants do not differ significantly. In conclusion, the kinetic values did not show any significant change between the control lysozyme and the glycated lysozyme at any of the time points studied indicating that the initial glycation of the protein did not affect its enzymatic activity.

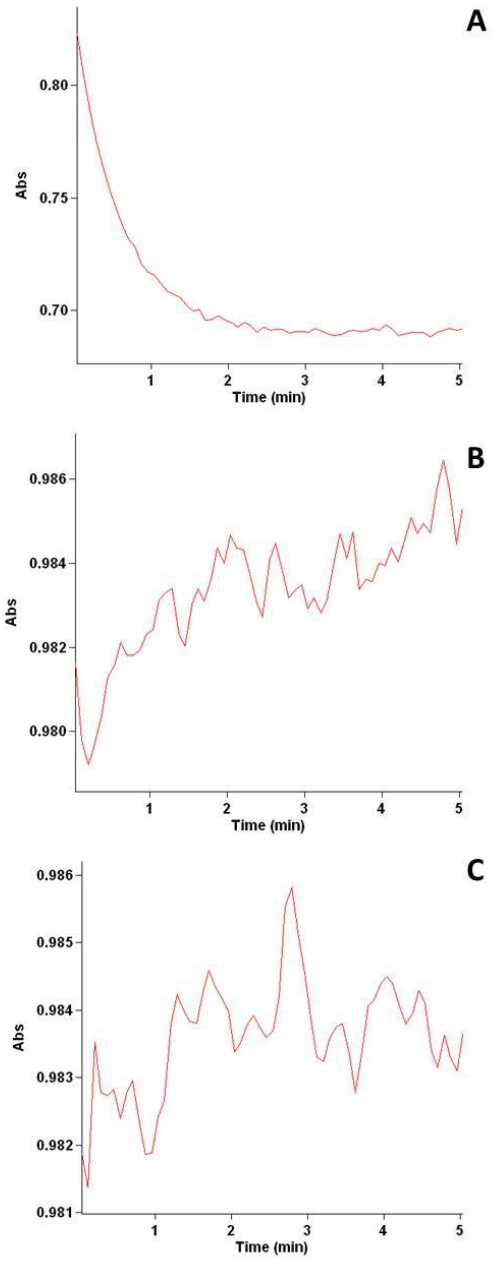

Figure 36: UV-VIS absorption profile of a suspended solution of *Micrococcus Lysodeikticus* bacterial cells at 450 nm over time in the presence of chicken lysozyme (A), D-galactose (B), and D-glucose (C).

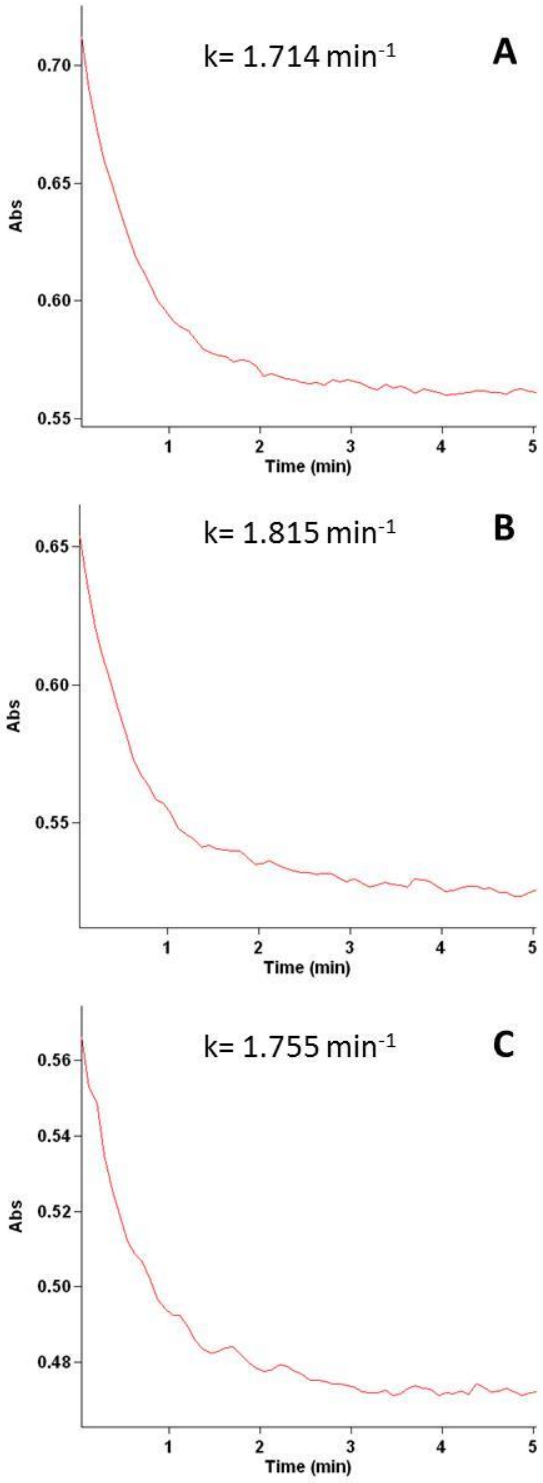

Figure 37: UV-VIS absorption profile of a suspended solution of *Micrococcus Lysodeikticus* bacterial cells at 450 nm over time after the addition of chicken lysozyme incubated in the absence of sugar (A), in the presence of D-galactose (B), and in the presence of D-glucose (C) for three days.

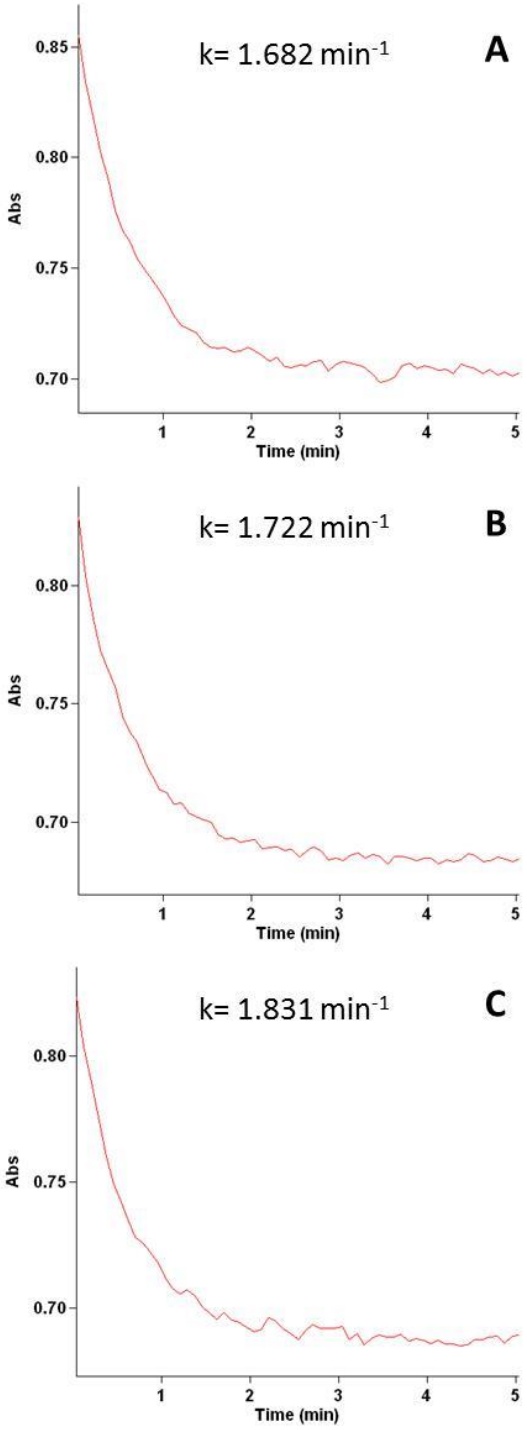

Figure 38: UV-VIS absorption profile of a suspended solution of *Micrococcus Lysodeikticus* bacterial cells at 450 nm over time after the addition of chicken lysozyme incubated in the absence of sugar (A), in the presence of D-galactose (B), and in the presence of D-glucose (C) for 14 days.

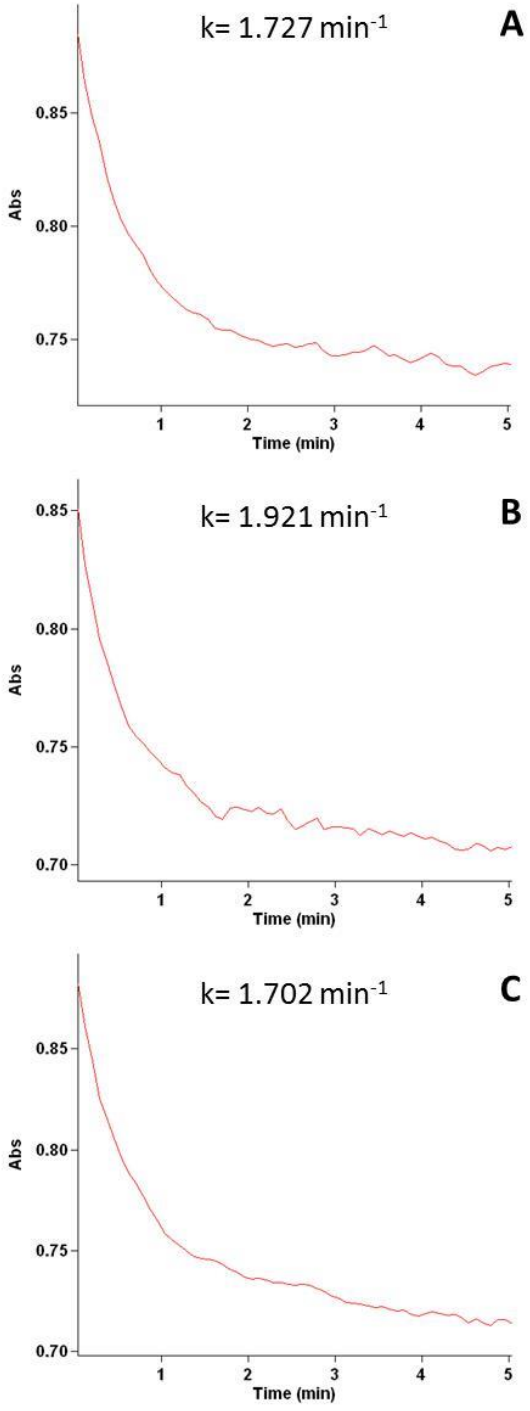

Figure 39: UV-VIS absorption profile of a suspended solution of *Micrococcus Lysodeikticus* bacterial cells at 450 nm over time after the addition of chicken lysozyme incubated in the absence of sugar (A), in the presence of D-galactose (B), and in the presence of D-glucose (C) for 33 days.

MARSHALL UNIVERSITY
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Office of Research Integrity

August 4, 2015

Wisam T. Joudah
1340 4th Ave., Apt #7
Huntington WV, 25701

Dear Wisam:

This letter is in response to the submitted thesis abstract entitled *"Glycation of Lysozyme."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

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