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ON THE PROTECTIVE PROPERTIES OF GLYCINE BASED OSMOLYTES IN A THIOL REDUCING ENVIRONMENT

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By

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To those who supported me and reminded me why I did it in the first place.

For the magic!

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Abstract

The protective properties of osmolytes have been studied intently for decades. Originally used to aid in the crystallization of proteins in x-ray diffraction studies, these cosolvents have been shown to reverse protein denaturation and aggregation. Osmolytes aid extremophiles in surviving harsh environments by preferentially excluding themselves from the surface of the protein, thus directing water molecules to the protein's surface. These osmolytes are naturally found in many health foods and also in many daily use products such as shampoo. Due to their osmoprotective effect their use in everyday consumer product is increasing. Consumers also supplement their diets with thiol-based antioxidants such as glutathione as part of a healthier life style. However, thiol-based antioxidants in high concentration have been known to cause un-intended health issues that relates to its disulfide reducing property. The disulfide-reducing agent can cleave the protein's disulfide-bonds and promote misfolding and aggregation. In this thesis, we investigated the influence of commonly consumed glycine-based osmolytes on providing stability to proteins against a disulfide-reducing agent. We chose glycine, sarcosine (N-methyl glycine), di-methyl glycine (DMG), and betaine (N,N,N-trimethylglycine) with increasing number of methyl groups. An added benefit to studying this class of osmolytes is to also investigate the impact N-methyl substitution has on the osmolytes' protective properties. We studied the effect of these osmolytes on protein aggregation using spectroscopic techniques such as UV-visible absorbance, intrinsic fluorescence, and extrinsic fluorescence measurements. In addition, we carried out non-reducing SDS-PAGE to check for higher order aggregates and characterized morphology of these

aggregates using scanning electron microscope. Overall, our results show that of all the osmolytes used, glycine was the best stabilizer followed by sarcosine. Betaine and dimethylglycine did not provide effective protection against disulfide-reducing influence.

1. Introduction

To better cope with environmental stresses such as high salt concentrations, extreme pH (acidic or alkaline conditions), and high temperatures etc. plants and micro-organisms accumulate low molecular weight compounds known as osmolytes[1-5]. The most common osmolytes are small organic or inorganic compounds like polyhydric alcohols (polyols), sugars, amino acids, salts, and their derivatives[2-7]. These osmolytes have been selected by nature and normally, do not affect enzymatic activity, are electrically neutral, and are preferentially excluded from the vicinity of cellular components so as not to affect biological processes[2 4 8 9]. At high concentrations these osmolytes are known to stabilize proteins by *preferential hydration* (a process by which osmolytes are excluded from the protein surface, structure the water molecule around protein, leading to protein stabilization; Figure 1.1)[10-15]. The osmolytes modulate protein stabilization by an intricate interplay of protein, water, and cosolvent interactions. The amount of water that is in constant flux, perturbing and being perturbed by the protein is termed *water of hydration*, W_H.[16] The amount of water molecules in contact of the protein is termed, water of *preferential hydration*, W_{PH}. This value can fluctuate if either the surface area of the protein changes or if another molecule is introduced into the solvent system. Several mechanisms for stabilization of proteins by preferential hydration in the presence of

osmolytes have been proposed: 1.) due to increase in '*surface tension'* (cosolvents are excluded from the protein-solvent interface, creating a cavity, leading to stabilization of proteins) e.g. sugars, polyols, salts, amino acids[4 10 11 12 17-25] 2.) due to '*solvophobic effect*' (increase in tendency of cosolvents to move away from water molecules and strengthen hydrophobic interaction) e.g. glycerol, TMAO[11 12 17 24 25], and 3.) by '*steric exclusion*' (depends upon the size of the cosolvent, that affects how close the compound can get to the protein surface region immediately surrounded by water) e.g. betaine[13].

To better understand how these cosolvents can lead to stabilization or can adversely affect stability (e.g. by binding and denaturing proteins), at the interplay of protein-water or protein-cosolvent interaction. The balance between cosolvent—protein-backbone interaction (leads to protein destabilization) or amino acid side chain—solvent interaction (leads to protein stabilization) determines the overall protein stability. If a protein becomes denatured, the surface area of the protein is modified and will thus change the water of preferential hydration. In a ternary solvent system, each locus of the protein surface area will be in contact with either the primary solvent (most concentrated solvent, usually water in biological systems) or with a cosolvent. These cosolvents can be either beneficial or detrimental to the protein's native state. A cosolvent can either increase the water of preferential hydration, thus making the protein more stable and rigid; or decrease the water of preferential hydration and cause the protein to precipitate out of the solution.

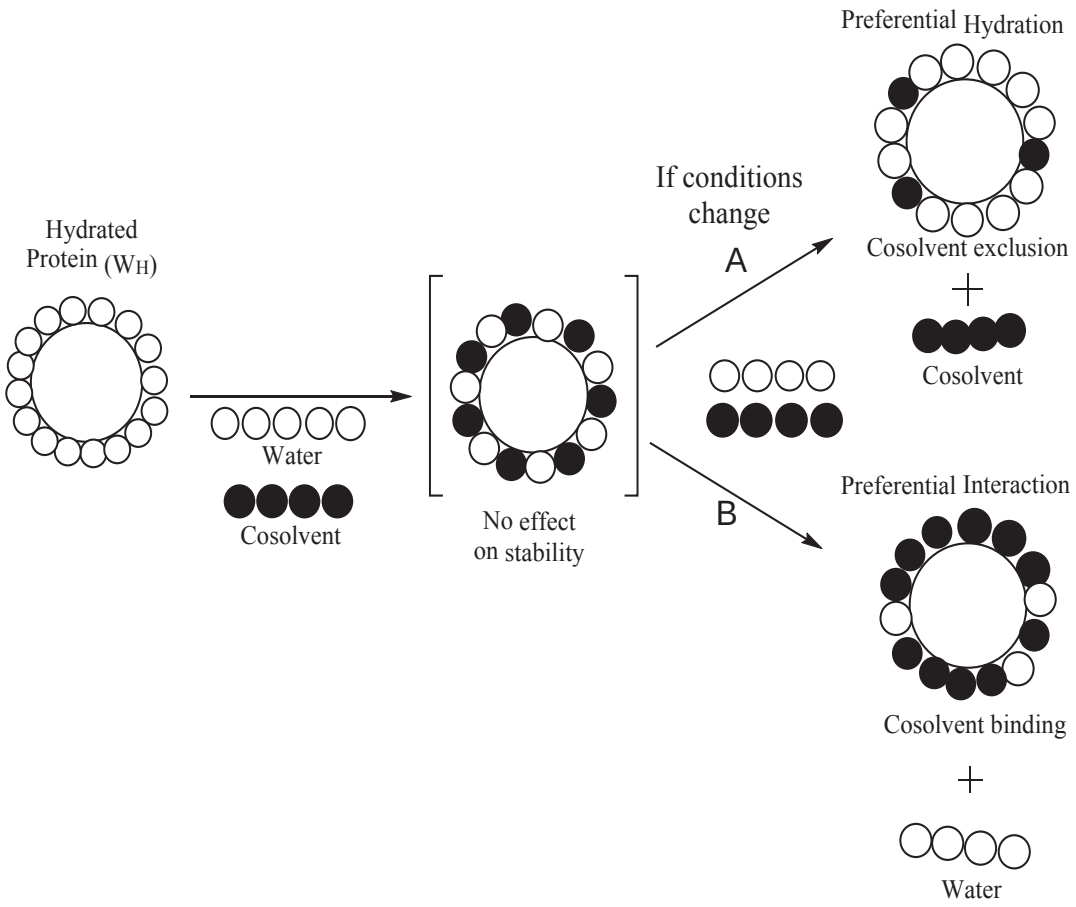


Figure 1.1. Mechanism for stabilization/destabilization of a protein molecule through cosolvent interaction. If cosolvent has little or no effect on the stability of the protein, then solvent and cosolvent evenly distribute across the protein surface. If the cosolvent does not favor interaction with the protein, it is excluded and structures water around the proteins surface. If the cosolvent, interacts favorably with the protein, it binds excluding water from the proteins surface. Adapted from Timasheff S.N. 2002.[26]

Cosolvents that promote preferential hydration and stabilize the protein's native state are called *osmolytes*, while cosolvents that decrease the preferential hydration are called *denaturants*. The most commonly used denaturants are urea and guanidinium

hydrochloride. These compounds cause denaturation of proteins by interacting favorably with the protein's backbone, dislodging the water molecule, thus decreasing preferential hydration.[27] The process by which water molecules are excluded and the cosolvent interacts with the protein surface area is called *preferential interaction*. Preferential interaction is the direct opposite of preferential hydration. Osmolytes on the other hand promote proteostasis by undergoing solvent exclusion, thus causing more water molecules to come into contact with the protein surface, thus increasing preferential hydration. This mechanism makes the protein more rigid; one consequence of such rigidity can be reduction of enzymatic activity if the osmolyte concentration is high.[28] The balancing act between preferential hydration and preferential interaction of cosolvent or ligands can be represented with the following equilibrium equation.[29]

$$P \cdot nH_2O + L \leftrightarrow P \cdot L + nH_2O$$

Whenever a ligand molecule interacts with a protein, water is displaced. Osmolytes will shift the equilibrium to the left while denaturants will shift this equilibrium to the right. These molecules have been widely used for many years to aid in protein crystallization as well as preservative for proteins.[30] Investigation into the protective properties of these osmolytes on proteins have been a major focus of research for decades. With a better understanding of how these osmolytes provide protection, different additive formulas or synthetic osmolytes can be designed, thus allowing for major contributions to several pharmaceutical and biotech industries. Osmolytes can be used to stabilize therapeutic proteins in the drug industry. Therapeutic proteins such as insulin, are small and only stable in vitro for short periods of time.[31] By increasing the shelf life and thermal stability

of therapeutic proteins, the cost of production, storage, and shipping would decrease, thus allowing for a more cost effective and consumer affordable medication.[32] [33] This use of osmolytes can be expanded to help improve the handling of proteins in a laboratory setting.

Osmolytes affect our everyday life as these are used as stabilizers in consumer goods such as foods, shampoos, toothpastes, and topical medications. Health foods, such as quinoa, spinach, fish, and whole grain products contain a significant amount of osmolytes. These osmolytes are found naturally in these food products at relatively high concentrations, and survive the baking process as these are very thermostable. Regardless of diet or lifestyle, many consumers enjoy the benefits of these osmolytes in everyday products that they use or consume regularly.

Extremophiles such as bacteria residing in deep sea thermal vents have also been observed to be stabilized by inorganic osmolytes.[34] These organisms live in extreme environmental conditions that is harsh and can affect proteins in their cell system. High pressure, temperature, salinity, and pH environments are detrimental to a proteins native structure. These osmolytes preferentially hydrate the protein protecting it from the harsh conditions and hence prevent its denaturation and/or loss of function. This osmoprotective role of these cosolvents has been the central focus of research in biotechnology, pharmaceutical, and academia. The goal is to increase shelf life of everyday use items such as toothpaste, shampoos and of food and medicine.

As people are becoming more health-conscious, everyday use of '*over-the-counter*' vitamins and supplements are on the rise. Use of antioxidant supplements into everyday

diet for either general health improvement or bodybuilding is becoming increasingly popular. Many of these antioxidants are thiol-based and are naturally occurring in the human body e.g. glutathione, N-acetylcysteine, cysteine, and lipoic acid. These commonly used thiol-based antioxidants are easily available as '*over-the-counter*' natural supplements. These compounds can attack conserved disulfide-bonds on proteins, leading to its destabilization.

Reduction of disulfide bonds can affect protein stability due to loss of anchoring effect of disulfide bonds in proteins making them more flexible and mobile.[35] This increase in structural flexibility (degrees of freedom), causes rearrangement of water molecules in contact with the protein surface promoting protein misfolding. As a consequence, hydrophobic amino acid residues that are normally buried within the bulk (core) of the protein become solvent exposed. This makes the protein more hydrophobic (decreasing the water of preferential hydration), making it sticky which can promote self-interaction and aggregation. In cells, it can lead to aberrant interactions with other proteins or cellular constituents. Studies involving muscle recovery and long term use of thiol-based antioxidants show that skeletal muscle recovery is decreased significantly when such antioxidants are taken consistently.[36] [37] These thiol-based supplements can increase the concentration of natural disulfide reducing agents in the body that may have a deleterious effect.

Health foods such as quinoa, spinach, and wheat bread have been on the rise and are encouraged to incorporate into a healthy diet for various nutritional benefits. Some health fanatics as part of healthy lifestyle, consume high amounts of thiol-based antioxidants in

combination with health foods. Studies show that these health food products contain high concentrations of osmolytes such as betaine and glycine. For example, spinach contains 645 mg/100g, wheat bran contains 1339mg/100g, eggs contains 251mg/100g, and wheat bread contains 201mg/100g of betaine.[38] Osmolytes can prevent the denaturation of proteins under harsh conditions such as high pH, salinity, temperature, and chemical denaturants.[1-5] Osmolytes have also been shown to reverse aggregation process of denatured proteins.[39 40] Therefore, it will be interesting to study how these glycine based osmolytes may modulate the overall deleterious impact of thiol-based antioxidants. Since, glycine-based osmolytes exist in such large amounts in commonly ingested food products, we wanted to study the protective effect of these osmolytes on proteins against disulfide-reducing influences.

2. Goals and Hypotheses

We *hypothesized* that glycine based osmolytes will provide protection to proteins against instability promoted by disulfide-reducing agents. The primary goal was to investigate the influence of commonly consumed glycine-based osmolytes on the stability of proteins against a disulfide-reducing agent. The disulfide-reducing agent can cleave the proteins disulfide-bonds and promote misfolding and aggregation as shown in model Figure 2.1.

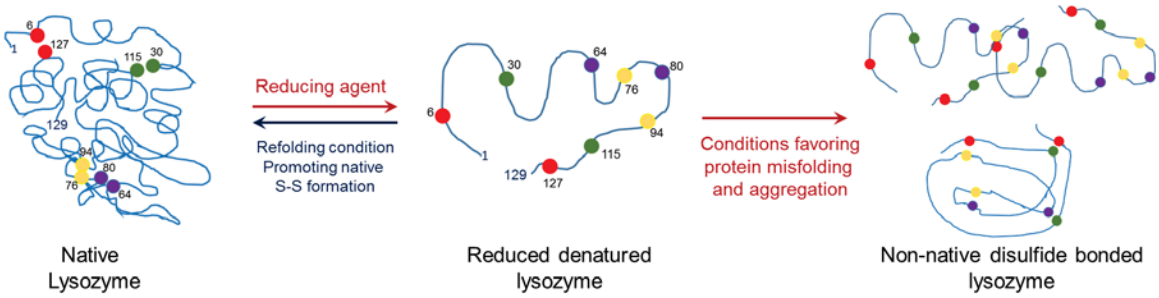


Figure 2.1. Effect of disulfide-bond reduction on protein unfolding and misfolding. Cleavage of these covalent bonds cause an increase in freedom of motion of protein domains, significantly increasing the probability of misfolding and aggregation. The different color dots and numbers represent Cys residues present on lysozyme that are disulfide-bonded in native state of the protein.

We chose a series of amino acids, viz., glycine, sarcosine (N-methyl glycine), di-methyl glycine (DMG), and betaine (N,N,N-trimethylglycine) with increasing number of N-methyl groups. Glycine has no methyl group, sarcosine has one, DMG has two, and betaine has three methyl groups attached to the amino terminal. Glycine, sarcosine, and betaine are naturally occurring osmolytes whereas DMG is a synthetic compound. We also chose dithiothreitol (DTT), a thiol based reducing agent that is commonly used in

protein stability and aggregation studies[41]. We carried out all experiments at conditions close to physiological pH and temperature (pH 7.2 and 37 °C) and monitored the proteins instability and aggregation by several techniques, such as, UV-visible spectroscopy, fluorescence (both intrinsic and extrinsic), electrophoresis, and scanning electron microscopy.

The *goal* of this project was to investigate the real world application of thiol-based antioxidant supplementation combined with a glycine-based osmolytes (present at high concentration in many health foods) on the stability of proteins. These experiments are designed to mimic the influence of consistent consumption of glycine based osmolytes in a typical healthy diet against the deleterious effects of thiol-based antioxidant supplementation. Interestingly, there are no reported studies that have looked at the beneficial effect of glycine based osmolytes against the noxious effect of disulfide-reducing agents on protein stability.

2.1. Justification for reagent use

Hen egg white lysozyme was used in this project since it is a well-characterized protein that behaves predictably *in vitro* in conditions close to physiological. This allows for minimal bias and complication in the investigation of osmolytes stabilizing influence in a disulfide-reducing environment. Lysozyme is a 14.3 kDa 129 amino acid globular protein containing four disulfide bonds. These disulfide bonds are located between C6-C127, C30-C115, C64-C80, and C76-C94. The C6-C127 disulfide bond is partially exposed to

the solvent, while the other three are buried and not accessible to the solvent (Figure 2.2). This arrangement allows for studying the protein in a partially reduced and a fully reduced state. In these two states, the degree of protection these osmolytes provide can be observed.

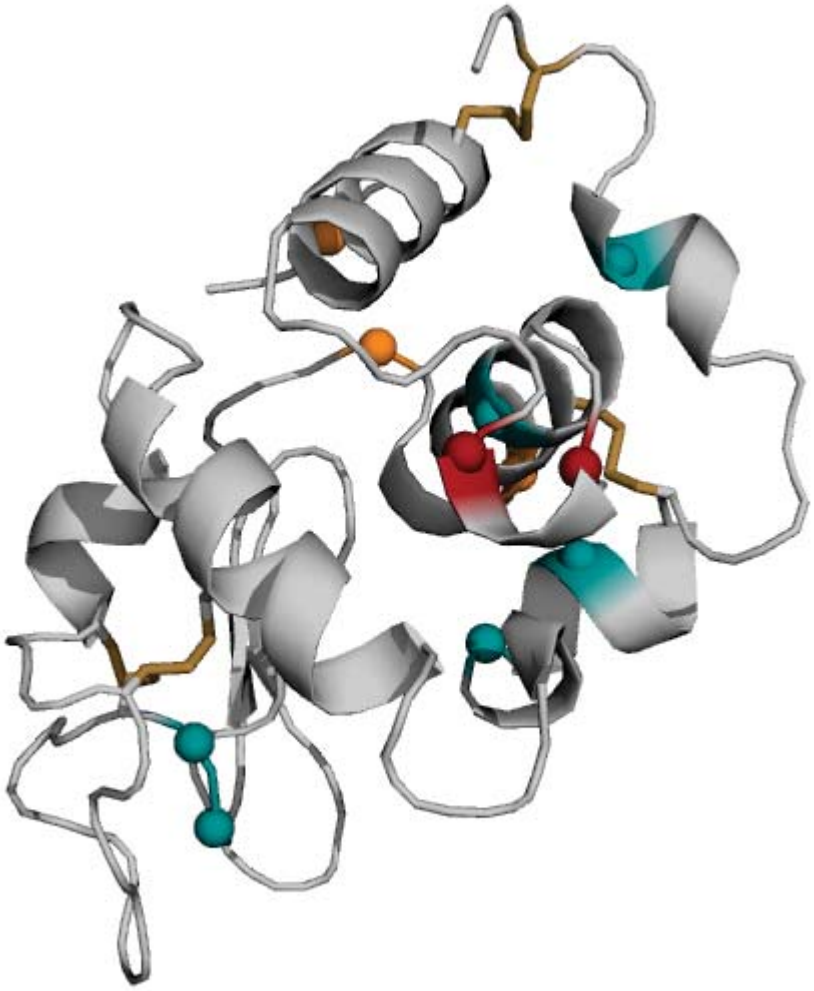
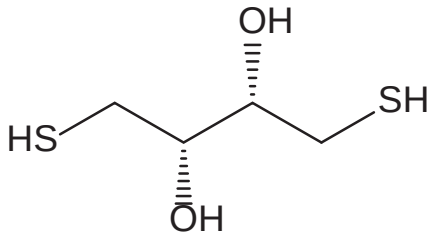


Figure 2.2. Structure of lysozyme generated using PyMOL 1.3 and PDB file 1UCO adapted from Nagendra HG et al (1996).[42]. Ribbon diagram highlights the disulfide bonds (S−S, sand-yellow) stick; the tryptophan (dark-teal), tyrosine (firebrick red), and phenylalanine residues (orange) as spheres. The backbone is shown in gray color.

Dithiothreitol (DTT) (Figure 2.3) is a thiol-based reducing agent that has been extensively investigated and the mechanism of its action is well known. This compound reduces proteins disulfide-bonds through two thiol disulfide exchanges.[43] The mechanism of action is shown in Figure 2.4. DTT was chosen as this reducing agent is commonly used for protein stability studies at pH 7.2 (near physiological), and has a structure similar to many thiol-based antioxidants.[41 44 45] It is hypothesized that partial reduction of the protein represents how thiol-based antioxidants may reduce lysozyme in a biological system. In summary, DTT is well positioned to be used as a reducing agent to investigate the protective power of osmolytes in an environment close to physiological.



Dithiothreitol

Figure 2.3. Structure of thiol-based reducing agent dithiothreitol (DTT).

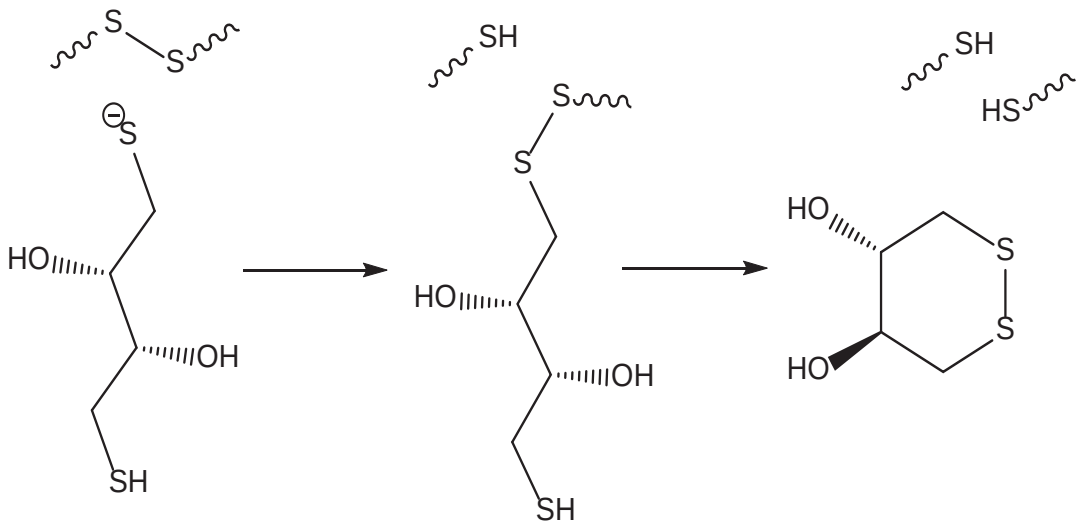


Figure 2.4. Dithiothreitol reduces disulfide bonds through two thiol disulfide exchanges.

Glycine based osmolytes exist in large amounts in everyday food products such as spinach, wheat bread, pretzels, and quinoa.[46] There is a growing consumer awareness and demand for healthy diet and lifestyle that has led to an increased consumption of health food such as quinoa and spinach. As shown in Figure 1.1, osmolytes stabilize proteins by directing more water molecules to the protein surface. Since previous studies have demonstrated the stabilizing effects of osmolytes on proteins under conditions of stress such as pH, temperature, etc., we hope to expand the knowledge base on the stabilizing effects of osmolytes on the influence of reducing agents on protein stability. There is a lack of research on the protective effects of osmolytes on a disulfide reducing stressor. This project provides insight into the stabilizing potential of glycine based osmolytes against a disulfide reducing influence and offers

an insight into the implication of consuming large amounts of these osmolytes in combination with thiol-based antioxidant supplementation.

An added benefit of studying this class of osmolytes is to investigate impact of N-methyl substitution on the osmolytes protective properties. It is hypothesized that glycine has the highest degree of solvent exclusion and thus will offer the strongest protection potential. This protection potential is also theorized to decrease as the degree of N-methyl substitution increases. We chose this series, as in addition to being at high concentrations in health foods providing beneficial effects, we wanted to know if number of N-methyl groups also played a role. If so, what is the relationship between number of methyl groups and the relative protection provided by these osmolytes? The four compounds pictured below, cover the full range of possible N-methyl substitutions for this class of cosolvents and studying them will provide a complete view of its influence under physiological conditions.

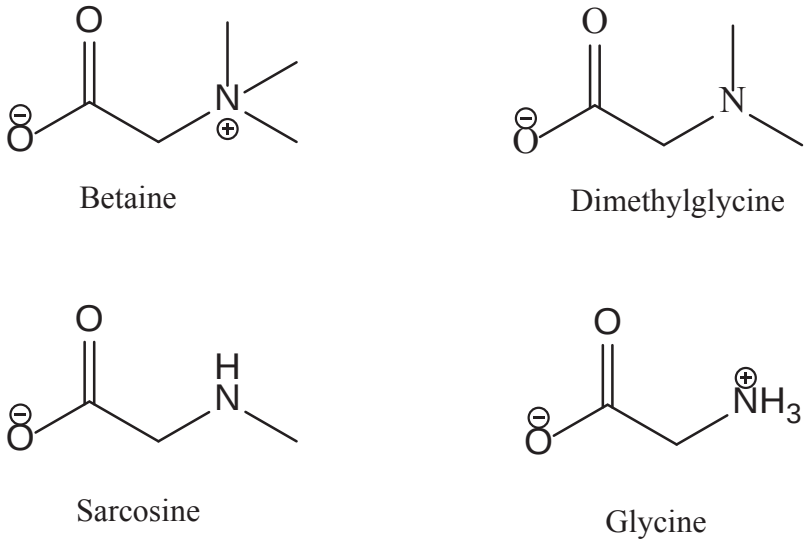
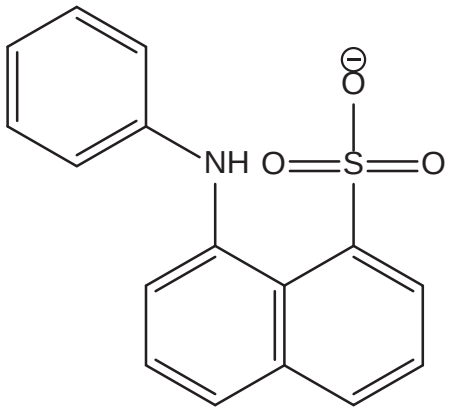


Figure 2.5. Glycine based osmolytes commonly found in consumer products.

An extrinsic fluorescence assay involving the dye 1-anilinonaphthalene-8-sulfonic acid (ANS) was chosen to monitor changes in hydrophobic exposure of lysozyme as the protein is exposed to a reducing environment. This dye was chosen as it is a well–established dye used to determine the hydrophobic exposure of proteins as they misfold.[47] The hypothesis, is that the fluorescence of the dye will be lower for proteins that are not reduced. Therefore, if the osmolytes protect the protein from disulfide reduction, then the fluorescence should be significantly lower than for the protein samples that were reduced successfully by DTT.



1-anilinonaphthalene-8-sulfonic acid (ANS)

Figure 2.6. 1-anilinonaphthalene-8-sulfonic acid (ANS) dye used to assess changes in proteins hydrophobic exposure as result of denaturation and misfolding.

2.2. Investigate the influence of osmolyte at 0.05M and 1M concentration on misfolding

When a weakly interacting ligand exists in solution at high concentration i.e. 0.2 M to 10 M, then the ligand is considered an equal solvent component to water. At this point the ligand is considered a cosolvent.[48] Studies have shown cosolvents having both positive

and negative effect on the proteins native structure depending on the environmental conditions. For example, betaine acts as an osmolyte at physiological pH but is a denaturant at acidic pH.[49] It is a known stabilizer against thermal stress and shows its protective property as a result of steric exclusion leading to preferential hydration. A protein is neutral thermodynamically to the interaction with water. By having a high concentration of osmolyte in solution (i.e. 1M), we hypothesize that the protein will be preferentially hydrated due to the solvent exclusion of the osmolyte. The samples were also enriched with a small concentration of osmolyte (0.05M) in order to determine if the osmolytes affect stability by direct interaction, since only a small amount of cosolvent would be required to observe such a destabilizing effect. In these experiments, osmolytes glycine, sarcosine, dimethylglycine, and betaine were used to assess the effect of N-methyl substitution on the stabilizing effect of the osmolytes against a reducing environment at pH 7.2 and at 37 °C.

3. Materials and Methods

3.1. Material and Sample Preparation

3.1.1. Lysozyme Stock Solution Preparation

All materials were purchased and used as supplied from Sigma unless otherwise indicated. Lyophilized lysozyme powder was dissolved in 20 mM sodium phosphate buffer (pH 7.2) and 150 mM NaCl. Lysozyme concentration was determined by UV-Vis spectrophotometry using extinction coefficient $\varepsilon_{280nm} = 2.64$ mg^{-1} mL cm^{-1}. Protein stock solutions used for this project had working concentrations of 40 μM (0.572 mg/mL) lysozyme, 20 mM sodium phosphate (pH 7.2) and 150 mM NaCl.

3.1.2. Material Preparation

Dithiothreitol solution was prepared by dissolving 38 mg dithiothreitol powder in 500 μL deionized water, producing a 500 mM final solution. ANS dye was prepared by dissolving 2.99 mg ANS powder in 1 mL of 100% ethanol. Betaine, dimethylglycine, and sarcosine working solutions were made by preparing a 3 M stock solution; then filtering through a 0.45 μm membrane before using. Glycine working solution as prepared similarly by preparing 3 M solution at 40 °C then it was filtered while warm to prevent crystallization.

3.1.3. Sample Preparation

Lysozyme sample solutions were prepared in nine different compositions in order to investigate the protective capability of glycine based osmolytes against thiol based antioxidants. Each solution consisted of 70 μL of 200 mM sodium phosphate buffer at pH 7.2, 27 μL of 40 μM lysozyme working solution, either 11.66 μL or 233 μL of 3 M osmolyte, either 0, 1.4, or 14 μL of dithiothreitol and diluted to 700 μL with deionized water. This produced a set containing nine different sample solutions. These sets were incubated for either 0, 0.5, 1, 2, 4, 24, 48, or 168 hours. This setup was repeated for each osmolyte, either betaine, dimethylglycine (DMG), sarcosine, or glycine).

3.2. Experimental Setup and Protocol

The following methods were used to investigate the rate of protein unfolding, misfolding, and aggregation of lysozyme caused by DTT, a thiol-based reducing agent in a glycine based osmolyte protected environment at pH 72 and 37 °C.

3.2.1. UV-Vis Turbidity Assay

Optical density measurements were taken using a PerkinElmer Lambda 35 UV/Vis spectrometer. Protein samples were incubated at their indicated time according to section 3.1.3 and a visible spectrum of each lysozyme sample was acquired between 380 to 700 nm. Each sample was mixed well via pipetting before measurements. Milli Q water was used in the reference cell. Absorbance measurements of each sample assayed was plotted

as incubation time vs. optical density at 600 nm. Absorbance values (pertaining to the same osmolyte) were divided by the largest absorbance value. This provided a percentage of protein denatured. The data sets were then normalized to incubation time vs. fraction denatured. All measurements were performed in triplicates.

3.2.2. Water Raman Spectrum

Before every fluorescence experiment, a water Raman spectrum was taken using MilliQ water. Fluorescence emission spectrum was taken between 365-450 nm with excitation at 350 nm. Emission and excitation bandwidths were both set to 5 nm. This was used for normalization of fluorescence data acquired on different days to avoid instrument variability.

3.2.3. Intrinsic Fluorescence Assay

Fluorescence experiments were performed using a Horiba Jobin Yvon spectrofluorometer (Fluoromax-4). Protein samples were incubated at their indicated time according to section 3.1.3. Intrinsic fluorescence emission spectra of the lysozyme samples were collected between 285-450 nm with excitation at 280 nm. Emission and excitation bandwidths were both set to 2 nm. The data was plotted at 345 nm as incubation time vs. counts per second divided by 1000 (CPS/1000). All experiments were performed in triplicate.

3.2.4.. ANS Extrinsic Fluorescence Assay

Immediately after collecting intrinsic emission spectra, 5 μL of 1-anilinonaphthalene-8-sulfonate (ANS) was added to each protein sample and was incubated on ice and in darkness for 15 minutes. Fluorescence emission spectra were collected between 400-700 nm with excitation at 385 nm. Emission and excitation bandwidths were both set to 2 nm. Fluorescence measurements were plotted at 345 nm as incubation time vs. counts per second divided by 1000 (CPS/1000). All experiments were performed in triplicates.

The following methods were used to investigate the hydrodynamic volume and the morphology of lysozyme after denaturing in an osmolyte stabilizing environment. These methods were used to provide evidence of the formation of high molecular weight species as well as provide insight into whether osmolytes influence disulfide scrambling.

3.2.5. Non-reducing SDS PAGE

Lysozyme samples containing 1 M osmolyte and 1 mM DTT were chosen for non-reducing SDS PAGE. These samples were incubated for either 4 hours or 48 hours at 37 °C. Then the samples were incubated with 5 mM iodoacetamide for two hours at room temperature to block any free –SH groups. These samples were then mixed with sample buffer (that has all the components of traditional SDS-PAGE except the reducing agent) and boiled for five minutes. A 12% SDS gel was cast and pre-run for 30 minutes before loading samples. Samples were loaded into the gel at 8.5 μg/lane and gel was run for two

hour and fifteen minutes at 80 V. Samples containing 0 M osmolyte with 0 mM DTT with 0 incubation time and samples containing 0 M osmolyte with 1 mM DTT incubated at either 4 or 48 hours were used as controls.

3.2.6. Field Emission-Scanning Electron Microscopic Imaging

Aggregate samples were prepared using a similar protocol to previous published work from our research group[41]. In order to remove osmolytes and low molecular weight species, the samples were diluted by a factor of ten, washed with distilled water three times (45 minute cycle each), aliquoted on Millipore Amicon Ultra centrifugal filters (3000 Da cutoff) and centrifuged at 7000 g at 4 °C. The samples were then aliquoted and allowed to dry on SEM stubs at ambient temperature. Samples were then sputter coated with 10nm of platinum. Each sample was imaged using a Hitachi S-4700 cold field emission high-resolution scanning electron microscope with an accelerating voltage of 5 kV and an emission current of 10 μA.

4. Data and Observations

In this project, protein samples were observed using UV-vis absorbance to determine the formation of aggregates and its associated kinetics. Do the osmolytes provide long term protection or only short term? Intrinsic fluorescence measurements were used to monitor the protein misfolding in presence of disulfide reducing agent. As the disulfide bonds reduce, the aromatic amino acids become surface exposed affecting their fluorescence properties. This can be monitored by shift in the wavelength with change in local environment of the amino acid (from non-polar (hydrophobic protein core) to polar (exposed to water/solvent)). ANS is an extrinsic fluorophore and provides information about protein misfolding, hydrophobic exposure and protein aggregation. The differential response observed in presence of osmolytes provides insight into their individual protective influence on protein incubated in specific set of conditions. Non-reducing SDS PAGE provides information on formation of high molecular weight protein species that may be a result of disulfide scrambling. Depending upon the samples incubated in presence of different osmolytes we expect to see variability in the different high molecular weight bands on gel. Scanning electron microscopy was used to characterize the morphology of these aggregates and see if the osmolytes affected the morphology or kinetics of aggregate formation. We acquired the data in presence of 0 M, 0.05 M, and 1 M of each osmolyte in presence of 0 M, 1 mM, and 10 mM of DTT at pH 7.2 and at 37 °C. However, in the main section we only included data for protein samples in presence of 1 mM DTT. Data for 0 mM and 10 mM DTT are available in the Appendix section. We did this for the following reasons: 1) 1 mM DTT is a mild reducing conditions that

maybe equivalent of concentrations reached with overuse of over-the-counter thiol-based antioxidants, 2) We expected to see variability in protective power of osmolytes under conditions that are relatively mild.

4.1. UV-Vis Turbidity

UV-vis absorption of protein at 600 nm in presence of 1 M glycine showed a significant difference in absorbance compared to 0.05 M and 0 M glycine as shown in Figure 4.1. The absorbance remains relatively flat and stable throughout the 168 hours of observation, suggesting protection from aggregation. Lysozyme containing 0 M and 0.05 M glycine exhibited very similar misfolding and aggregation rates; the fraction denatured lysozyme increased rapidly for the first 24 hours then absorbance was stable for the rest of the 168 hours. This suggests failure of glycine to protect lysozyme at 0.05 M concentration. The fraction denatured lysozyme increases minimally in presence of 1 M glycine in 1 mM DTT sample up to 24 hours. Glycine shows long term protection potential throughout the course of 168 hours of incubation.

Sarcosine enriched samples followed a near identical trend to that of glycine enriched samples. In Figure 4.2, lysozyme at 1 M sarcosine and 1 mM DTT showed no turbidity for the first four hours of incubation while the 0.05 M and 0 M both at 1 mM DTT showed gradual increases in turbidity. Sarcosine showed protective effect for lysozyme samples similar to that seen with glycine (Figure 4.2).

In Figure 4.3, 1 M dimethylglycine showed protection from aggregation in the first 4 hours, then the lysozyme showed increased aggregation with time that was very similar to

samples with 0 M or 0.05 M of DMG. The overall fraction denatured lysozyme is slightly lower for samples containing 1 M DMG compared to protein samples having 0 M or 0.05 M DMG; thus showing a limited protecting potential at 1 M concentration of DMG. Surprisingly, betaine did not show any protective effect on disulfide reduced proteins even at 1 M concentration (Figure 4.4). This osmolyte was the least protective when compared to other osmolytes under same conditions. There was no difference in turbidity between the 0 M, 0.05 M, and 1 M betaine enriched samples (Figure 4.4). This trend was consistent throughout the 168 hours of incubation.

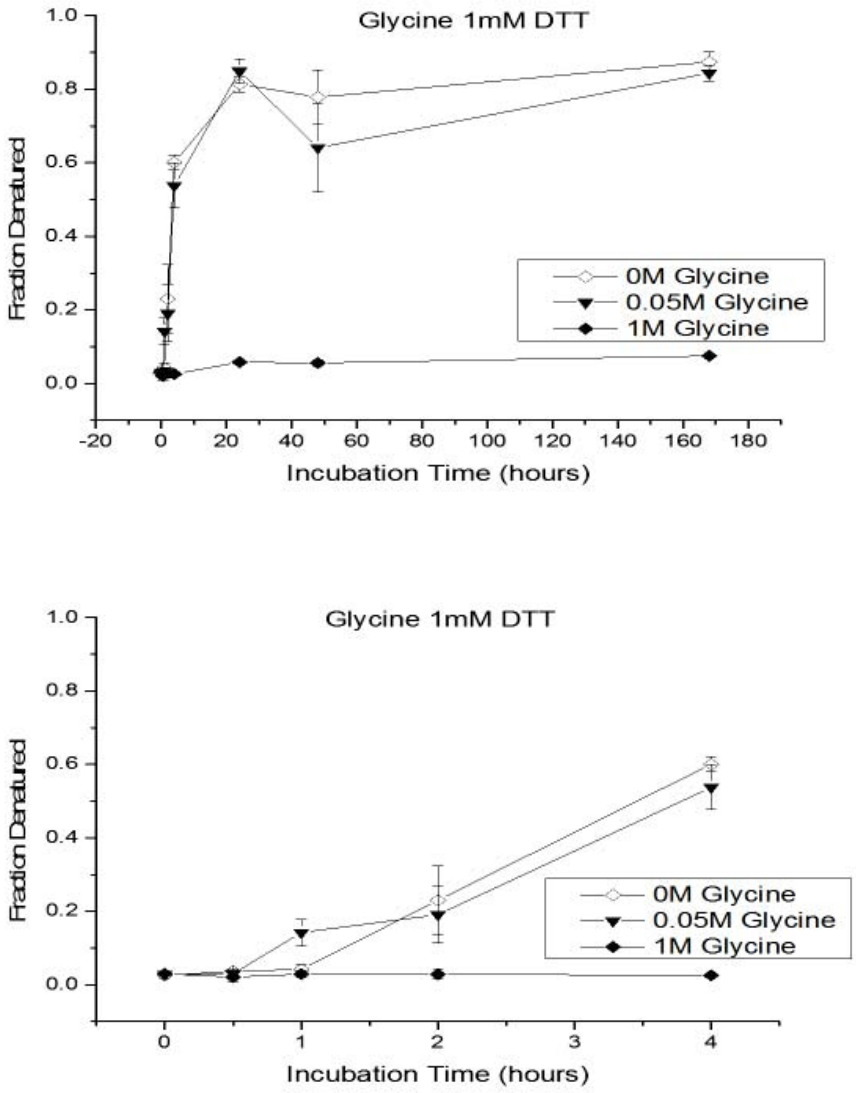


Figure 4.1. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

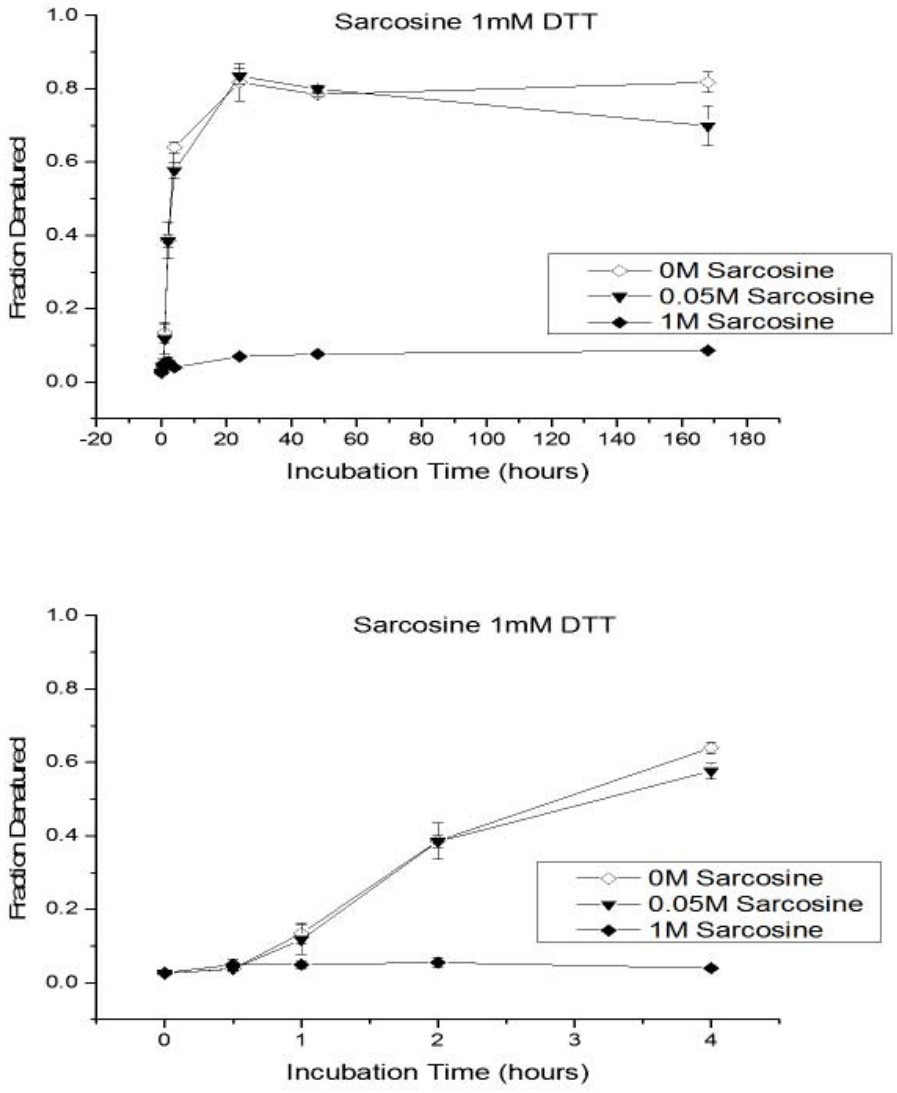


Figure 4.2. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

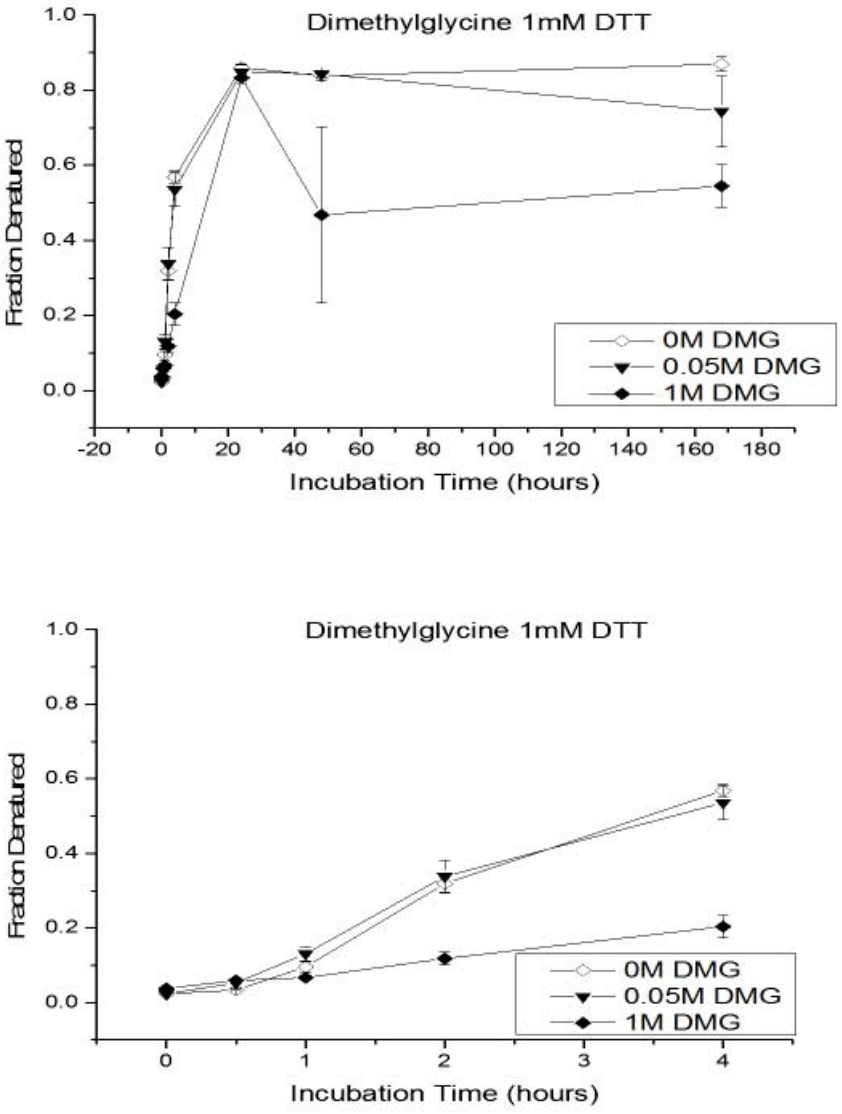


Figure 4.3. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

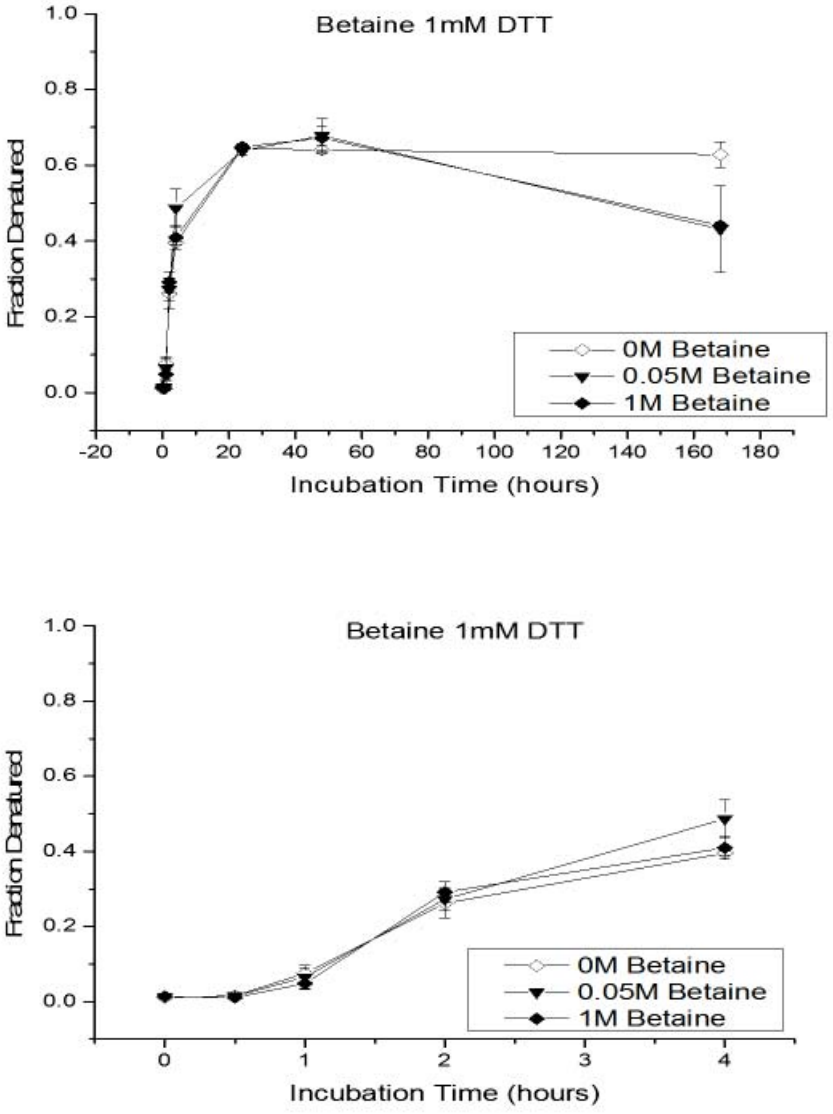


Figure 4.4. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

4.2. Intrinsic Fluorescence

In Figure 4.5, the intrinsic fluorescence for samples at 1 M glycine concentration was noticeably lower than that for proteins incubated in presence of 0 M or 0.05 M glycine. There was no perceptible difference in fluorescence for the samples in 0 M or 0.05 M throughout the 168 hours. The difference in intrinsic fluorescence for lysozyme samples at 1 M glycine compared to proteins incubated at 0 M and 0.05 M glycine was more up to 48 hours of incubation and that gap decreased upon longer incubation.

Protein samples containing sarcosine showed similar trend as that observed for samples containing glycine but still showed a little more protection for protein at 1 M concentration compared to glycine (Figure 4.6). The peak cps value for samples containing 1 M sarcosine stayed consistently low while the fluorescence increased gradually for the samples containing 0 M and 0.05 M sarcosine. After 48 hours, the samples showed a consistent decrease in fluorescence for the length (168 hours) of the incubation.

Samples containing dimethylglycine showed a noticeable difference in fluorescence for samples with 1 M DMG compared to samples with 0 M or 0.05 M DMG (Figure 4.7). This pattern remained consistent throughout the duration of the 168 hours incubation period (Figure 4.7).

Again no difference in fluorescence of samples incubated in presence of betaine was observed (Figure 4.8). This indicates that betaine has no osmoprotective effect on

proteins incubated in its presence. This indicates that betaine is unable to stabilize the protein against reducing influence where both glycine and sarcosine are effective.

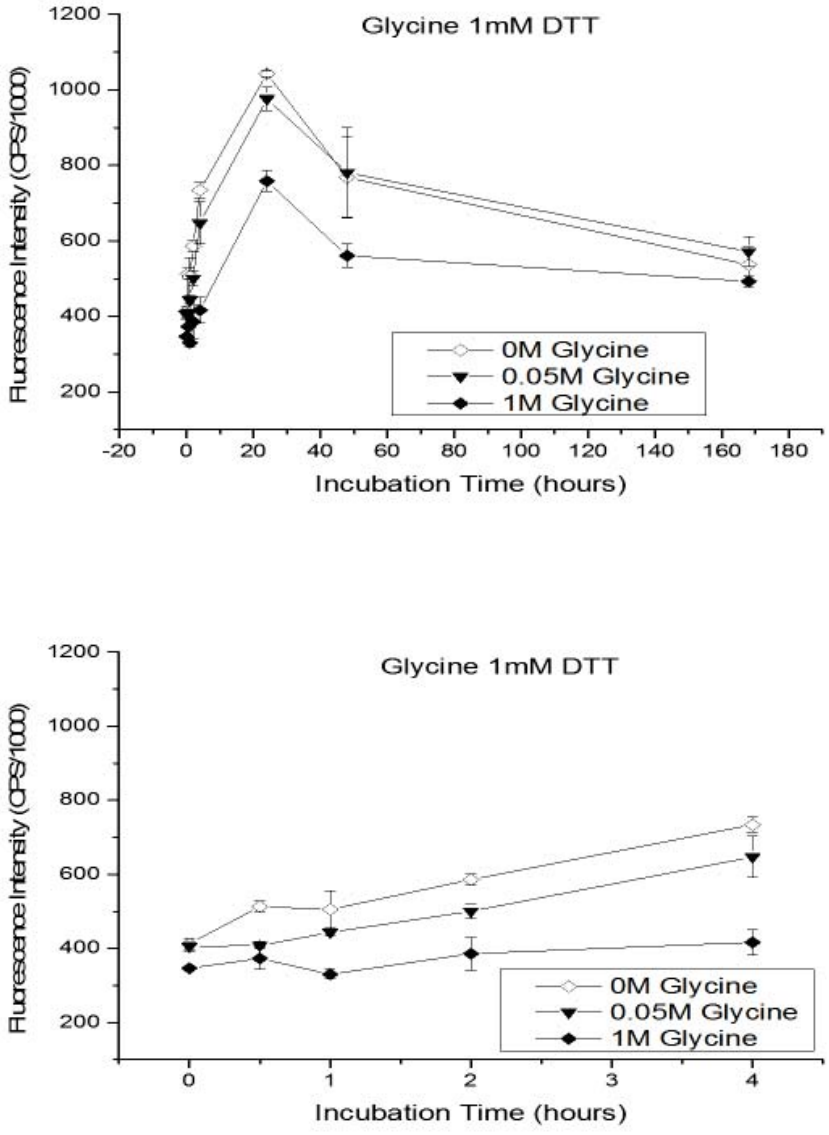


Figure 4.5. Intrinsic fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

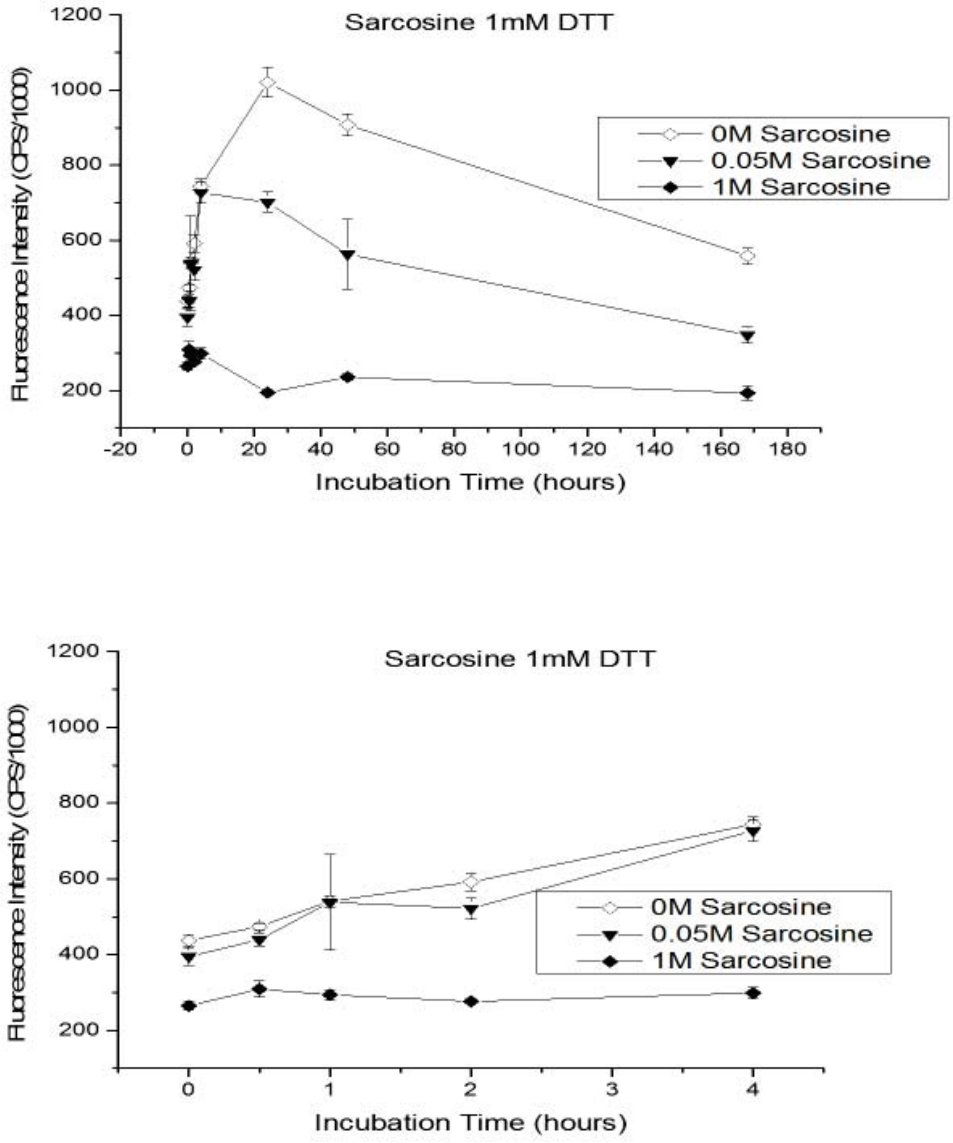


Figure 4.6. Intrinsic fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

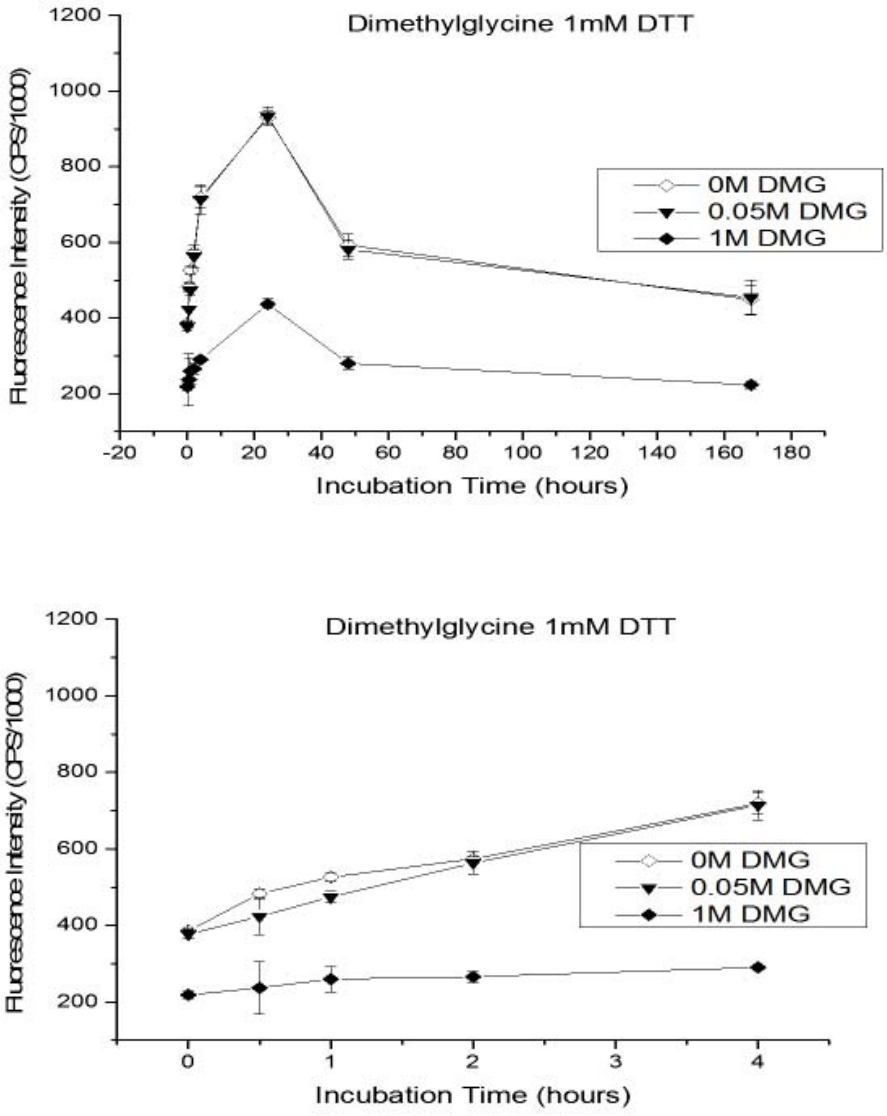


Figure 4.7. Intrinsic fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

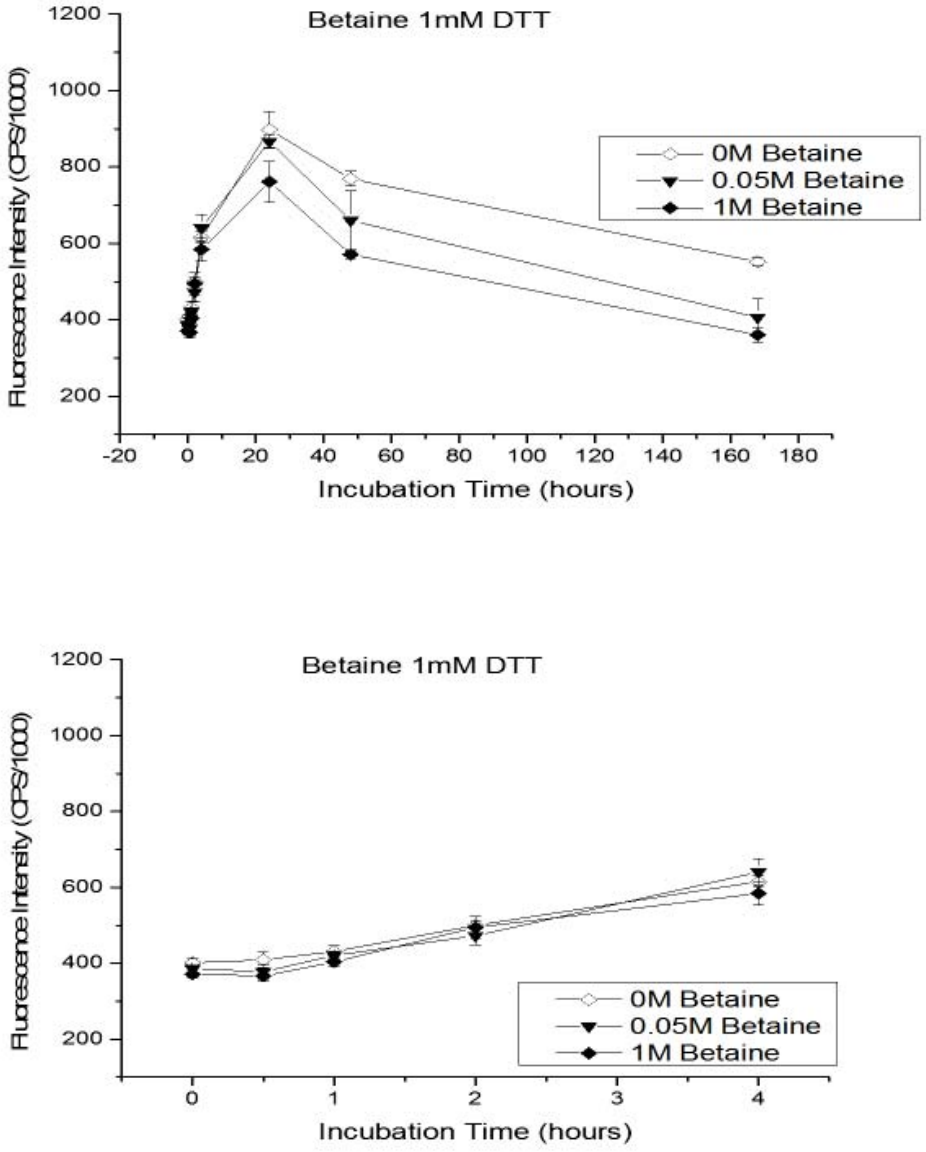


Figure 4.8. Intrinsic fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

4.3. ANS Fluorescence

In Figure 4.9, proteins in presence of 1 M glycine showed a decreased emission only in the first 4 hours compared to samples incubated in presence of 0 M or 0.05 M glycine. Interestingly, upon longer incubation there was not much difference in emission peak of samples at 168 hours of incubation irrespective of the concentration of glycine.

Sarcosine shows a measurable protection for proteins incubated in presence of 1 M osmolyte compared to proteins incubated with 0 M or 0.05 M osmolyte (Figure 4.10). This result is in line with other measurements done (intrinsic fluorescence and UV-visible turbidity assay) for samples containing sarcosine.

The ANS binding for samples in presence of DMG did not vary much with change in osmolyte concentration (Figure 4.11). In contrast, the turbidity data and intrinsic fluorescence data for DMG showed a protective effect at 1 M concentrations of osmolyte.

Again proteins incubated in presence or absence of betaine (Figure 4.12) did not show any difference in ANS fluorescence suggesting betaine do not protect proteins against reducing influence.

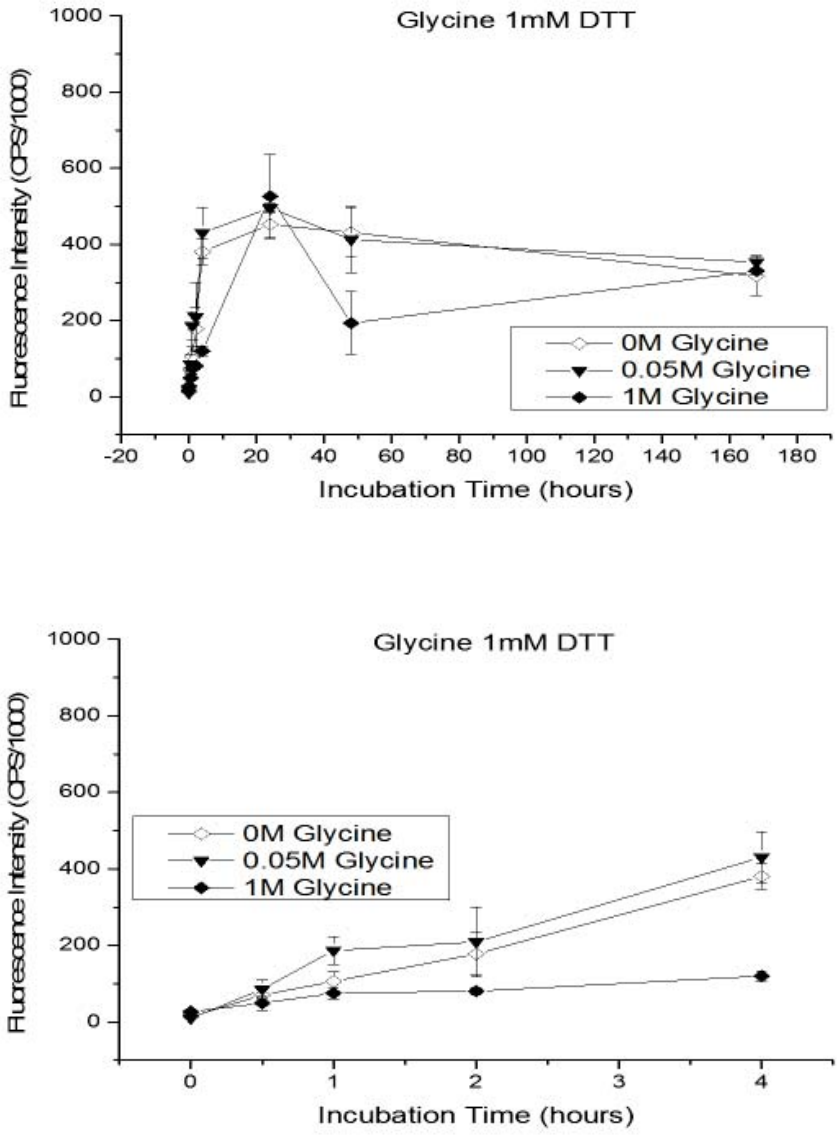


Figure 4.9. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

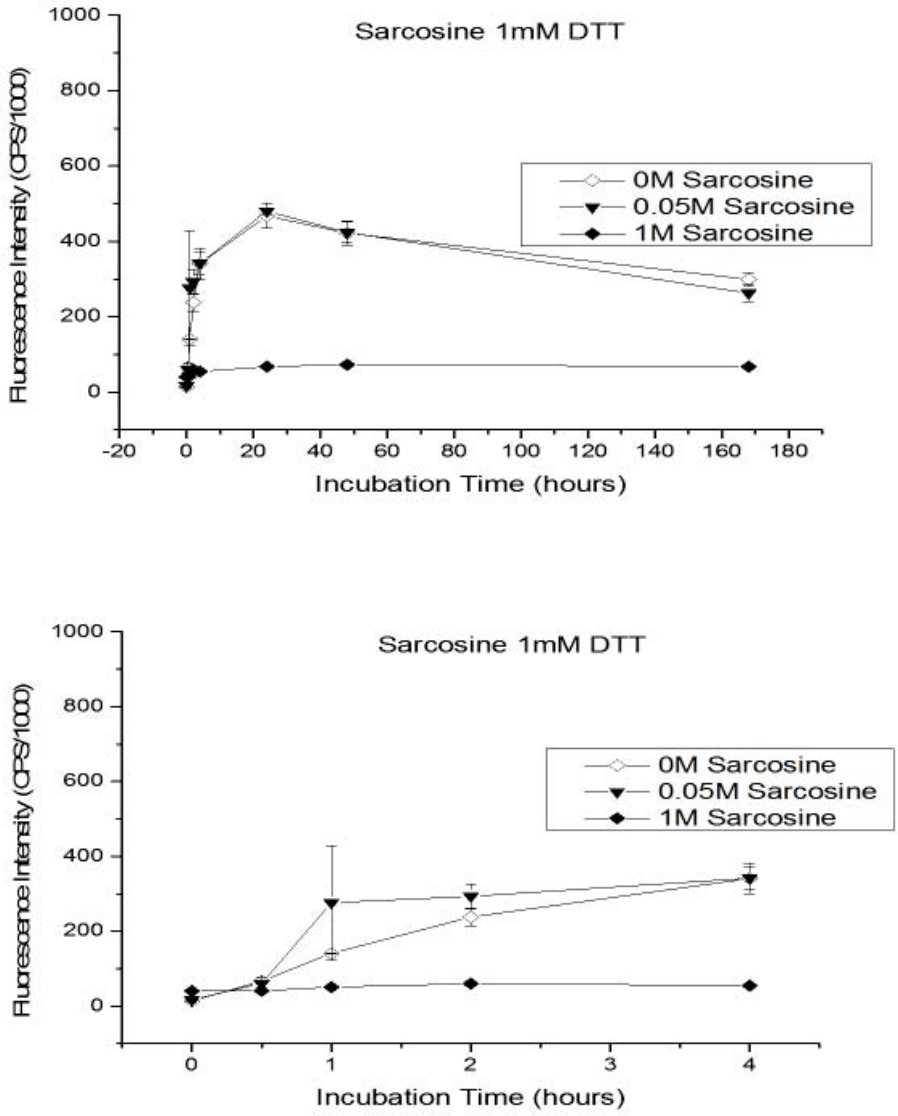


Figure 4.10. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

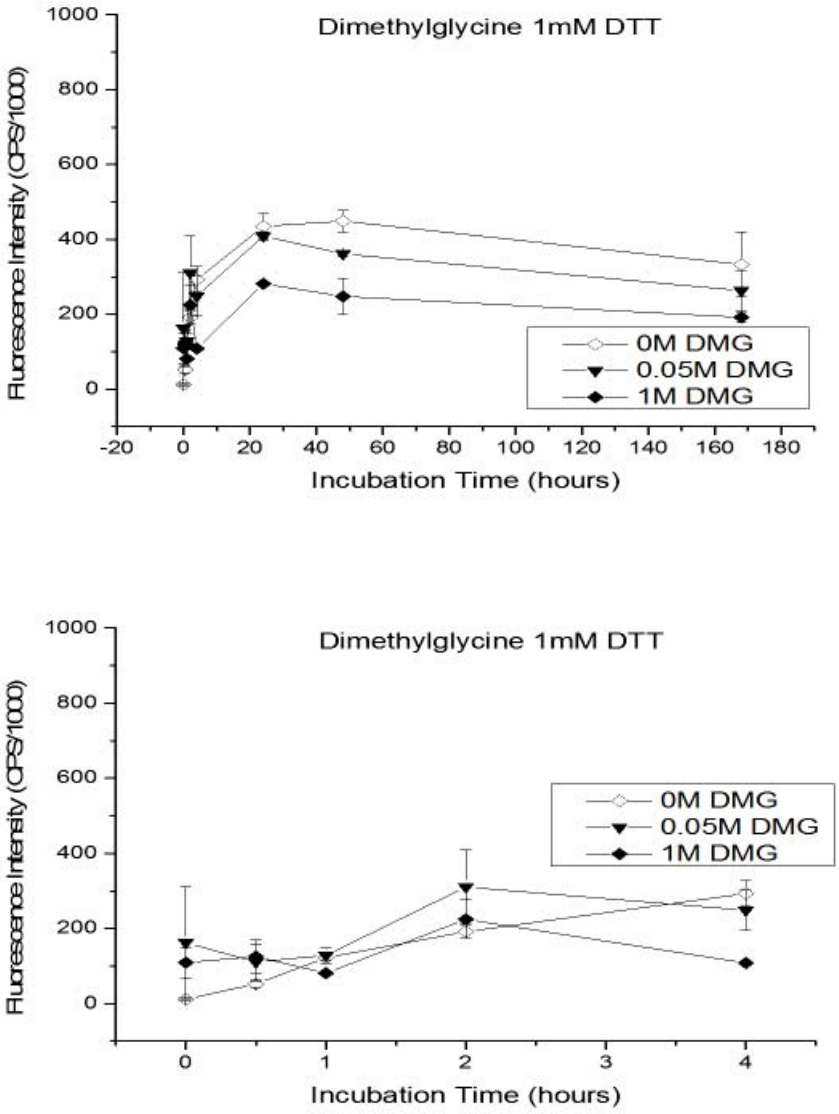


Figure 4.11. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

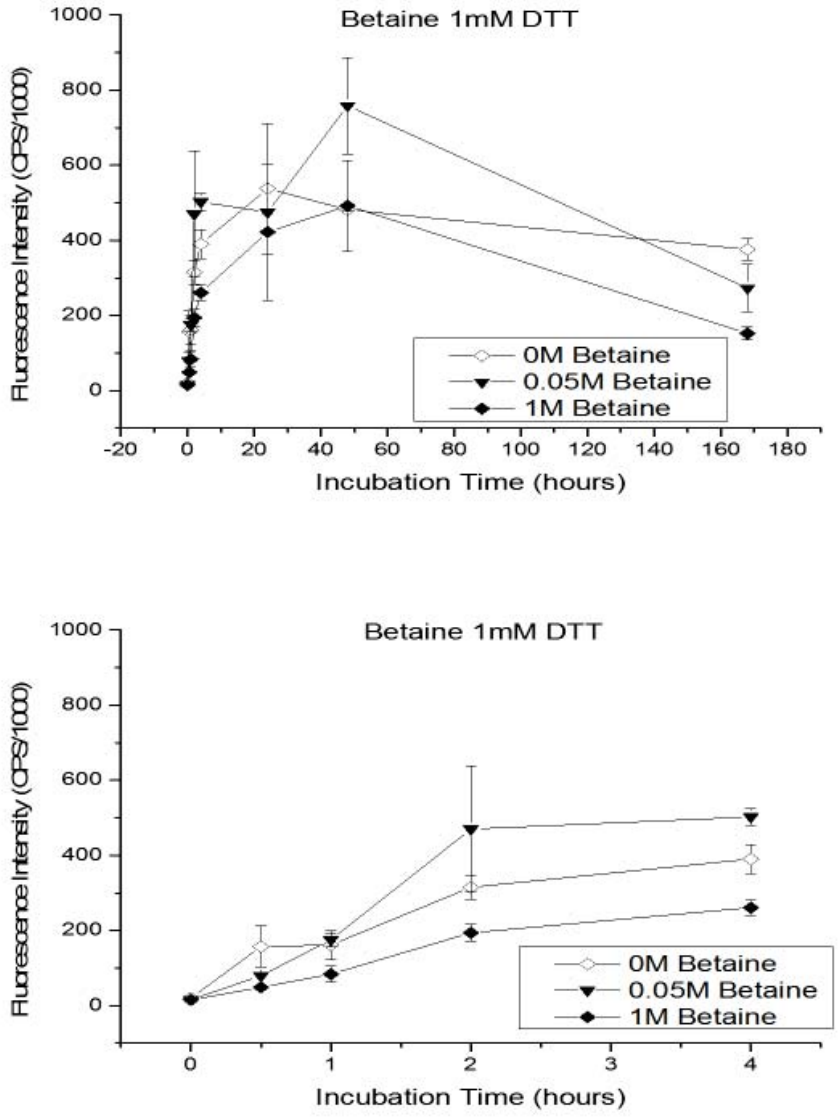


Figure 4.12. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 1mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

4.4. Non-reducing SDS PAGE

Non-reducing SDS-PAGE was carried out to check for the presence of high molecular weight species that may be present in samples incubated in absence or presence of 1 M concentration of all four osmolytes in buffer containing 1 mM DTT at pH 7.2 and incubated at 37 °C. Based on spectroscopic data (both UV-visible absorbance and fluorescence) samples containing 1 M osmolyte were run on gel for 4 hours and 48 hours incubation times (Figures 4.13 and 4.14). The 4 hours gel shows strong presence of lysozyme dimers for samples incubated with betaine that are similar to lane of protein incubated similarly (lane 5) but in absence of any osmolyte (Figure 4.13). Samples incubated in presence of DMG (lane 9) shows a very faint band for the dimeric species. Interestingly, no higher molecular weight species were observed for proteins incubated with sarcosine. Glycine (lane 13) showed a faint band, indicative of dimeric species. For samples incubated for 48 hours showed very strong dimeric band for samples in absence of osmolyte (lane 5) and presence of betaine (lane 7), no higher molecular weight species were observed in presence of DMG (lane 9), and a faint dimeric band for glycine (lane 13) (Figure 4.14). Sarcosine showed increased protection with absence of any dimeric band even after 48 hours of incubation of protein samples (Figure 4.14).

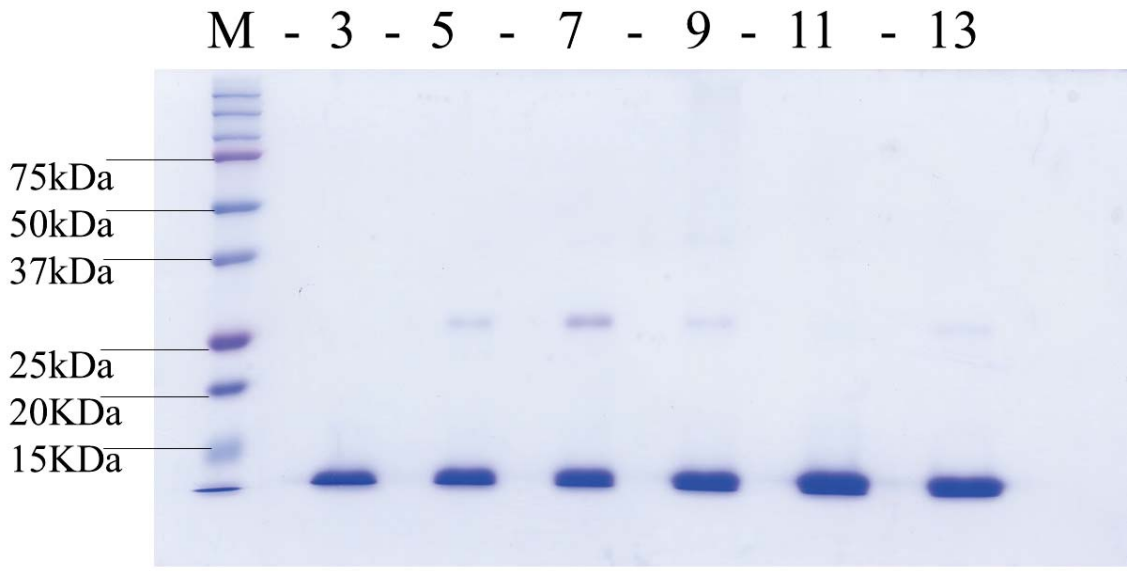


Figure 4.13. Non-reducing SDS PAGE of lysozyme samples incubated for 4 h at the conditions indicated. For samples in different lanes the following incubation conditions were used: (3) 0 M osmolyte, 0 mM DTT (Fresh control sample), (5) 0 M osmolyte in presence of 1mM DTT, (7) 1 M Betaine in presence of 1mM DTT, (9) 1 M DMG in presence of 1 mM DTT, (11) 1M Sarcosine in presence of 1 mM DTT, (13) 1M Glycine in presence of 1 mM DTT. All samples were incubated at 37 °C for 4 h before preparing for non-reducing PAGE as detailed in method section. Samples were run on a 12% SDS-PAGE gel at 80 V for 2 h and 15min. Lane marked (M) is SDS marker proteins.

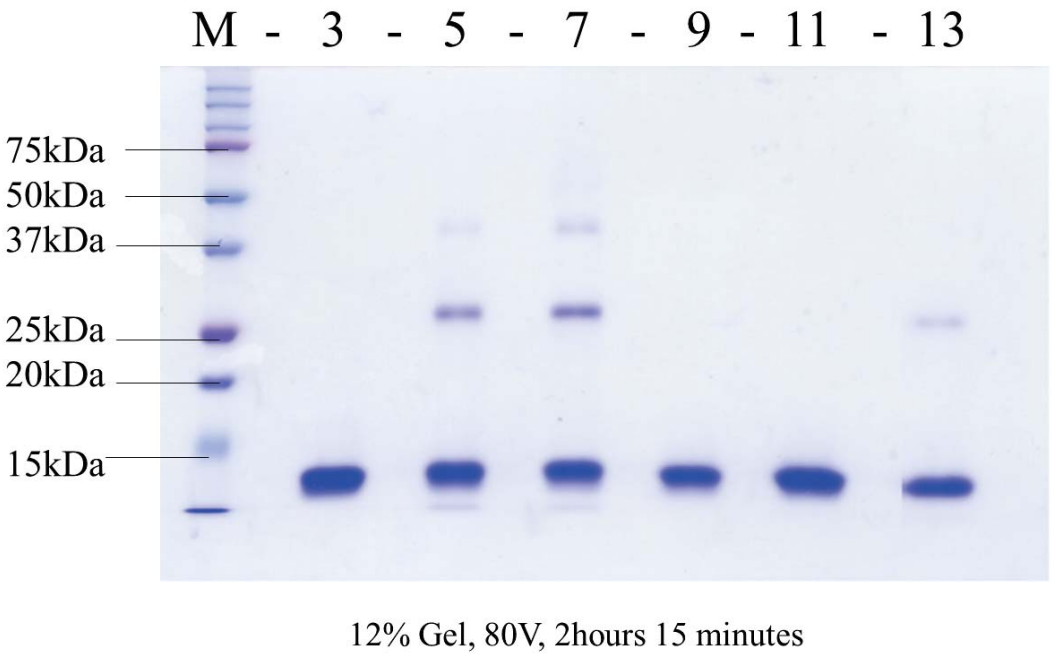


Figure 4.14. Non-reducing SDS PAGE of lysozyme samples incubated for 48 h at the conditions indicated. For samples in different lanes the following incubation conditions were used: (3) 0 M osmolyte, 0 mM DTT (Fresh control sample), (5) 0 M osmolyte in presence of 1mM DTT, (7) 1 M Betaine in presence of 1mM DTT, (9) 1 M DMG in presence of 1 mM DTT, (11) 1M Sarcosine in presence of 1 mM DTT, (13) 1M Glycine in presence of 1 mM DTT. All samples were incubated at 37 °C for 48 h before preparing for non-reducing PAGE as detailed in method section. Samples were run on a 12% SDS-PAGE gel at 80 V for 2 h and 15min. Lane marked (M) is SDS marker proteins.

After 48 hours of incubation, the dimeric population for both the 0 M osmolyte and that for samples containing 1 M Betaine in presence of 1 mM DTT showed an increase in relative amount of the protein (Figure 4.14). For these samples, a small population of higher molecular weight species were also observed. This is most likely due to formation of higher order aggregates via aberrant interaction and disulfide scrambling.

4.5. Field Emission Scanning Electron Microscopy

Field emission scanning electron microscopy was used to characterize the morphology of the protein aggregates formed after 4 hours and 48 hours of incubation (Figures 4.15, 4.16, and 4.17). The images show that lysozyme in presence of glycine-based osmolytes form amorphous aggregates that look slightly different than the amorphous aggregates formed in absence of osmolytes for proteins similarly incubated (Figures 4.16 and 4.17). Since the aggregates appear to be amorphous at both 4 hours and 48 hours of incubation, it can be concluded that these osmolytes do not alter the morphology of the protein at least in the length of time samples are incubated. Figure 4.15 shows image of a control lysozyme sample in the absence of both DTT and osmolyte. No aggregate is visible in this sample thus proving that aggregates did not form in the absence of reducing agent.

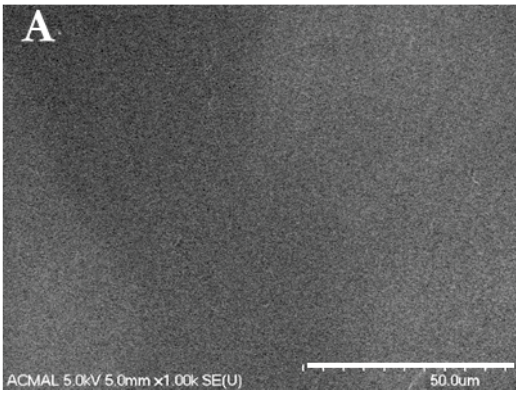


Figure 4.15. 40 μM lysozyme incubated in absence of DTT or osmolytes for 4 h. A cold field emission high-resolution Hitachi S-4700 scanning electron microscope was used with an accelerating voltage of 5 kV and an emission current of 10 μA. Scale bar = 50 μm.

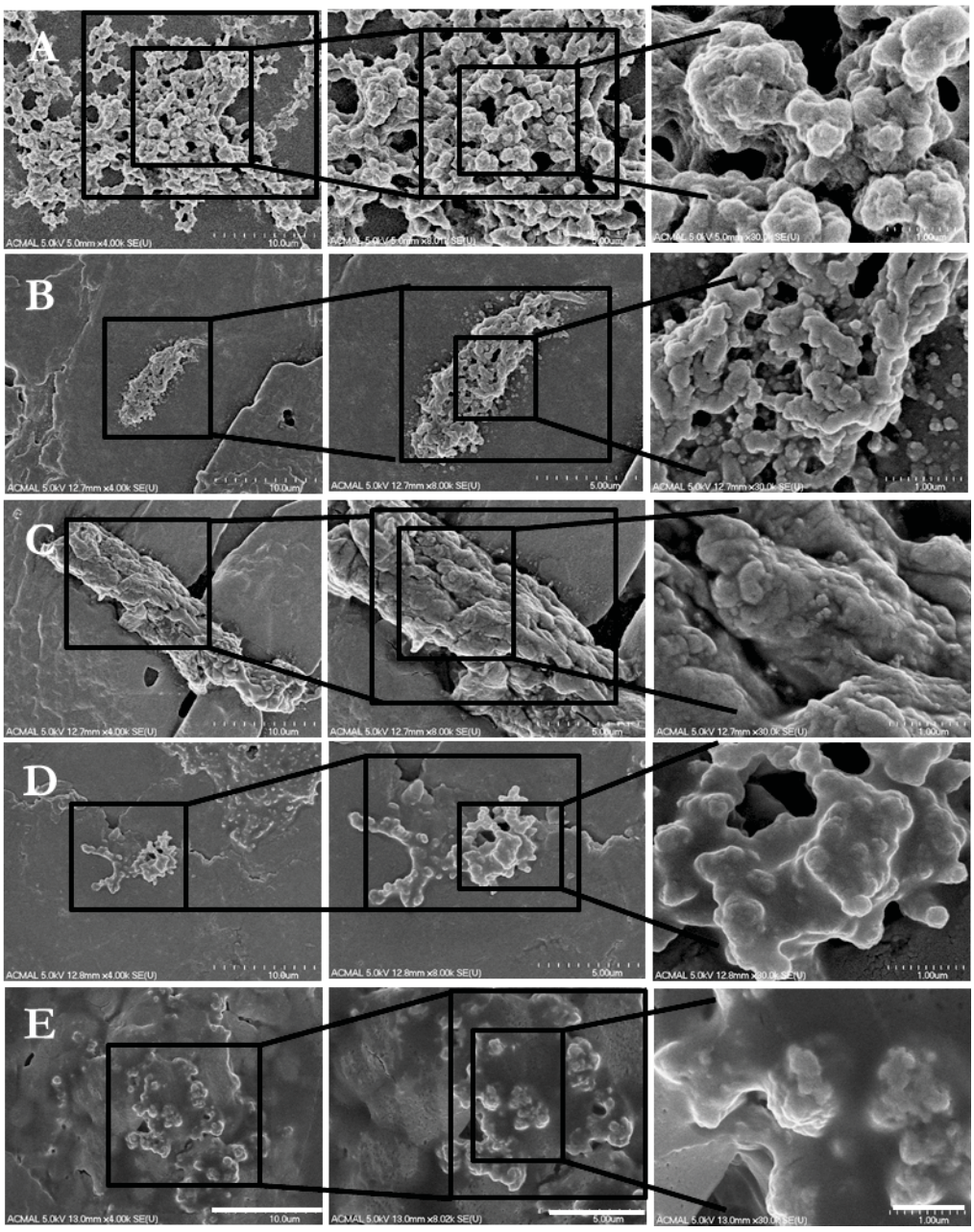


Figure 4.16. SEM images showing lysozyme (40 μM) aggregates observed in buffer containing 1 mM DTT at 37 °C for 4 h in presence of indicated osmolytes (A) 0 mM osmolytes (B) 1M glycine (C) 1 M sarcosine (D) 1 M DMG, and (E) 1M Betaine. Scale bars are 10, 5, and 1 μm from left to right.

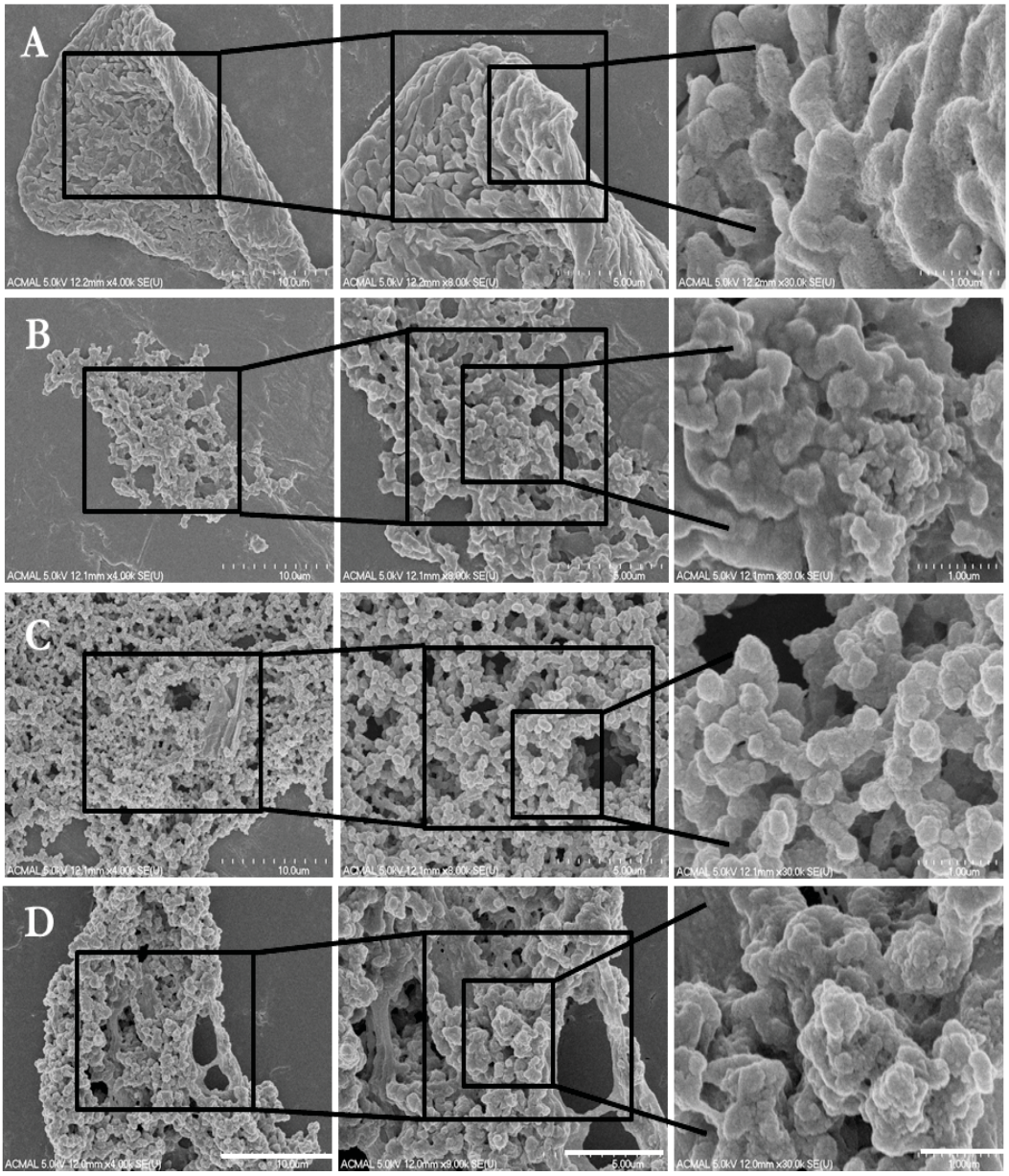


Figure 4.17. SEM images showing lysozyme (40 μM) aggregates observed in buffer containing 1 mM DTT at 37 °C for 48 h in presence of indicated osmolytes (A) 1M glycine (B) 1 M sarcosine (C) 1 M DMG, and (D) 1M Betaine. Scale bars are 10, 5, and 1 μm from left to right.

5. Discussion

In general osmolytes protect proteins from environmental stresses to preserve their functionality.[1-9, 28 50] The mechanism by which most cosolvents protect proteins from denaturing stressors is *preferential hydration*.[10-15] Health foods, such as quinoa, spinach, fish, and whole grain products naturally have a significant amount of osmolytes such as betaine and glycine.[51] These compounds are highly thermostable and survive the baking process.[52 53] As a result, consumers who eat these healthy foods ingest glycine-based osmolytes regularly. Consuming regular amount of betaine have shown to have beneficial influences on the human body, especially when exercising.[54 55] Thiol-based antioxidants are also ingested by consumers in order to improve their health.[56 57] However, these antioxidants can have deleterious effects if taken improperly. As, glycine-based osmolytes exist at high concentrations in commonly ingested health food products, we wanted to study the protective effect of these osmolytes on proteins against disulfide-reducing agent.

We chose a series of glycine based osmolytes with varying N-methyl substitutions. Glycine has no methyl group, sarcosine has one, di-methyl glycine (DMG) has two, and betaine has three methyl groups attached to the amino terminal. Glycine, sarcosine, and betaine are naturally occurring osmolytes whereas DMG is a synthetic compound. We also chose dithiothreitol (DTT), a commonly used thiol based reducing agent in protein stability and aggregation studies.[41] All the studies discussed below had 0, 0.05, or 1 M of osmolyte in buffer that had 1 mM DTT and was incubated for varying periods of time. The pH and temperature of incubation were close to physiological (pH 7.2; temperature

37 °C). The objective of this study was to determine if commonly consumed glycine based osmolytes were able to provide protection to proteins (or contribute to denaturation) when exposed to different concentrations of thiol based reducing agent. If so, was it by directly binding (low concentration study at 0.05 M) or through preferential hydration (high concentration study at 1 M)? It is well known and studied that when proteins disulfide bonds are cleaved, the protein destabilizes and begins to unfold.[35] As a consequence of unfolding, hydrophobic core of the proteins opens up and gets solvent exposed.[41] This makes the protein sticky that can then self-associate or associate with other proteins and cellular constituents leading to aggregation.[58] [59] We used UV-visible absorbance spectroscopy and monitored proteins aggregation at 600 nm (Figures 4.1 to 4.4). This is a useful assay to monitor the protein aggregation as it denatures. The data shows that when proteins are incubated in presence of 1 M osmolytes, the maximum protection against aggregation is shown by glycine and sarcosine. DMG shows some protection against aggregation but betaine fails to provide any protection against reducing influence. This is interesting as betaine is a well-known stabilizer against thermal denaturation but in this study it does not provide any protection from disulfide-reducing agent. Previous studies have shown that betaine stabilizes proteins through a mechanism called steric exclusion. However, in our experimental conditions (highly reducing) the proteins fails to show any protection by betaine. This may be because as disulfide bonds are reduced, proteins unfold and methyl substitutions instead of structuring water around protein may be directly interacting with the proteins hydrophobic residues. However, to conclusively determine this, further experiments would be required to determine its affinity constant with the unfolded protein. Glycine and sarcosine (glycine has no methyl

substitution and sarcosine has only one) were the best stabilizers. These may be providing stability directly through well-known mechanism of preferential hydration. This observation is further corroborated by the data presented in the intrinsic fluorescence assay and the ANS extrinsic fluorescence assay (Figures 4.5. to 4.8 and 4.9 to 4.12). As the protein denatures, fluorophore residues such as tryptophan and tyrosine become exposed to the solvent thus allowing for detection via fluorescence measurements due to wavelength shift in the emission spectra. ANS is used to monitor the changes in hydrophobic exposure caused by protein unfolding. The greater the fluorescence observed for ANS with protein, the more unfolded/loose state of the protein. In presence of osmolyte we expect the water to structure better around the protein and as a consequence see decrease in fluorescence. The quantum yield of the dye increases as it comes in contact with a hydrophobic surface. All three spectroscopic techniques provide complementary information into the stabilizing influences of these glycine-based osmolytes. Low concentration of osmolytes (0.05 M) did not provide any measurable protection from the denaturing influence of reducing agent. This suggests that these osmolytes do not preferentially interact with the proteins.

We used non-reducing SDS PAGE to confirm presence of high molecular weight species in protein samples incubated in presence of osmolytes (Figures 4.13 and 4.14). We observed appearance of dimer and higher order aggregates for samples incubated in presence of betaine that was in line with spectroscopic data suggesting no protection against protein aggregation under disulfide-reducing influence. Dimethylglycine (has two N-methyl substitutions) also showed appearance of dimer for 4 hour samples. This

indicates that the osmolytes are not directing non-native disulfide bond formation but betaine and dimethylglycine are failing to preferentially hydrate the protein enough to maintain its native state or are directly interacting with the unfolded protein through their methyl substitutions.

Scanning Electron Microscopy (SEM) imaging showed the morphology of the protein aggregates. As data at 4 h and 48 h showed a major change in values for spectroscopic measurements (both UV-vis absorbance and fluorescence); we imaged samples at these two time points (samples incubated for 4 h and 48 h) to see their structural morphology (Figures 4.16 and 4.17). Samples, both at 4 and 48 hours in presence of osmolytes showed amorphous aggregates for each of the four osmolytes studied. Moreover, these aggregates appear very similar to aggregates formed in the absence of osmolyte. This suggests that osmolytes do not directly interact with protein or influence aggregation (if initiated) pathway of the protein.

In summary, glycine and sarcosine showed increased protection against disulfide reducing agent. Betaine and DMG did not show much protection against disulfide-reducing influence. And, the amount of protection against reducing influence increases as the number of methyl substitutions decrease on the osmolyte.

6. Future Work

This section provides insight into opportunities for further investigation on the subject of osmolyte stabilization influence vs. disulfide reduction.

6.1. Thioflavin T assay

There are many opportunities for the further investigation of the protective properties of glycine based osmolytes against thiol-based antioxidants. The use of thioflavin T dye using a similar protocol to the ANS extrinsic fluorescence assay outlined in section 3.2.4. would provide insight on the nature of lysozyme aggregates. Thioflavin T is a well-established fluorescent dye used in many works to determine if a protein is amyloid in nature. Even though the FESEM images provided evidence of the morphology of the protein, thioflavin T would provide more detailed structural information; such as whether glycine-based osmolytes of differing degrees of saturation influence the compactness of protein aggregates.

6.2. Variety in osmolyte and reducing agent concentration

In this project, only two concentrations of osmolyte were investigated. Further experimentation would involve investigating the protective properties of osmolytes between 0.05 M and 1 M concentration. This information would allow us to determine if there is a minimum concentration of osmolyte needed to protect a protein from denaturation by thiol reducing agent.

6.3. Investigation of other osmolyte

Future phases of this project would expand to investigating other frequently used osmolytes and molecular crowders. In particular, trehalose, polyethylene glycols (PEGs), and glycerol. These cosolvents are well established and have been investigated intently for decades. However, there is little to no information on their influence on denaturation via disulfide reduction.

6.4. Investigate osmolyte protection against reducing agents under extreme conditions

All experiments in this project have been under near-physiological conditions i.e. pH 7.2, 37 °C, and 150 mM NaCl. Evidence exists of osmolytes having different protection properties at different pH, salinity, and temperatures. An important next step in this investigation would be to modify the experimental environment to high and low temperatures, higher salinity levels, and high and low pH values. This information would provide useful information to drug industries that produce therapeutic proteins and the food industry in order to better preserve food. This information would help biologists better understand the mechanisms of osmolytes involved in extremophile homeostasis.

6.5. Apply to other proteopathic proteins

Lysozyme was chosen in this experiment as a model protein since it behaves very well *in vitro* and would provide minimal complications in this preliminary investigation. Once the protective properties of osmolytes, in particular glycine-based osmolytes, are well investigated then the ultimate goal for this project would be to apply these concepts to other proteins. Consumers ingest thiol-based antioxidants as supplements which diffuse into the circulatory system where they eventually come into contact with other proteins, in particular hormones. Hormones, such as insulin, are small proteins that contain a high concentration of disulfide bonds. Disulfide bonds, exposed to solvent, can be reduced naturally by glutathione. Glutathione exists naturally in the human body; however, consumers also ingest glutathione as a supplement to reduce oxidative stress. This increased concentration of glutathione could have a negative impact on the stability of proteins. Investigating the protective influence of glycine-based osmolytes on known proteopathic proteins would be beneficial in understanding its unfolding mechanism in vivo. This may even provide insight on the onset of certain neurodegenerative diseases.

7. Conclusion

To conclude, glycine based osmolytes are able to protect lysozyme from disulfide reduction to an extent. At near physiological conditions, these osmolytes are able to preferentially hydrate the protein to an extent at both 1 mM DTT and 10 mM DTT. The amount of aggregation was reduced as the number of N-methyl groups decreased. This information indicates that in a disulfide reducing environment, the less methylated the osmolyte (for glycine-based) the greater the solvent exclusion of the osmolyte. This was confirmed through a series of assay of UV-vis absorbance measurements, and fluorescence assay, both intrinsic and extrinsic with ANS. Non-reducing SDS-PAGE confirmed the formation of aggregates for betaine and dimethylglycine in which betaine especially mimicked samples not enriched by osmolyte. SEM imaging presented the formation of amorphous aggregates, which did not change as incubation time increased. These experiments provide an initial investigation into the protective properties of glycine based osmolytes on proteins when exposed to disulfide reducing stressors. This information can be applied to consumers ingesting an improper amount of thiol-based antioxidants while consuming and using glycine based products.

8. References

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Appendix

Data for 0 mM and 10 mM DTT were placed in the Appendix for the following reasons: 1) 1 mM DTT is a mild reducing conditions that may be equivalent of concentrations reached due to over use of over-the-counter thiol-based antioxidants, 2) We expected to see variability in protective power of osmolytes under conditions that are relatively mild. Data obtained at 0 mM DTT served as a control, while data obtained at 10 mM DTT represented extreme reducing conditions.

This appendix is broken down into four sections: 1. UV-vis Turbidity Supplemental data, 2. Intrinsic Fluorescence Supplemental Data, 3. ANS Extrinsic Fluorescence Supplemental data. The figures within each section are organized first by concentration of DTT used, i.e. 0mM DTT data then 10mM DTT data, and then by osmolyte used (same order as Results section, i.e. glycine, sarcosine, dimethylglycine, betaine). Section 4 has a high contrast image of the gel Figures 4.14 and 4.15 showing the high molecular weight species observed for the cross-linked proteins.

A.1. UV-vis Turbidity Supplemental Figures

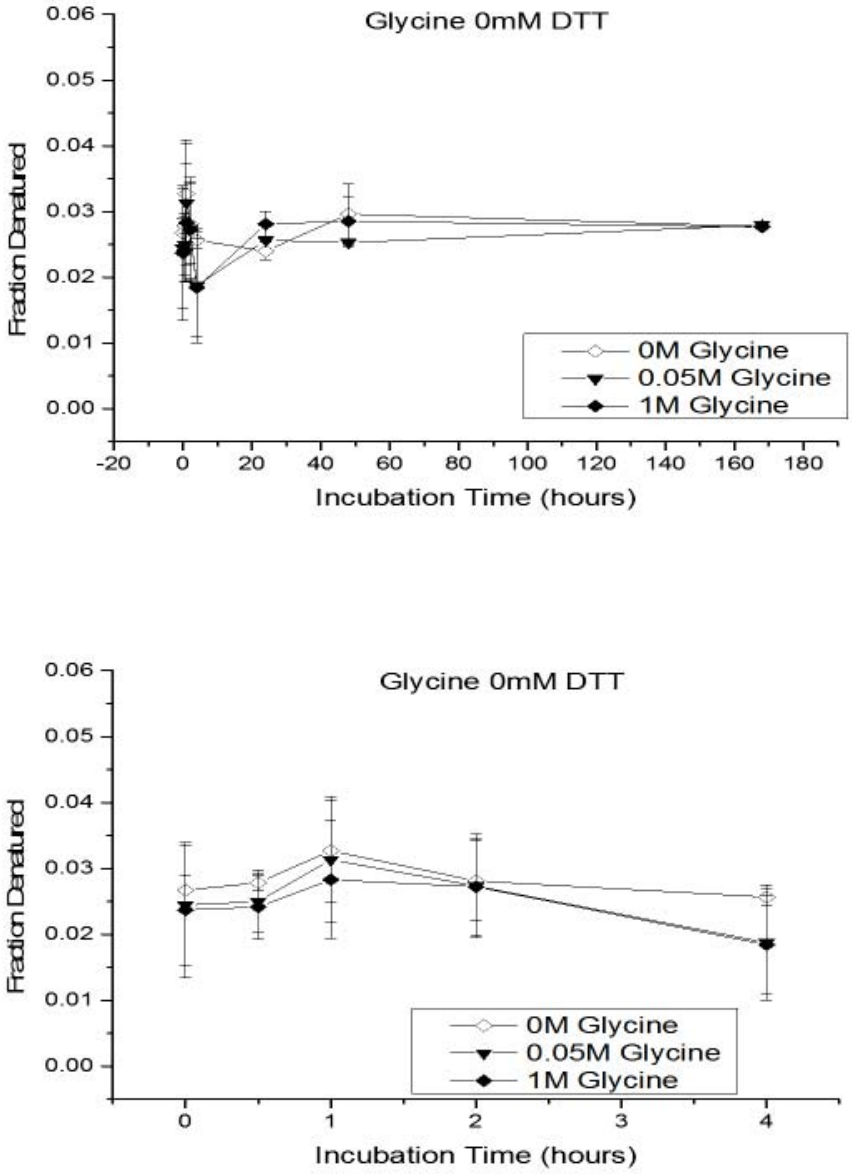

Figure A.1. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

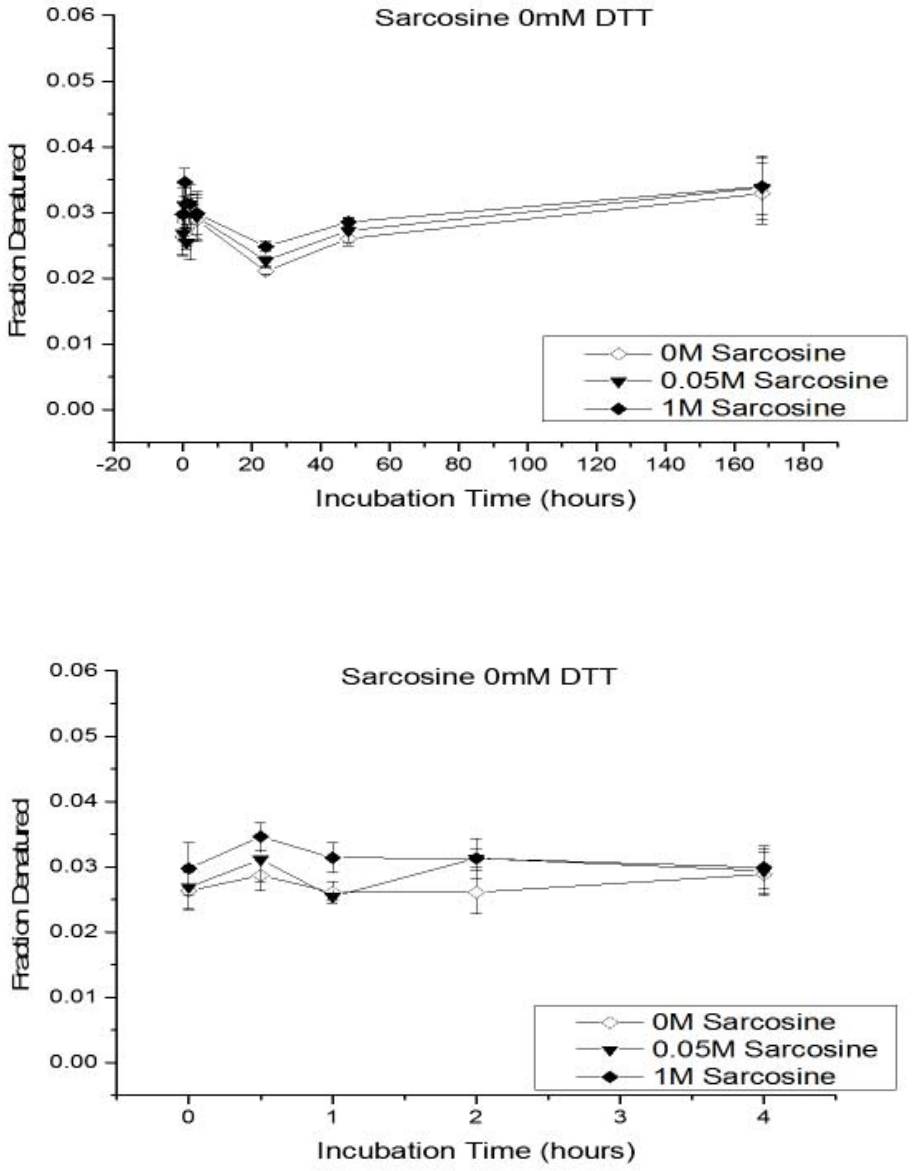

Figure A.2. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

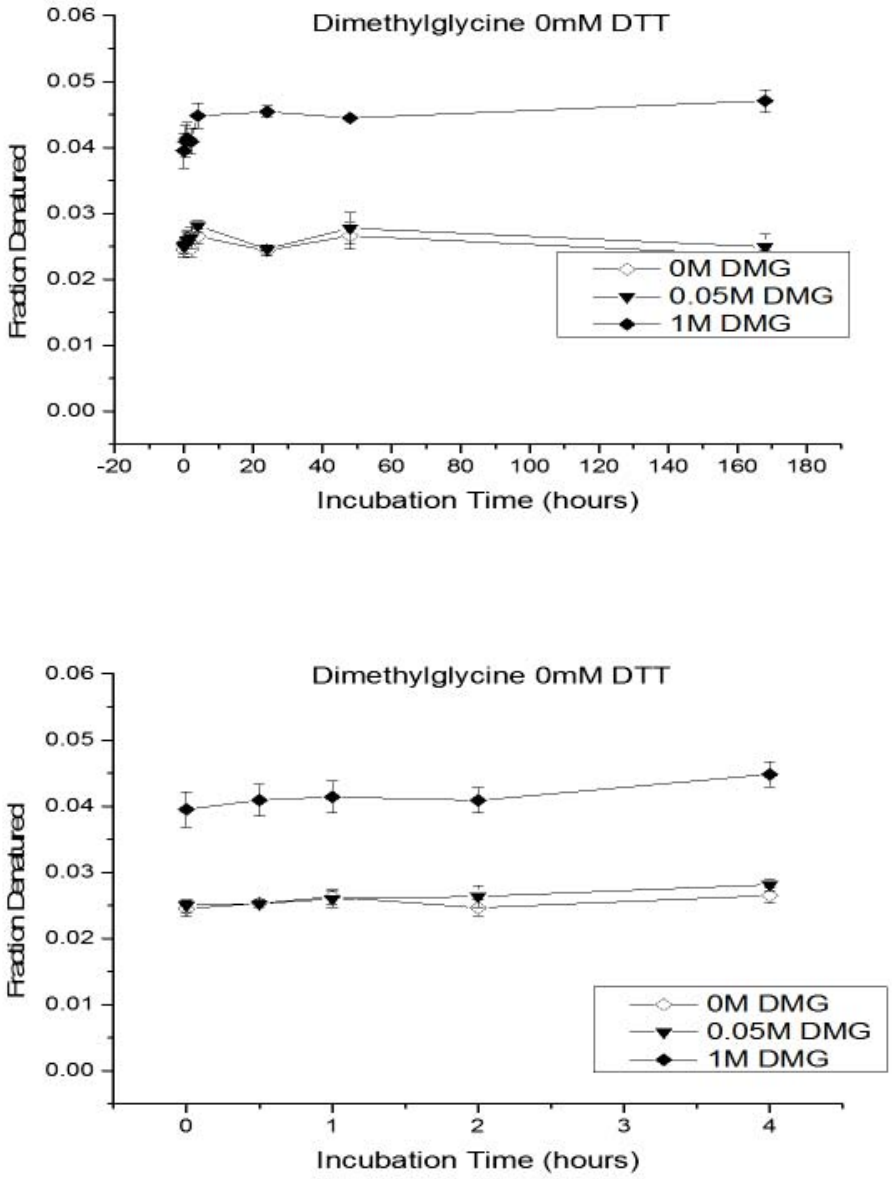

Figure A.3. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

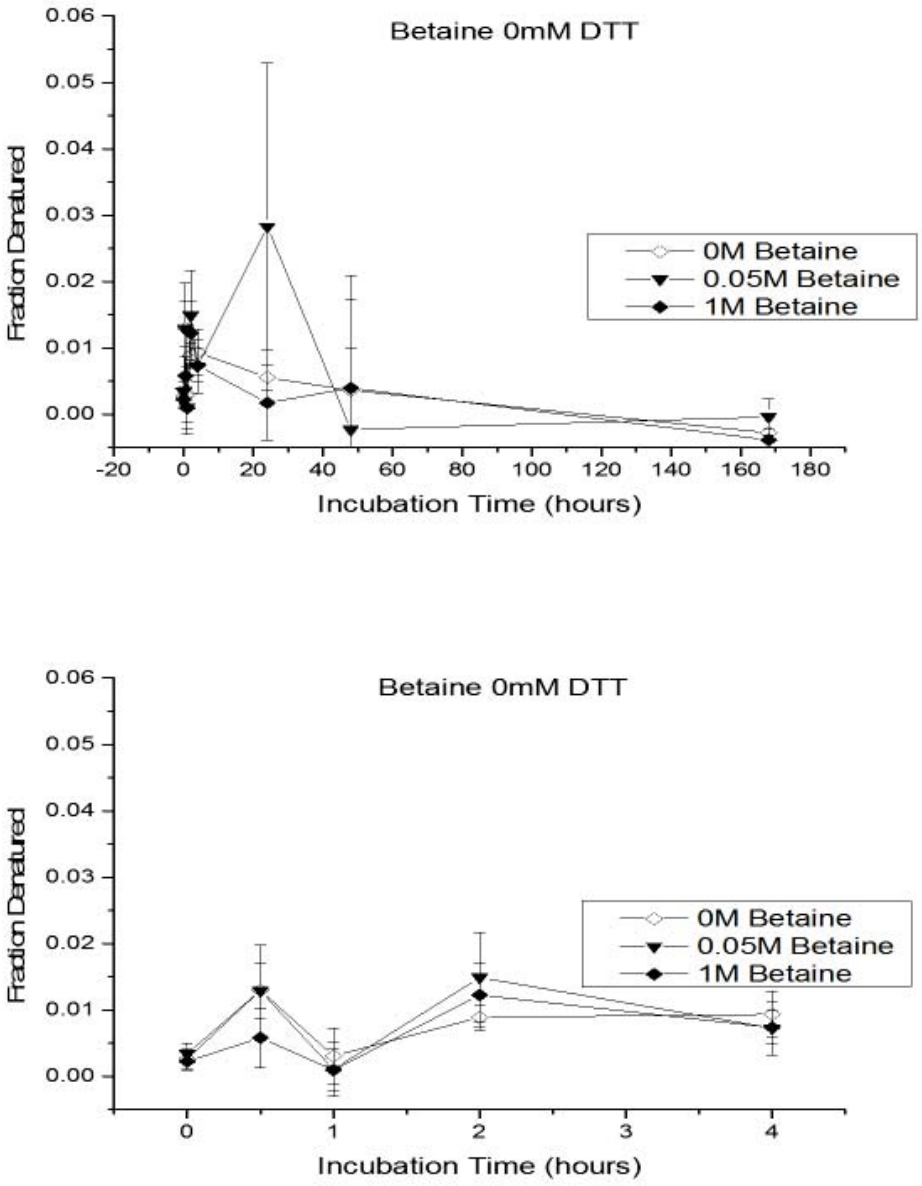

Figure A.4. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

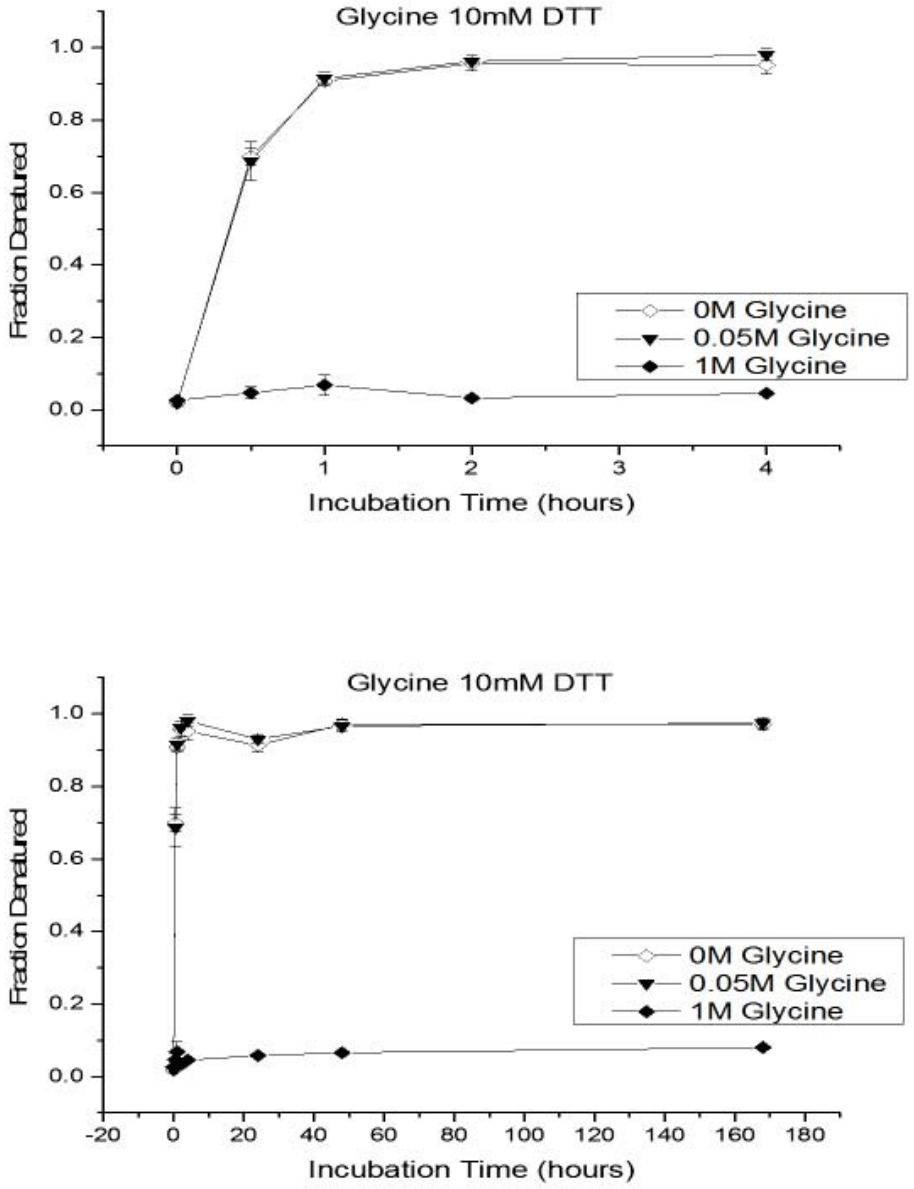

Figure A.5. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

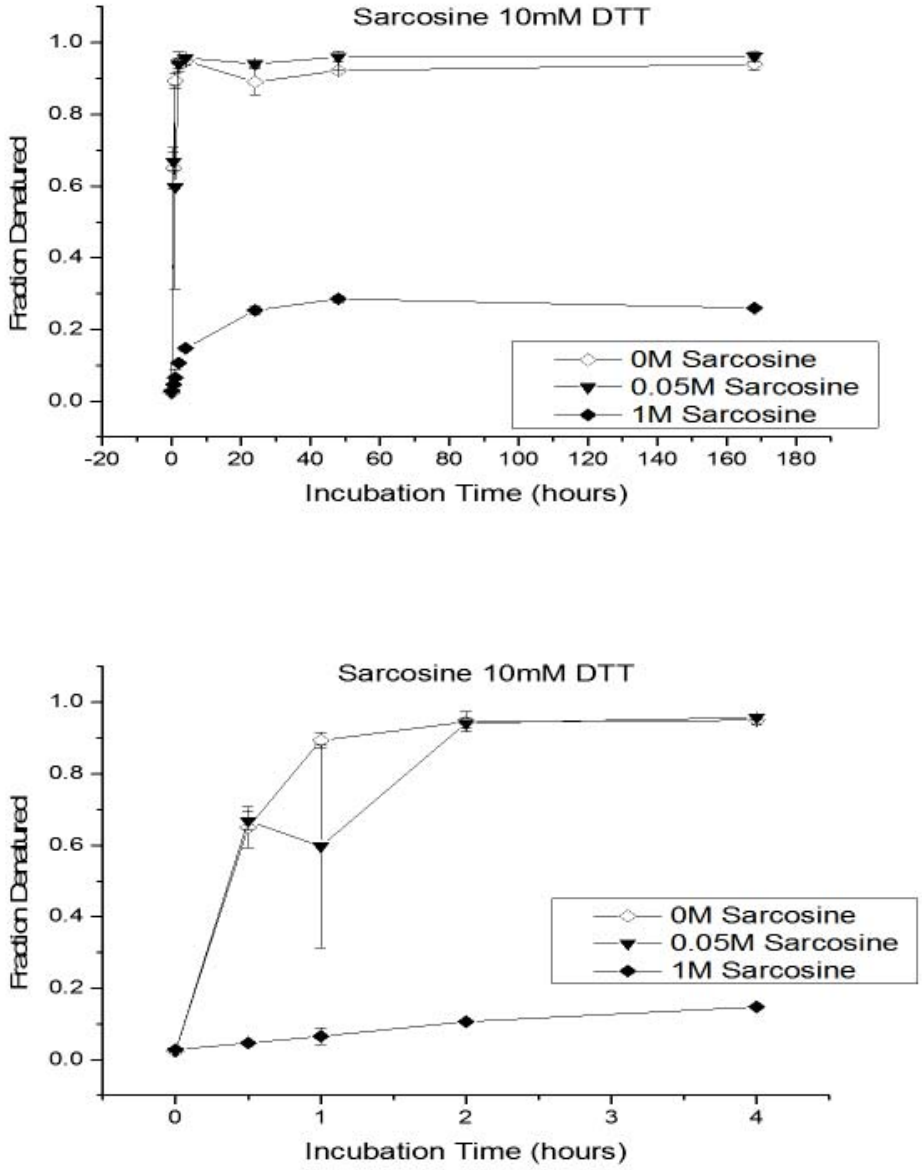

Figure A.6. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

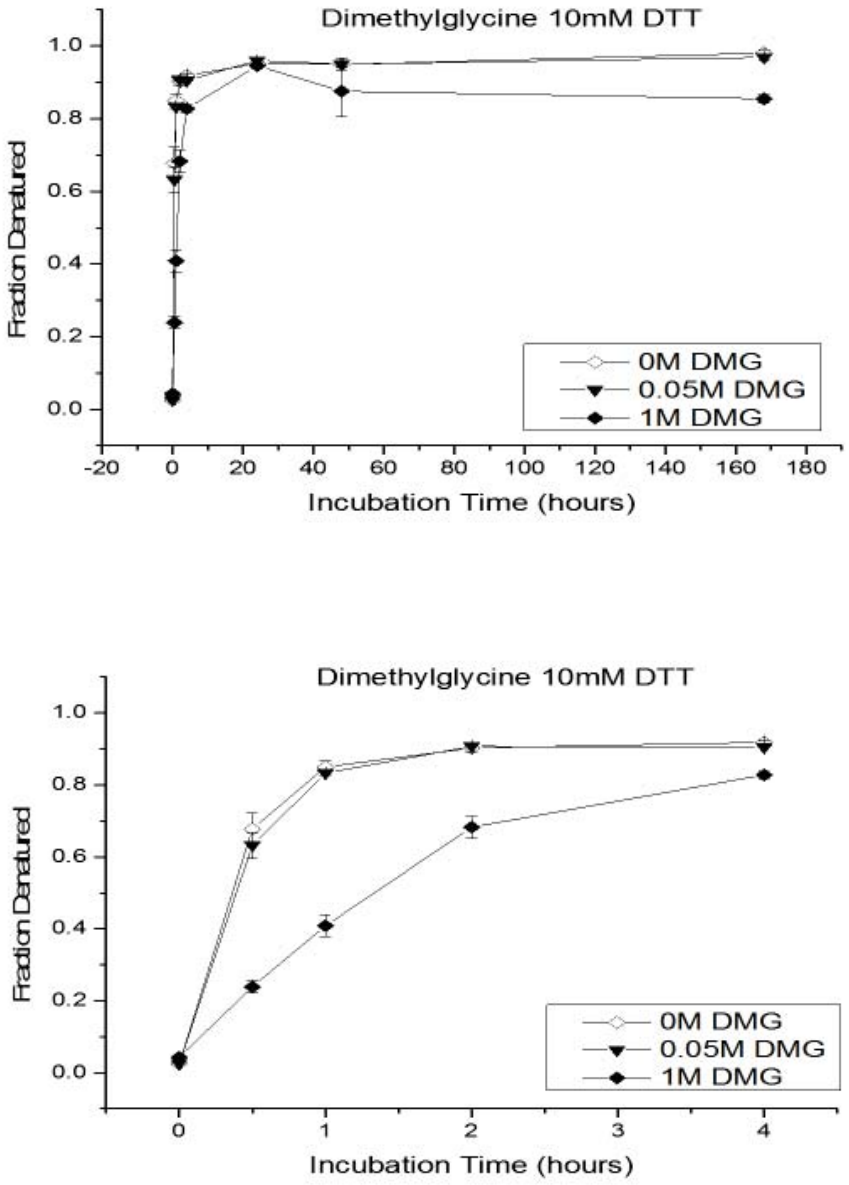

Figure A.7. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

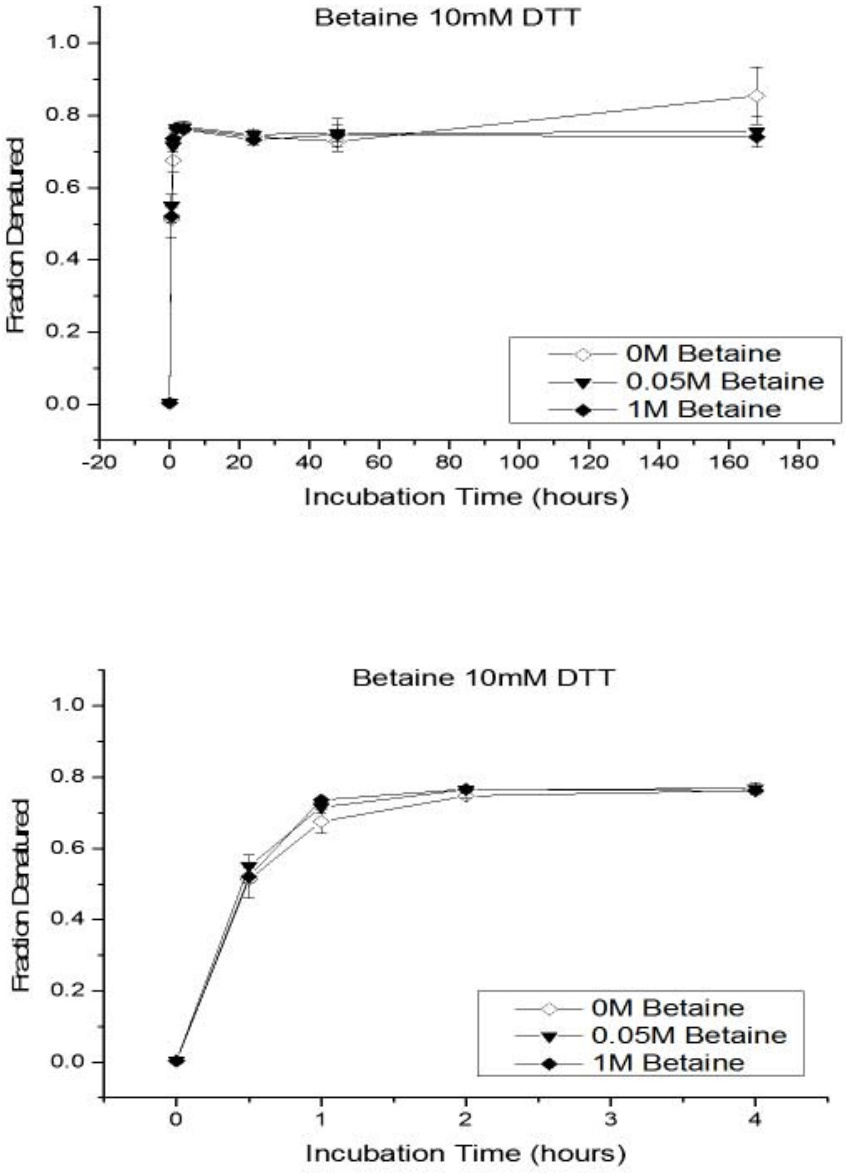

Figure A.8. UV-vis absorbance for lysozyme showing fraction denatured protein as a function of time. Lysozyme was incubated at 37°C in the presence of varying concentrations of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time. The plot for 4 h incubation is shown below the 168 h incubation. Fraction of protein denatured was determined by UV-vis absorbance at 600 nm. Error bars indicate ±SEM.

A.2. Intrinsic Fluorescence Supplemental Figures

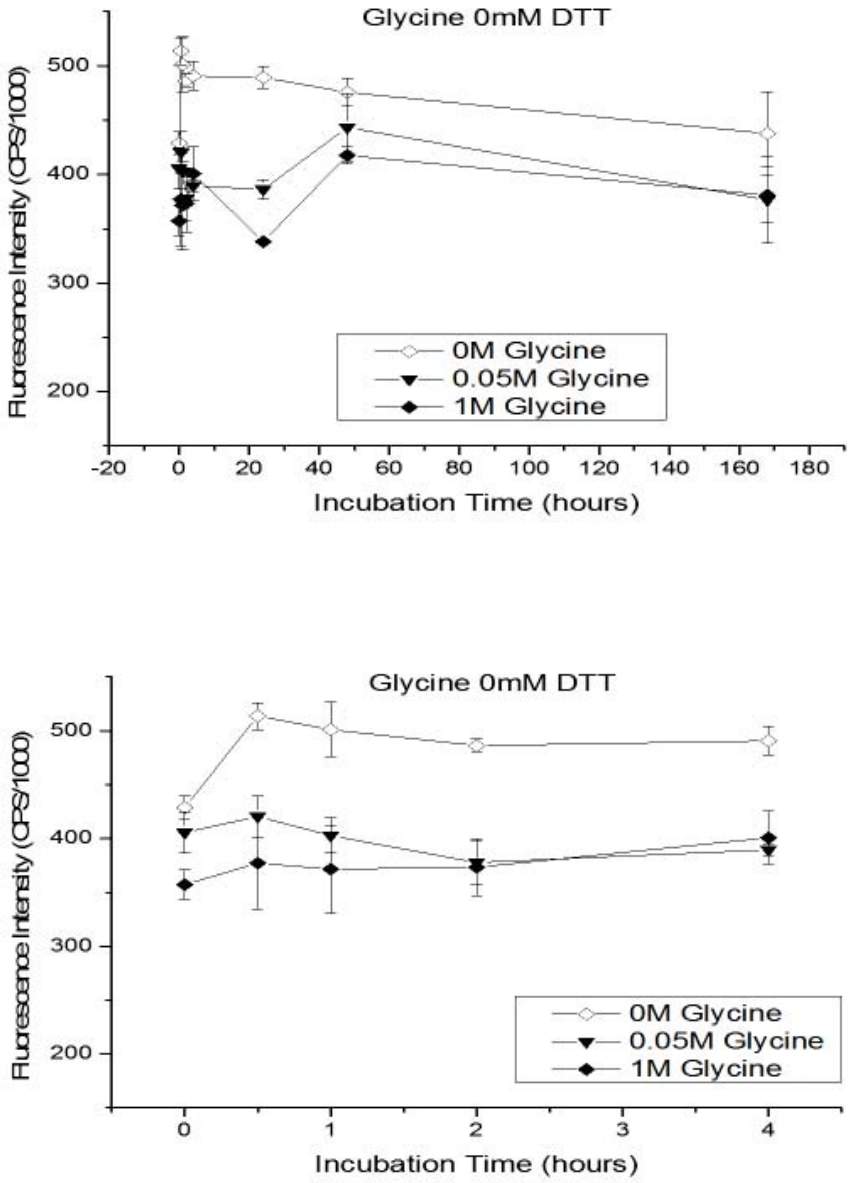

Figure A.9. Intrinsic fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

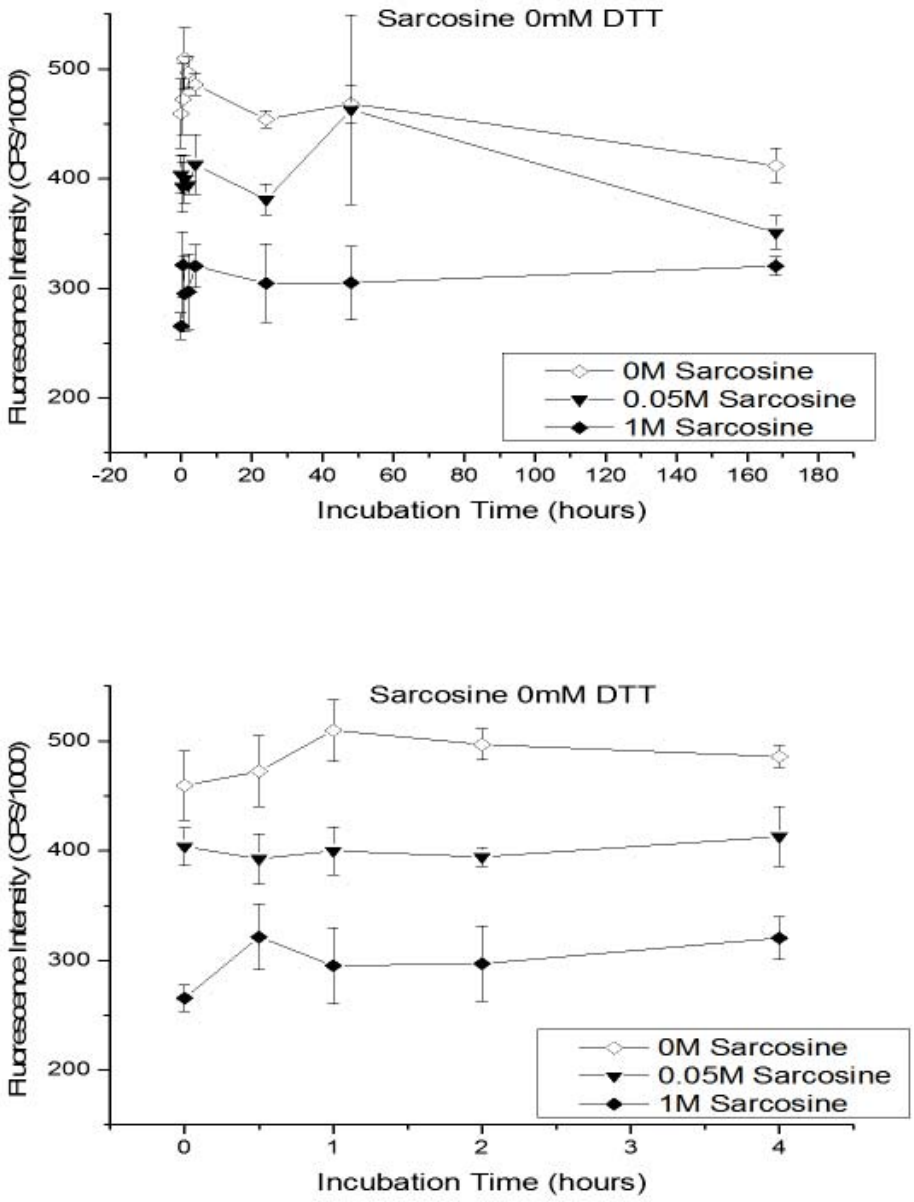

Figure A.10. Intrinsic fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

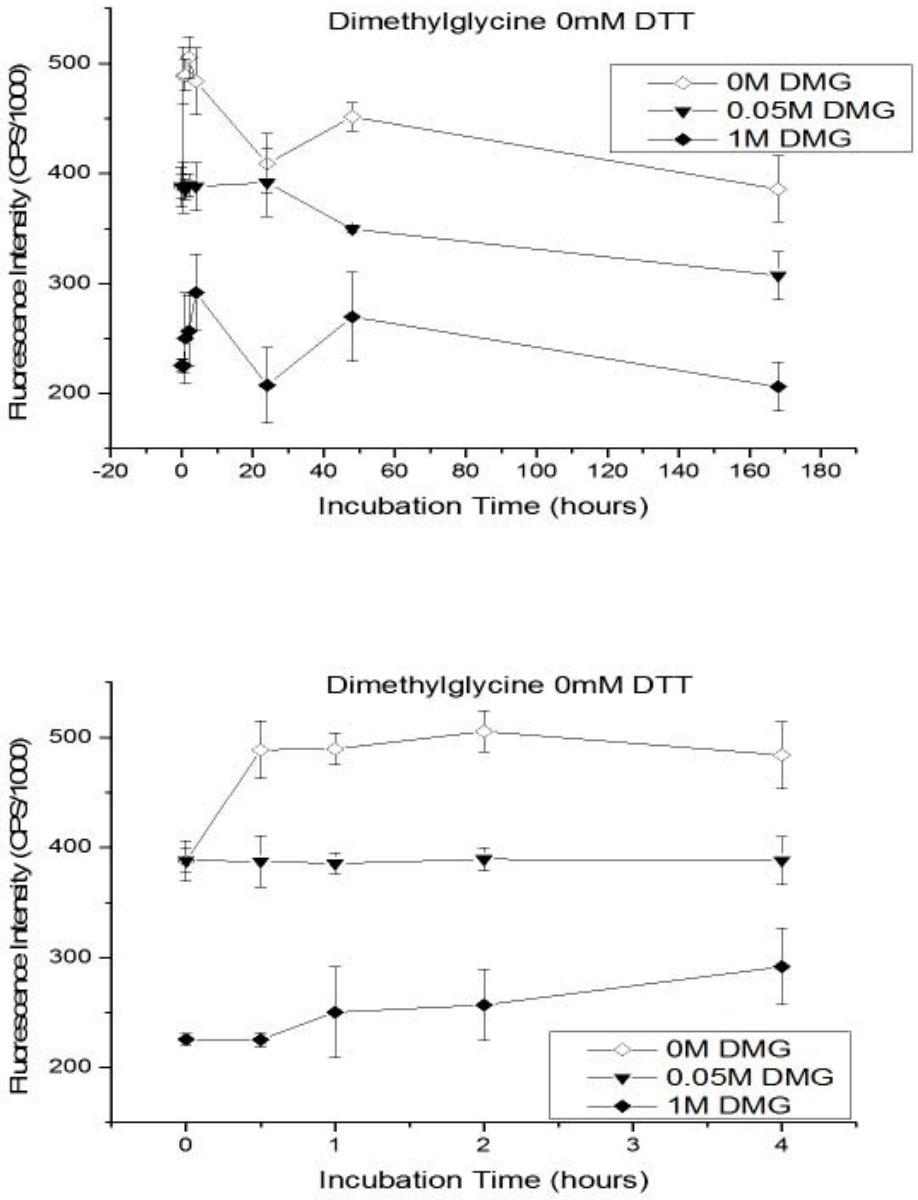

Figure A.11. Intrinsic fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

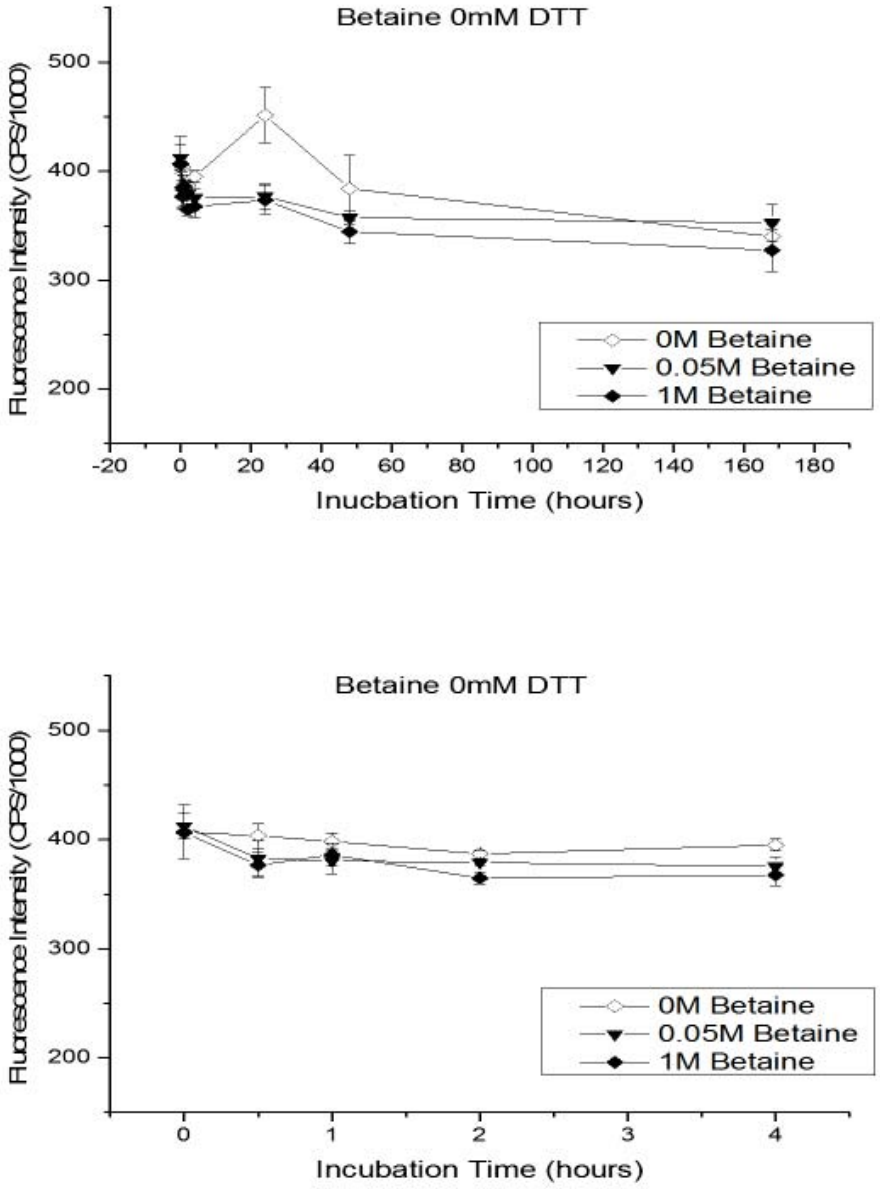

Figure A.12. Intrinsic fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

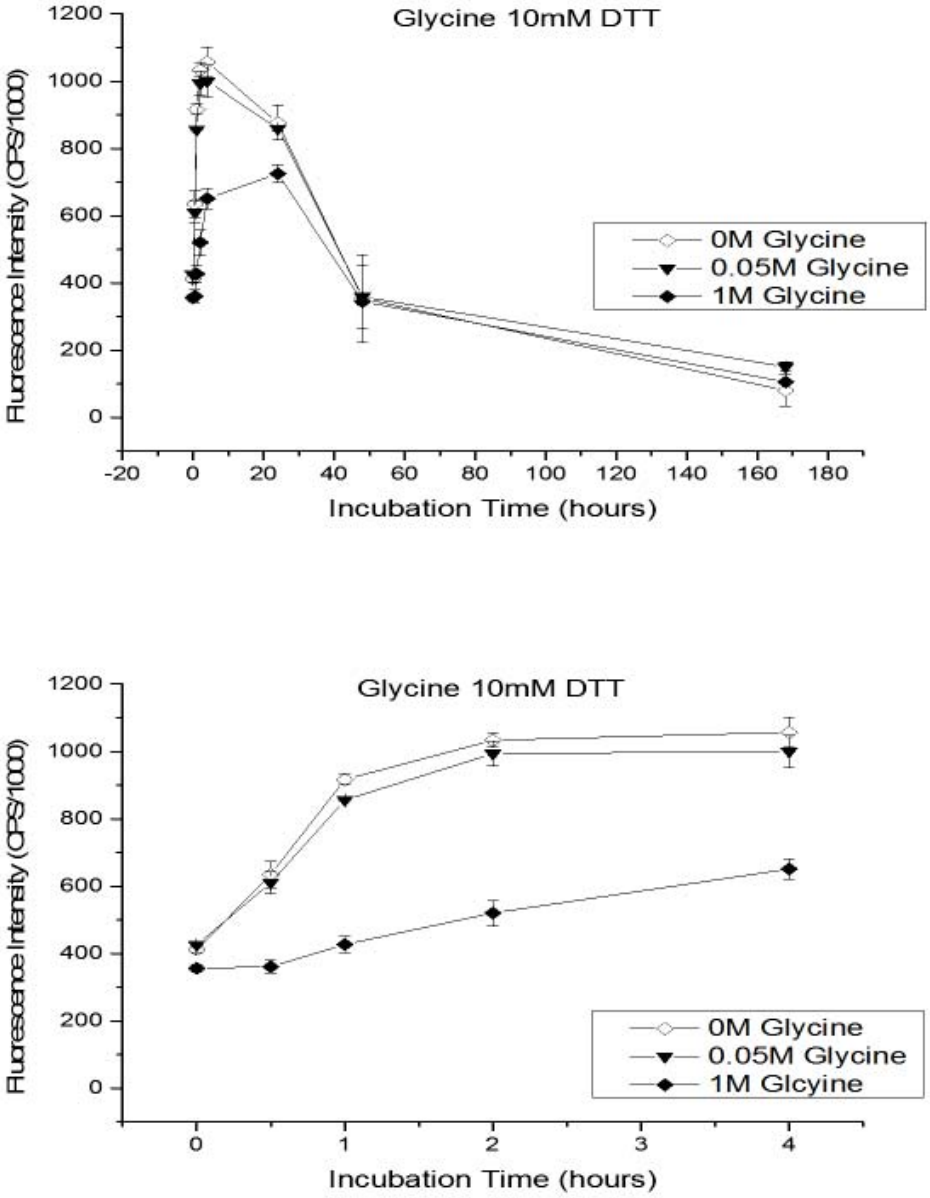

Figure A.13. Intrinsic fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

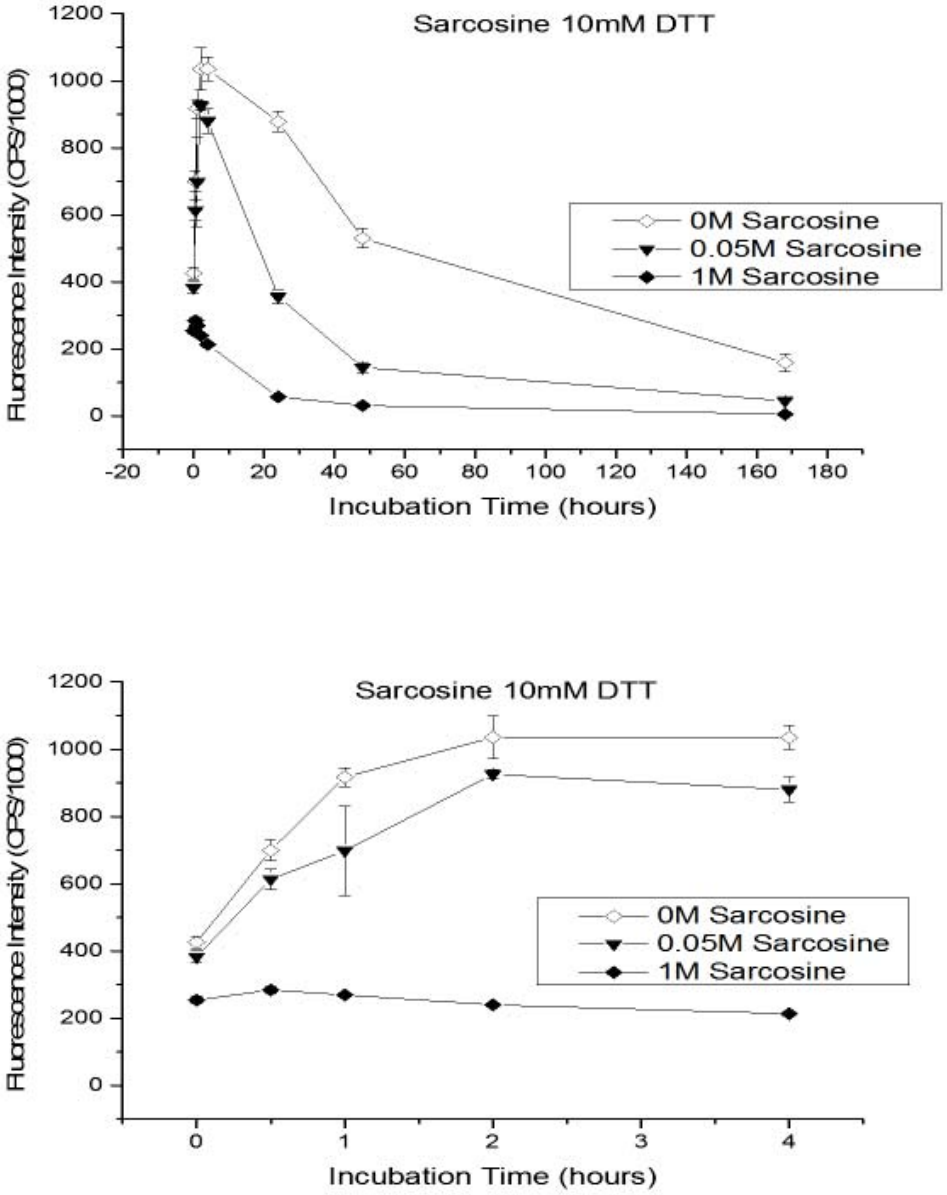

Figure A.14. Intrinsic fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

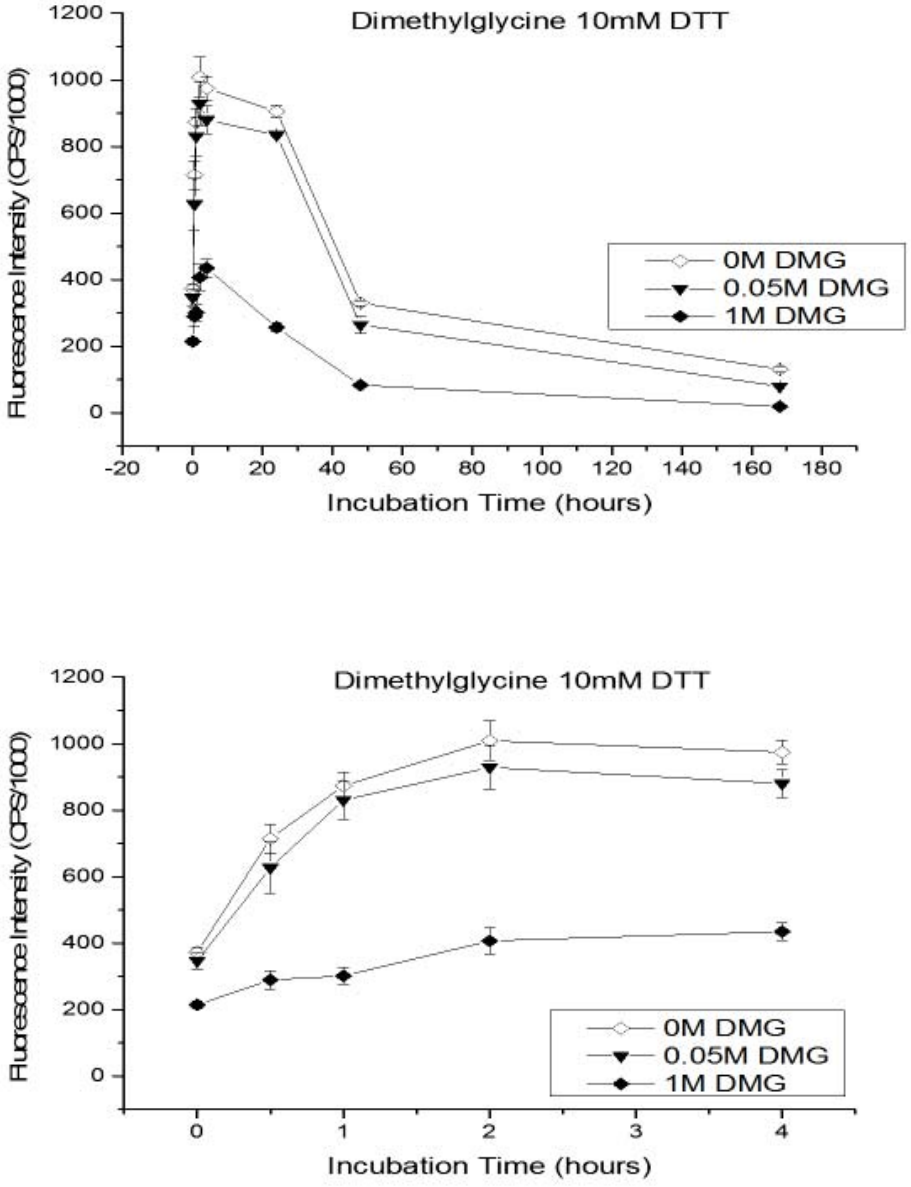

Figure A.15. Intrinsic fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

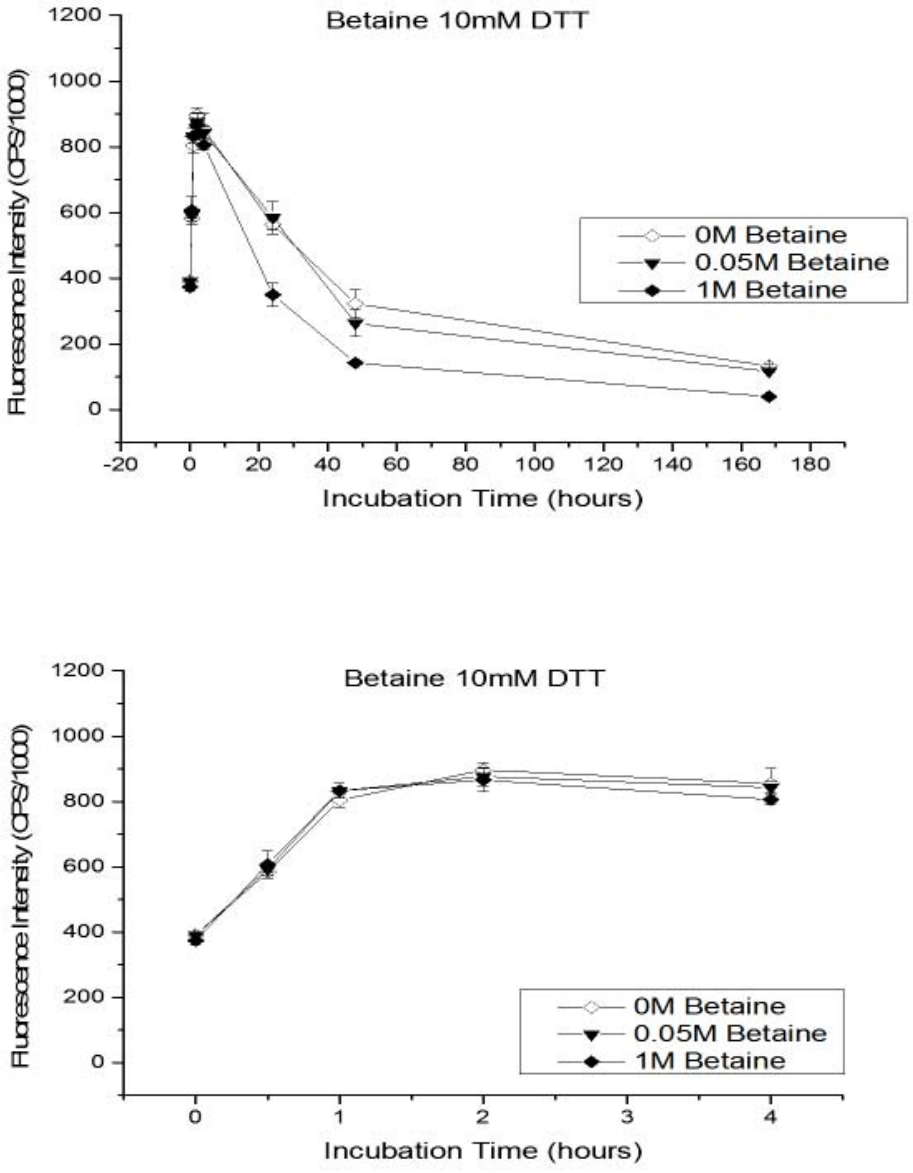

Figure A.16. Intrinsic fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C. Fluorescence emission spectra was collected from 285-450nm with excitation at 280nm. Peak intensity at 346 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

A.3. ANS Extrinsic Supplemental Figures

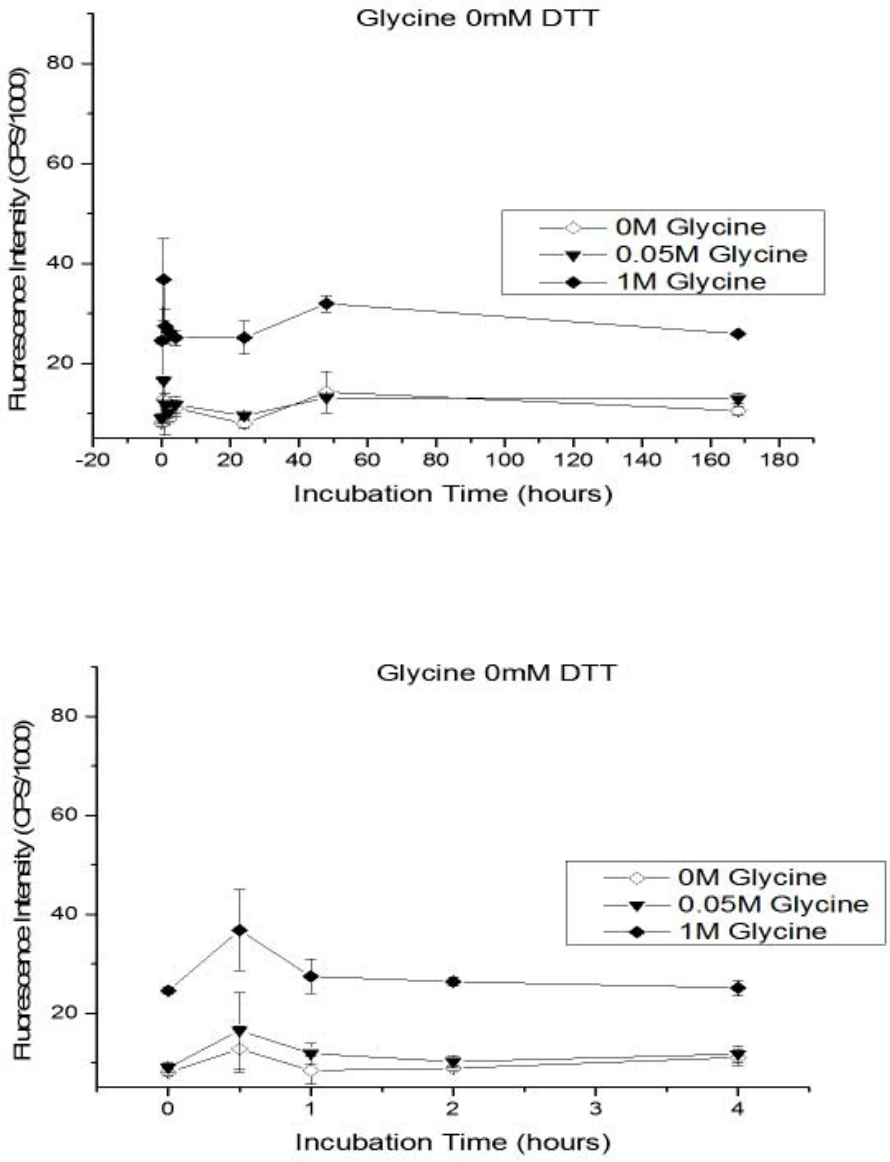

Figure A.17. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

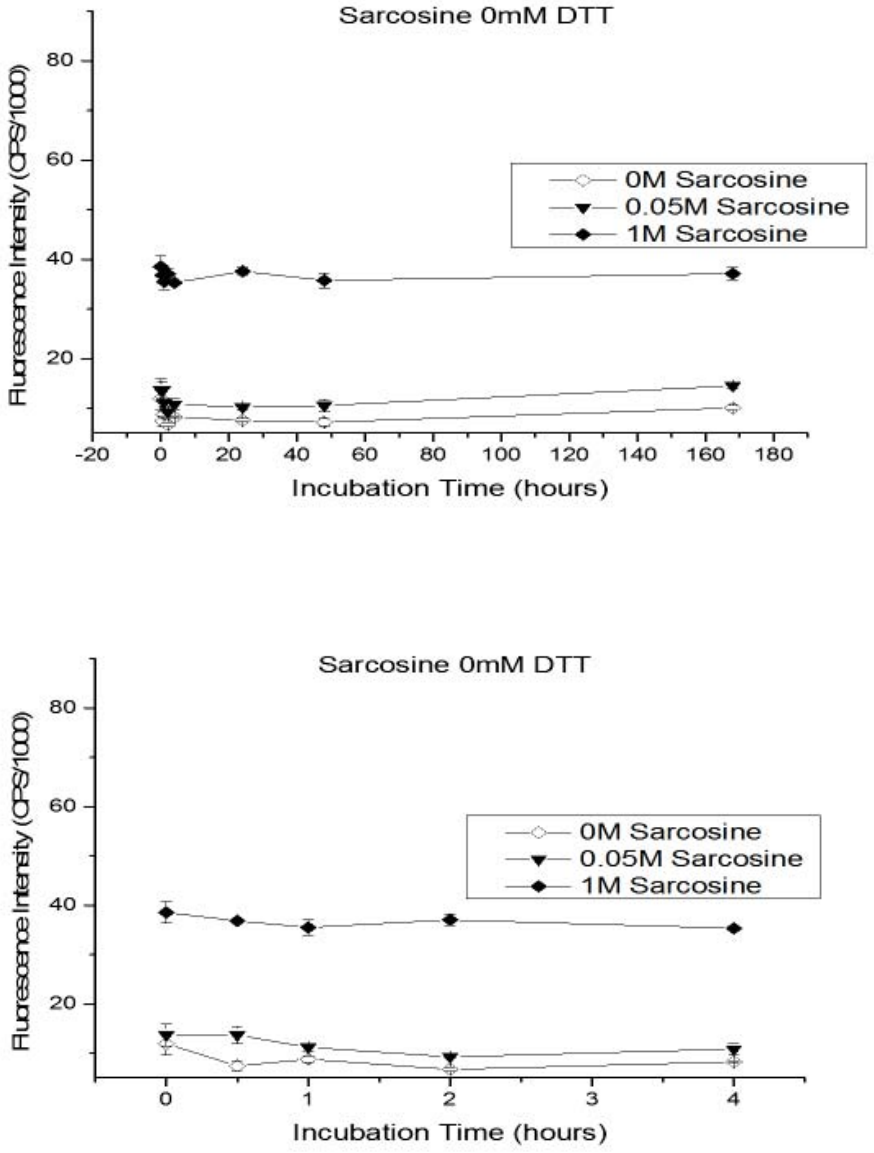

Figure A.18. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

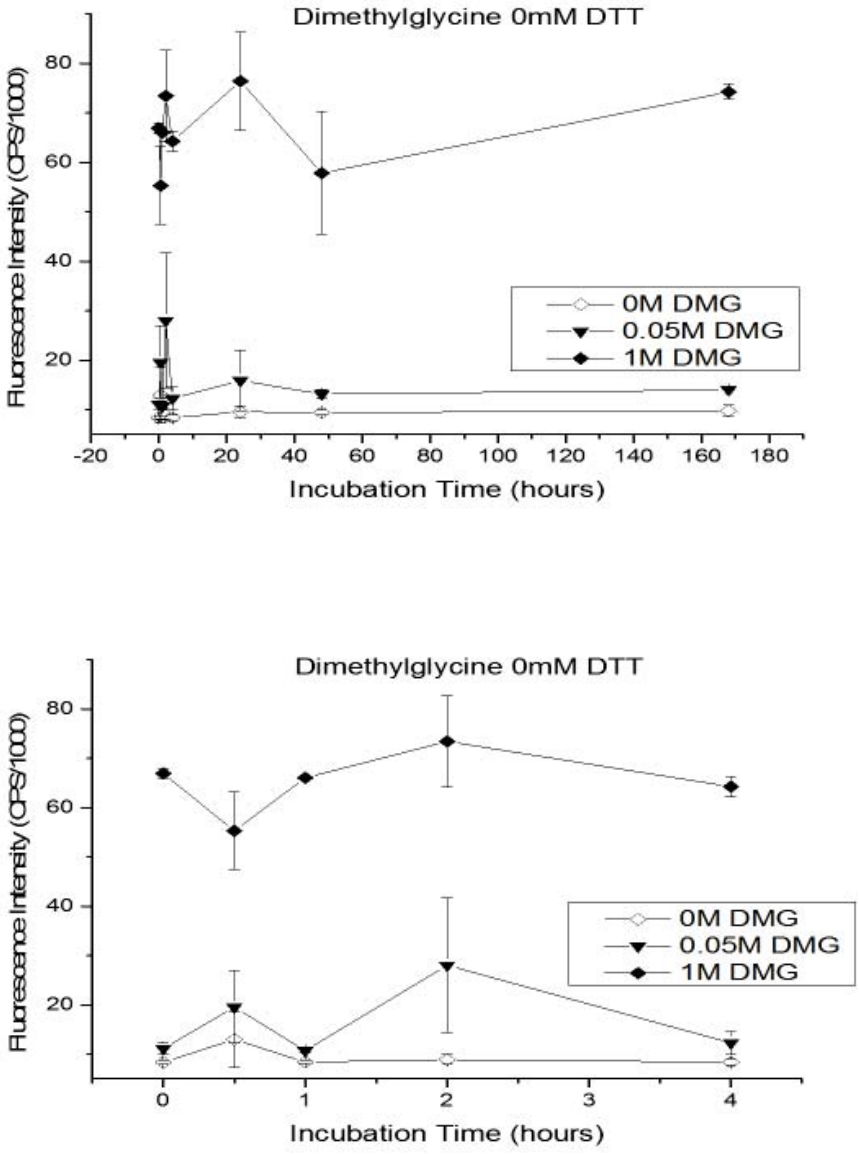

Figure A.19. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

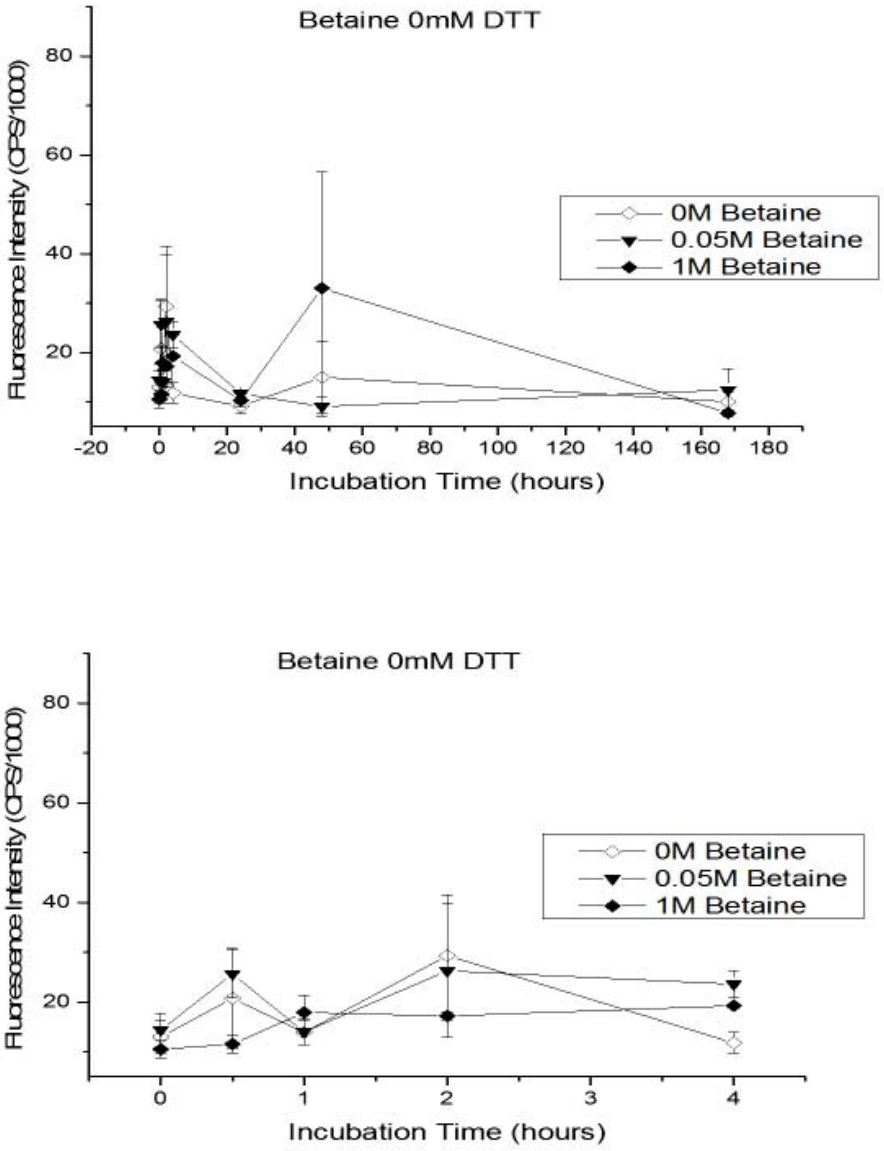

Figure A.20. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 0mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

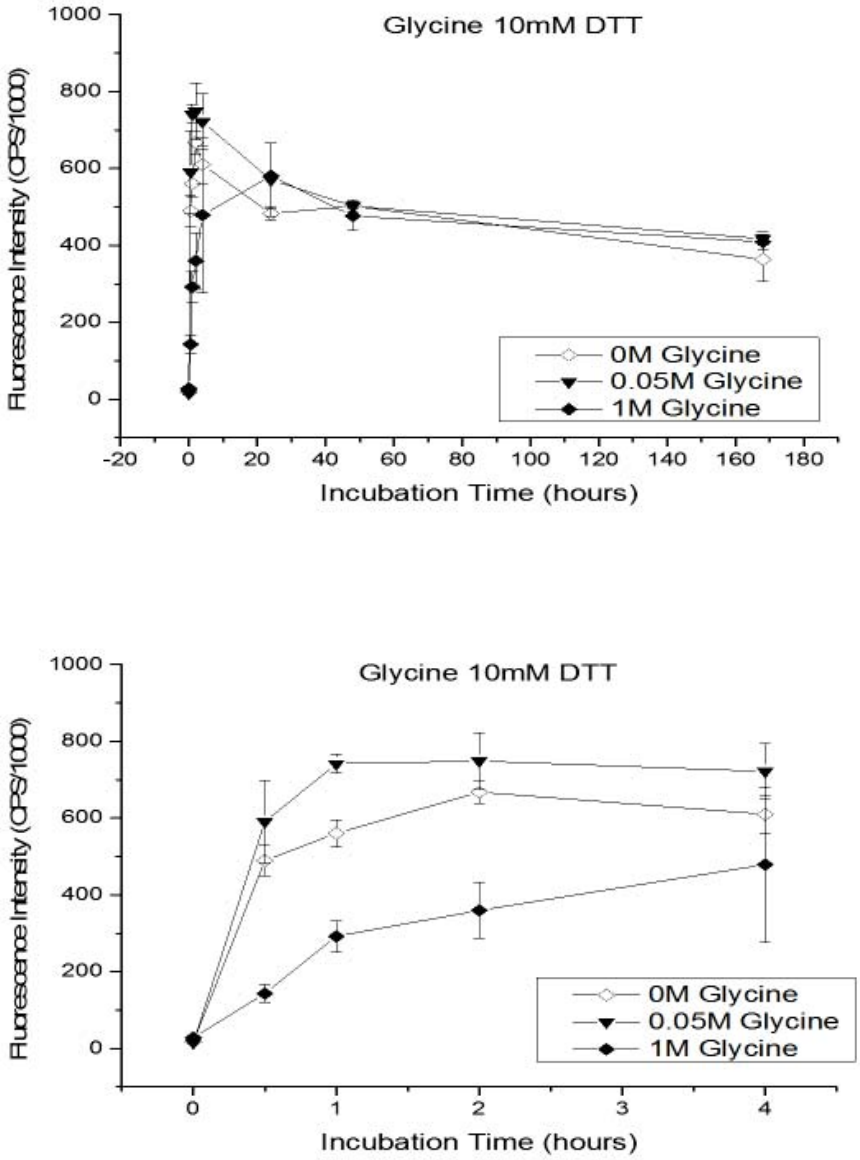

Figure A.21. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of glycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

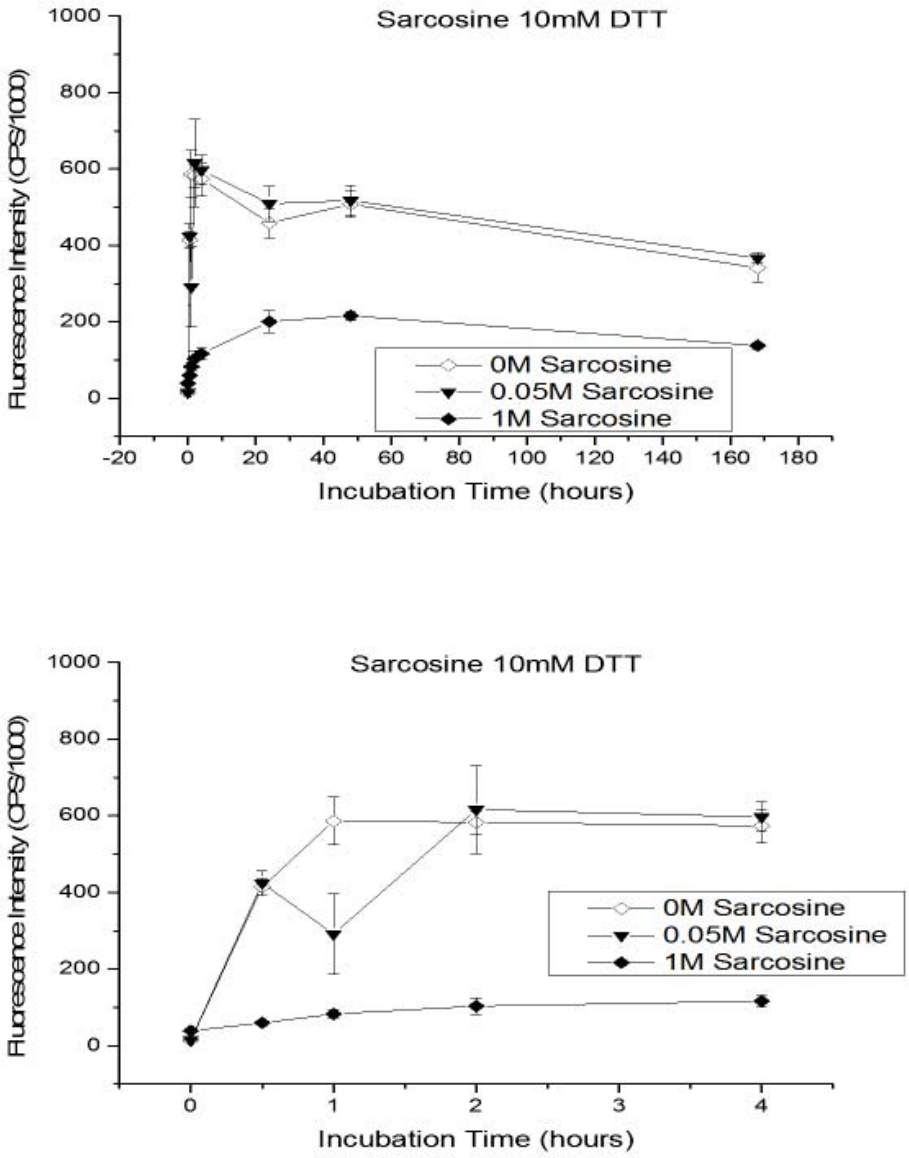

Figure A.22. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of sarcosine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

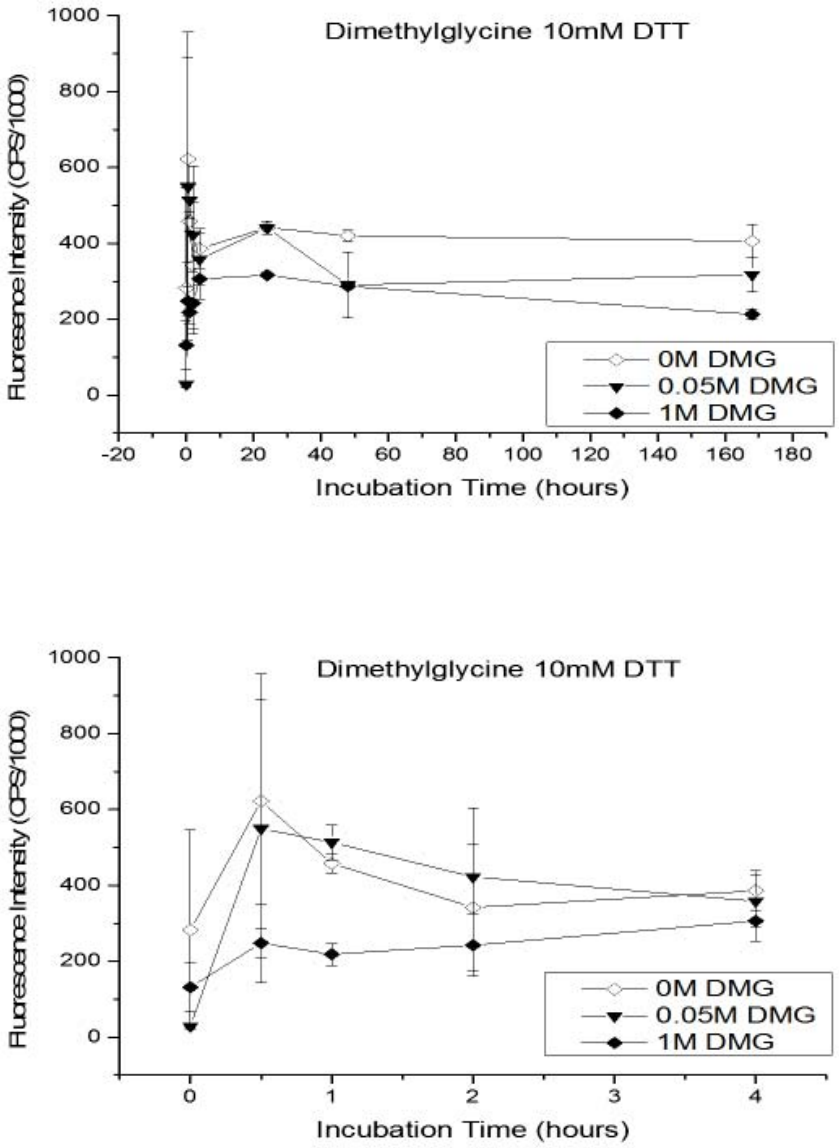

Figure A.23. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of dimethylglycine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

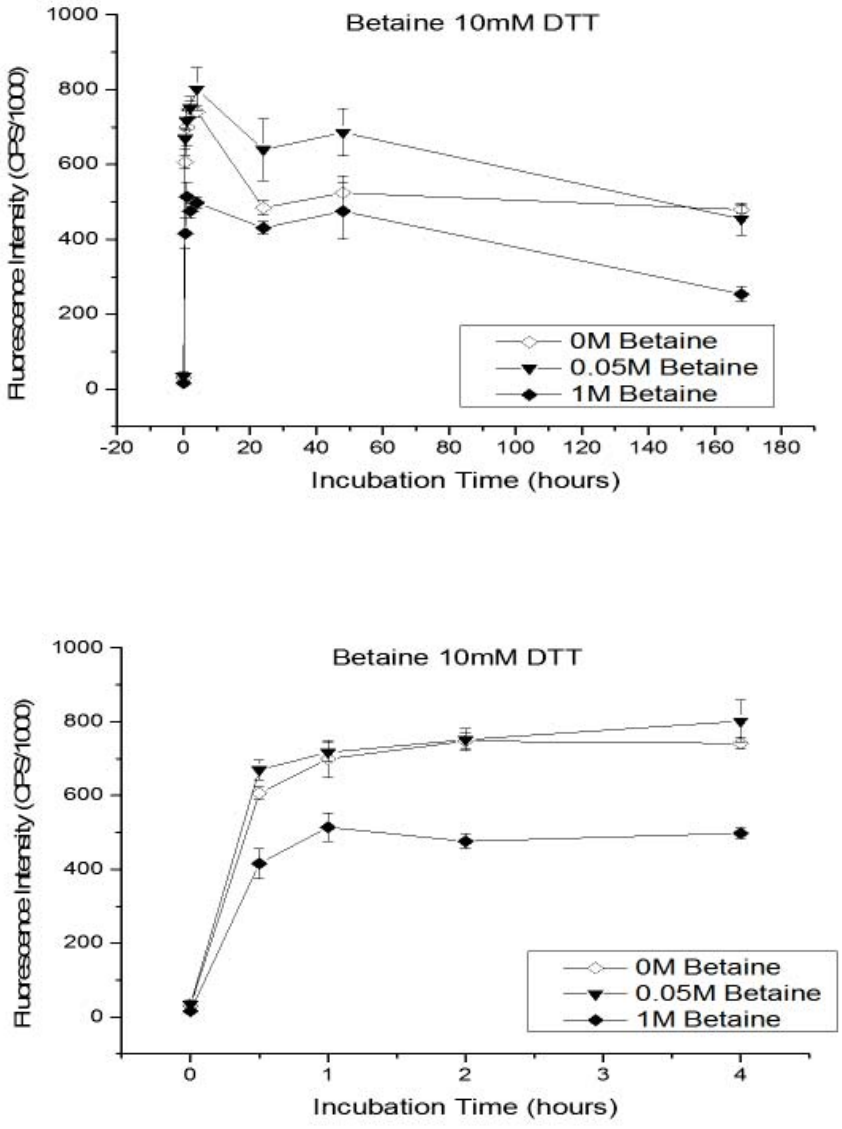

Figure A.24. ANS was used to monitor increase in hydrophobicity as a result of lysozyme misfolding and aggregation. ANS fluorescence measurements for lysozyme in presence of betaine (0 M, 0.05 M, or 1 M) in buffer (pH 7.2) containing 10mM DTT for the indicated periods of time at 37 °C were carried out. Fluorescence emission spectra was collected from 400-700 nm with excitation at 380 nm. Peak intensity at 471 nm were plotted as a function of time. The plot for 4 h incubation is shown below the 168 h incubation. Error bars indicate ±SEM.

A.4. Non-reducing SDS PAGE gel Supplemental Figures

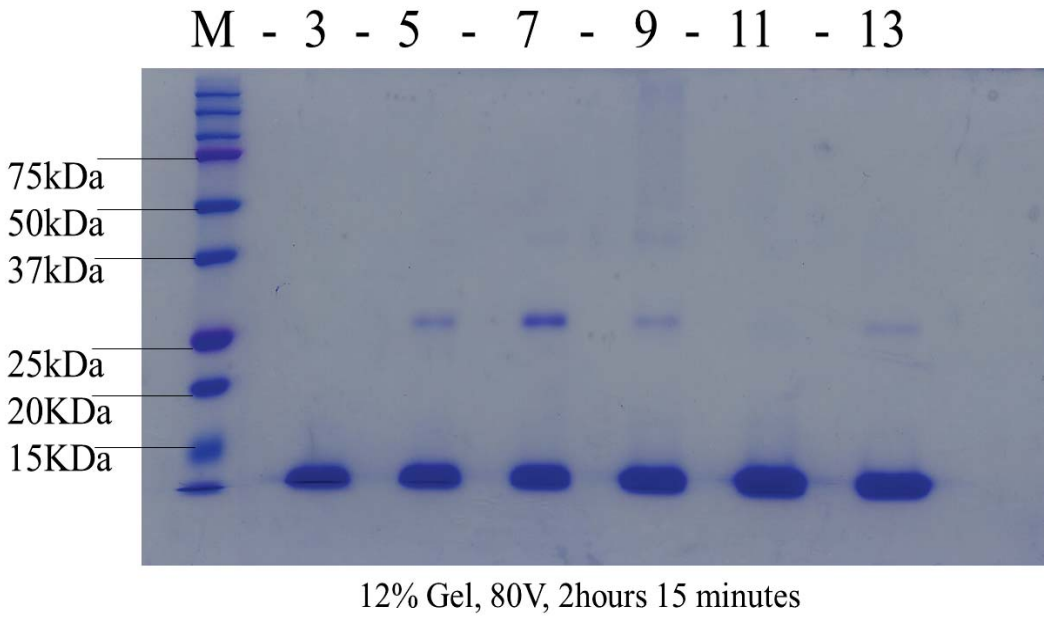

Figure A.25. This is high contrast image of the gel Figure 4.13 (page 45) showing the high molecular weight species observed for the cross-linked proteins in lanes 5, 7, 9, and 13. Non-reducing SDS PAGE of lysozyme samples incubated for 4 h at the conditions indicated. For samples in different lanes the following incubation conditions were used: (3) 0 M osmolyte, 0 mM DTT (Fresh control sample), (5) 0 M osmolyte in presence of 1mM DTT, (7) 1 M Betaine in presence of 1mM DTT, (9) 1 M DMG in presence of 1 mM DTT, (11) 1M Sarcosine in presence of 1 mM DTT, (13) 1M Glycine in presence of 1 mM DTT. All samples were incubated at 37 °C for 4 h before preparing for non-reducing PAGE as detailed in method section. Samples were run on a 12% SDS-PAGE gel at 80 V for 2 h and 15min. Lane marked (M) is SDS marker proteins.

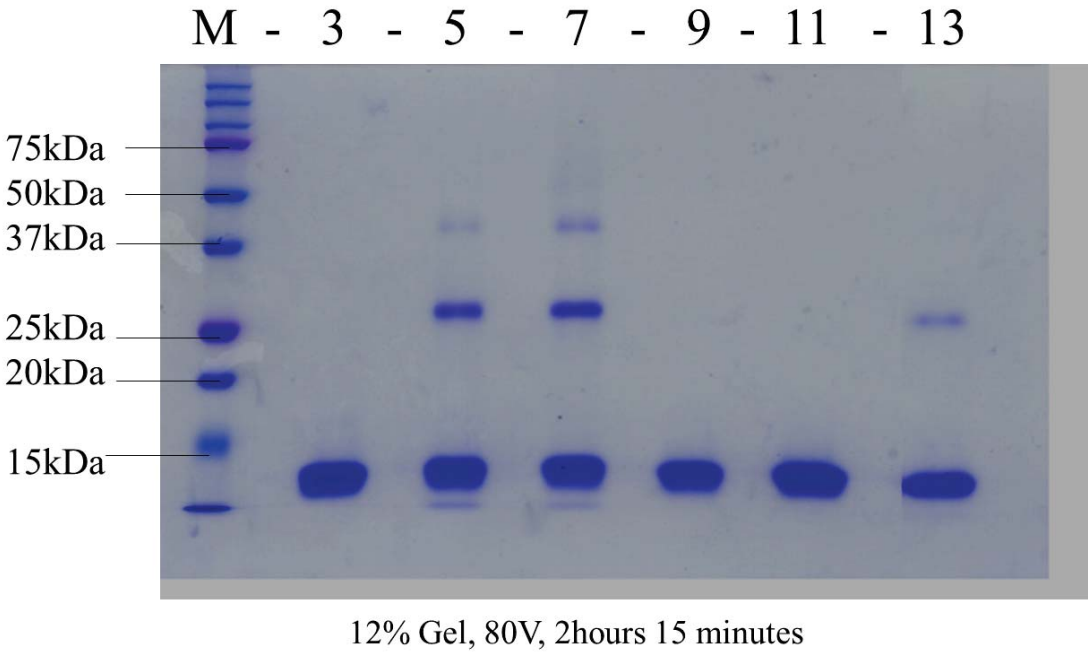

Figure A.26. This is high contrast image of the gel Figure 4.14 (page 46) showing the high molecular weight species observed for the cross-linked proteins in lanes 5, 7, and 13. Non-reducing SDS PAGE of lysozyme samples incubated for 48 h at the conditions indicated. For samples in different lanes the following incubation conditions were used: (3) 0 M osmolyte, 0 mM DTT (Fresh control sample), (5) 0 M osmolyte in presence of 1mM DTT, (7) 1 M Betaine in presence of 1mM DTT, (9) 1 M DMG in presence of 1 mM DTT, (11) 1M Sarcosine in presence of 1 mM DTT, (13) 1M Glycine in presence of 1 mM DTT. All samples were incubated at 37 °C for 48 h before preparing for non-reducing PAGE as detailed in method section. Samples were run on a 12% SDS-PAGE gel at 80 V for 2 h and 15min. Lane marked (M) is SDS marker proteins.