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Modifications of Lysozyme by Substituted Benzoquinones

by Hendrik J. Greve

Departmental Honors Thesis

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Abstract

Amyloidosis, which involves the precipitation of mis-folded protein aggregates, is a prominent process that occurs in many neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease; the protein aggregates being amyloid-β for Alzheimer’s disease and α-synuclein for Parkinson’s disease. This study has focused on lysozyme modifications induced by metabolites of the commonly found pollutants known as polycyclic aromatic hydrocarbons (PAHs). PAHs are found in substances ranging from grilled meats to cigarette smoke to cosmetics, and they can be metabolized into a family of biological toxins known as benzoquinones. The molecules of interest for this study were 1,4-benzoquinone (pBQ), 2-chloro-1,4-benzoquinone (CBQ), and 2-methyl-1,4-benzoquinone (MBQ). The effect these benzoquinones had on lysozyme was studied by first creating samples through time- and concentration-dependent incubations in physiologic conditions. These samples were then examined through SDS-PAGE analysis, fluorescence assays, and UV-Vis spectroscopy to determine the products created through this modification. Our findings revealed the effective oligomerization and aggregation of lysozyme modified by benzoquinones.

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Glossary

APS .... Ammonium Persulfate

Ar .... Argon Gas

CBQ 2-Chloro-1,4-Benzoquinone

Da .... Dalton (equal to 1 amu)

EtOH .... Ethanol (Ethyl Alcohol)

hr .... Hour

kDa .... Kilodalton (equal to 1000 amu)

Lys .... Lysine

MBQ 2-Methyl-1,4-Benzoquinone

MeOH .... Methanol (Methyl Alcohol)

Min .... Minute

PAH .... Polycyclic Aromatic Hydrocarbon

PBQ 1,4-Benzoquinone

RNase .... Ribonuclease

ROS .... Reactive Oxygen Species

SDS .... Sodium Dodecyl Sulfate

SDS-PAGE .... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TEMED .... *N,N,N',N'*-tetramethyl-ethane-1,2-diamide

Chapter I

Introduction

In 1854, the physician-scientist Rudolph Virchow introduced the term *amyloid* to describe necrotic plaques (Figure I) seen in the cerebral corpora amylacea of some of his patients [1]. Virchow wrongly concluded that the substance must be made of cellulose, but it was determined to be a protein structure in 1859, and thus the study of the pathology of protein mis-folding and modification came into existence [1]. Over a hundred and fifty years later, amyloids and amyloidogenesis, a process in which the secondary conformations of a protein changes into pleated β-sheets, resulting in self-aggregation into precipitated (and often necrotic) plaques, are known to play a central role in several prominent and crippling neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease [2-4].

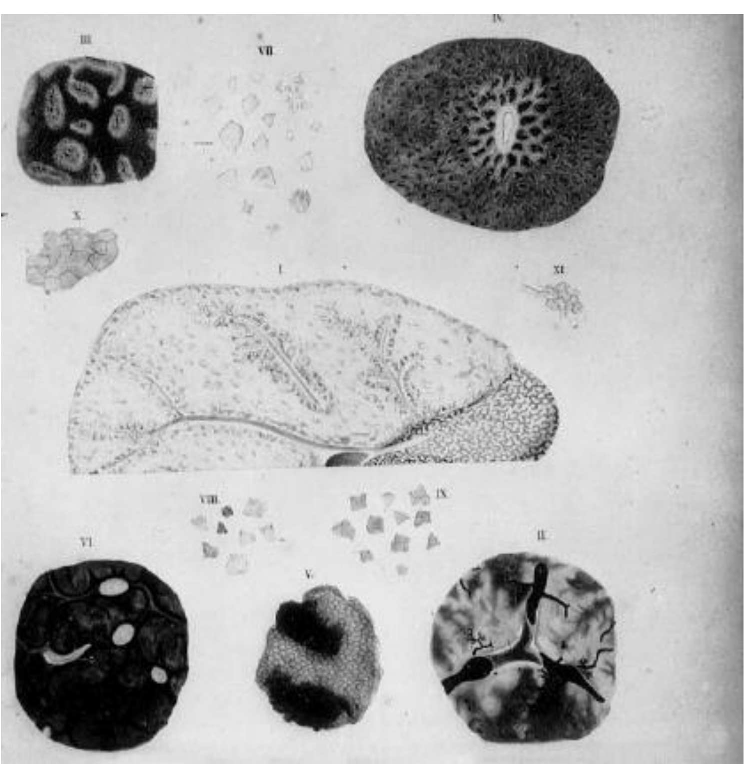

Figure 1.1: Amyloidal deposits in cellular samples at low magnification [1]

Proteins are essential to all organisms, eukaryotes and prokaryotes alike, and interruption of their normal functions can be very detrimental to life at the cellular level [5]. Proteins can be modified by a number of factors, from genetic errors, to translational and post-translational modifications, to toxicological and chemical modifications by environmental factors [5-10]. Because of this, proteopathies, pathological mis-folding and aberrant polymerization of proteins, have become central in the study of many cerebral and neurological diseases because their causes are often very difficult to elucidate or involve many pathological pathways [11-14]. One such area of study focuses on environmental toxicants and their subsequent effects on proteins and cellular functions [9, 10].

Polycyclic aromatic hydrocarbons (PAHs) are a prevalent class of environmental pollutants that can be produced by many industrial processes [9, 13, 14]. PAHs are characterized by condensed aromatic ring systems, and they are commonly found as environmental pollutants in the soil, atmosphere, and other sources that include cigarette smoke, diesel fuel, cosmetics, grilled foods, and in the disinfectants for public water supplies [9, 10, 13, 14]. PAHs are of concern because they can be metabolic precursors to cancer-causing molecules, as well as precursors to protein modifying molecules [9, 13-15]. PAHs arise mostly from the partial or incomplete combustion of other hydrocarbons, which is a process that consumes hydrogen faster than carbon, so the carbon is left in the more thermodynamically stable state of an aromatic ring system [9, 10]. Benzo(a)pyrene is a commonly studied PAH because its partially oxidized and metabolized form is a strong carcinogen that can easily bind to DNA in humans and other animals [9]. A few common PAH and PAH-like molecules are displayed in Figure 1.2.

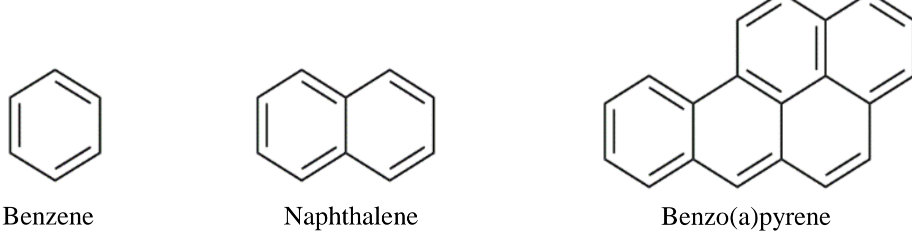

Figure 1.2: Common Polycyclic Aromatic Hydrocarbons

PAHs exhibit their cytotoxicity through their metabolites, which are most often quinones in either their reduced and oxidized forms [13, 14]. Quinones can cause toxic damage to a cell through a variety of processes, including DNA modifications, oxidative damage, lipid modifications, and protein modifications [13-17]. PAHs begin their biological journey in the smooth endoplasmic reticulum of a cell, where they undergo an epoxidation reaction via the enzyme cytochrome P450 [5, 6, 13, 17-20]. Cytochrome P450 takes its name from its λ_{max} at 450 nanometers, and it belongs to a superfamily of heme-containing enzymes that are found in almost all living organisms [5, 6]. From this point, a PAH can either spontaneously form a phenol or form a dihydrodiol via an enzyme called epoxide hydrolase [17-20]. The phenol can form a hydroquinone under biological conditions, while the dihydrodiol can undergo another enzyme catalyzed reaction via dihydrodiol dehydrogenase to form a diol [17-20]. The hydroquinone can spontaneously form a benzoquinone under physiological conditions, and this product is in equilibrium, whereas the diol undergoes a final enzymatic reaction with tyrosinase to also form a quinone [17-20]. Several quinones can arise from the metabolic processing of PAHs, and it is the quinones that exhibit the largest amount of toxicity to the organism

[13-15, 17-21]. A stepwise mechanistic summary of the metabolism of PAHs into benzoquinones from benzene (the simplest PAH) is given in Figure 1.3.

Figure 1.3: Metabolism of PAHs into Benzoquinones.
A) 1,4-hydroquione (HQ). B) 1,4-benzoquinone (PBQ) [13, 17]

The resulting benzoquinone from this metabolism is toxic and highly detrimental to a cell in a number of ways [13]. Quinones can exhibit their hazardous effects through cytotoxicity, immunotoxicity, and carcinogenesis [15]. Benzoquinones have long been studied for their effects on various biomolecules, especially polypeptides and nucleic acids [14, 18, 19, 22]. 1,4-benzoquinone (PBQ) has been shown to cause acute myelogenous leukemia, aplastic anemia, myelodysplasia, and immunotoxicity; and it is through this metabolite that benzene is thought to exhibit part of its toxicity [9, 13, 15, 16, 22-24]. Benzoquinones are Michael acceptors, and it has long been observed that they can demonstrate their toxicity to DNA and proteins through alkylation of large biomolecules [13-15, 25]. Adduct formation by PBQ can involve multiple pathways, such as a cyclized diquinone-lysine adduct or an adduct of PBQ and glutamic acid [13, 17, 25-27]. The most studied mechanism of benzoquinone toxicity, however, is through oxidative-reductive cycling, or simply redox cycling [13-15, 17, 28]. Benzoquinones are extremely active redox molecules in biological systems, and they can redox cycle with their semiquinone radicals (quinones being the oxidizing agent in the cycle), which can

then lead to the formation of reactive oxygen species (ROS) [13-17]. The ROS formed through redox cycling of benzoquinones can cause a massive oxidative stress in a cellular environment by reacting with DNA, lipids, and proteins to form oxidized macromolecules, as well as reacting with molecules in various signaling pathways to cause their activation, such as protein kinase C [15-19, 28, 29]. Both the process of adduct formation and redox cycling are thought to occur simultaneously with quinones in cellular environments [15]. A final pathway that can contribute to benzoquinones toxicity arises from the cross-linking of proteins, which directly affects the functionality and integrity of the polypeptide [13, 14, 17]. Figure 1.4 displays the three major pathways by which benzoquinones can affect cellular proteins.

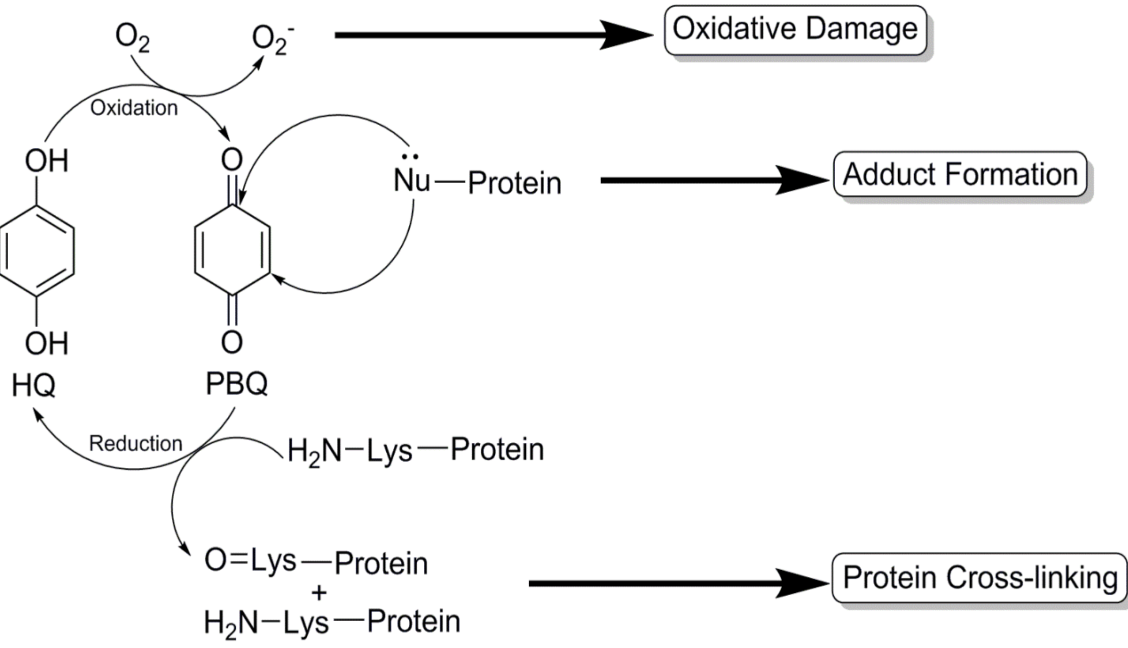

Figure 1.4: Proposed Mechanism of Quinone's Action [13, 14, 17].

Few studies have been performed on the particular area of protein cross-linking induced by PAH quinones, and it is this pathway that is the focus of this thesis. Protein cross-linking is thought to occur when a benzoquinone reacts with a lysine residue on a protein, resulting in a lysine oxidation, where the amine group is replaced with an aldehyde [13, 14]. This modified lysine, called allysine, can then react with an intact lysine residue on another protein, forming a covalent bond between the two [30-33]. This condensation of lysine and allysine is seen as a natural and normal process in the well-known protein, collagen, but it can be a detrimental process if it occurs in some other proteins (Figure 1.5) [30-36].

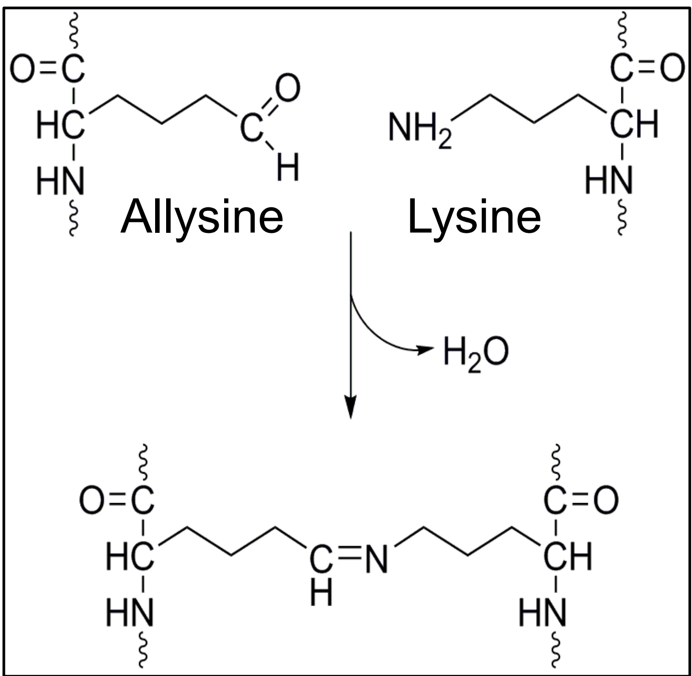

Figure 1.5: Condensation of Lysine and Allysine Residues [30-34].

If this cross-linking process continues, oligomerization of multiple proteins can occur, with the possibility of the formation polymeric aggregates [13, 14, 17]. Protein

aggregation, a process where multiple mis-folded proteins assemble into large accumulated groups, is a common event in biological systems [13, 37-40]. Protein aggregation and mis-folding have become of particular interest in recent years due to large amounts of scientific evidence associating the processes with neurodegenerative diseases [2, 4, 13, 14, 37, 41-44]. In particular, the process of amyloidosis, in which protein aggregates precipitate out of solution in the form of plaques, is central to current studies of both Alzheimer's disease and Parkinson's disease [4-6, 41-47]. Table 1.1 displays several neurodegenerative diseases and their associated amyloidal and mis-folded protein.

Table 1.1: Neurodegenerative Diseases and Associated Proteins [4, 5, 48, 49].

Disease	Protein
Alzheimer's Disease	Amyloid-β from APP
Parkinson's Disease	α-synuclein
Huntington's Disease	Huntingtin protein with polyglutamine expansion
Amylotrophic Lateral Sclerosis	Unknown

Because of its strong association with many human diseases and disorders, it is important to understand protein aggregation and mis-folding, especially that of the amyloidogenic types. This thesis attempts to study PAH-quinones as a potential environmental toxin that can contribute to protein aggregation. In order to study this effect, the model protein, lysozyme, was used. Lysozyme was chosen because its aggregation and its link to amyloidosis is well-researched [13, 38-40, 50-55].

The protein lysozyme is a model protein for protein chemistry and structural biology. This is due to the fact that lysozyme easily crystalizes and the crystals that it forms diffract very well [56]. In 1963, the complete primary structure of hen egg white lysozyme was elucidated [57]. Lysozyme became an important protein in the study of protein mis-folding and aggregation when it was shown that point mutations in human lysozyme correlated heavily with the hereditary disease non-neuropathic systematic amyloidosis [58]. Human lysozyme functions as an antibacterial agent that hydrolyses the glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in Gram-positive bacteria [59]. The structure of lysozyme is presented in Figure 1.6 with the lysine residues highlighted. The lysine residues are of particular interest due to the possibility of lysine-allysine bonds serving as a contributing factor of aggregation.

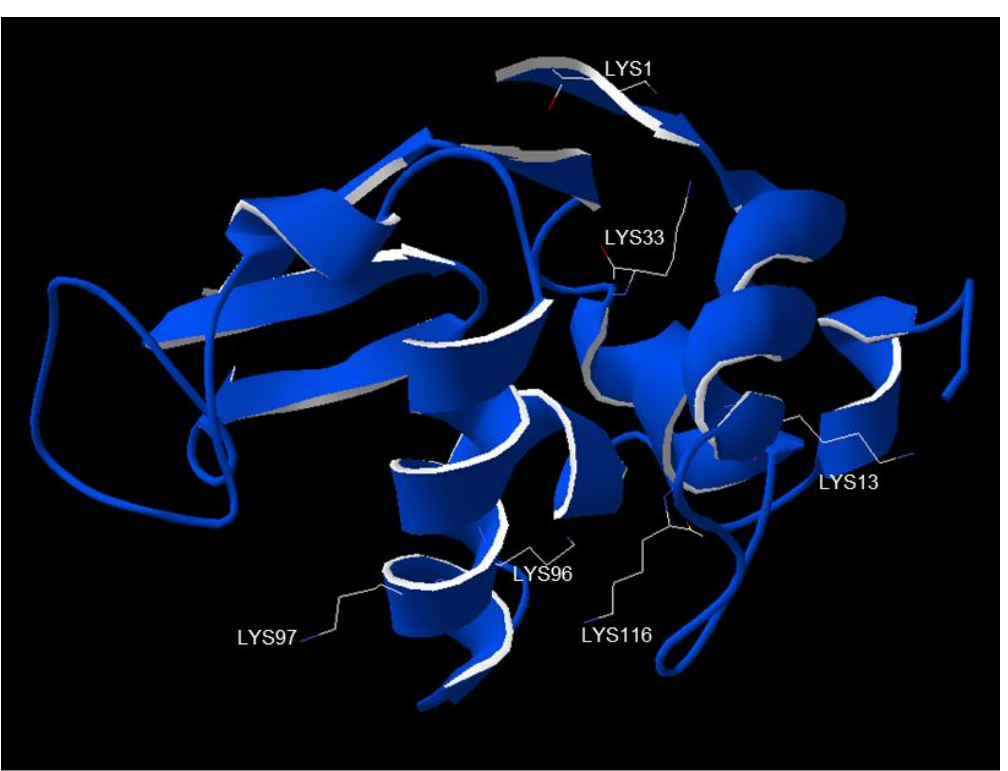

Figure 1.6: Structure of Lysozyme with lysine residues labeled [60].

Lysozyme was chosen not only because of its history as a model protein in studies of amyloidosis and protein aggregation, but also because of its similarities to ribonuclease A (RNase). RNase is similar in amino acid residue content to lysozyme, and our laboratory has performed studies with RNase previously. An important distinguishing factor, however, is that RNase is non-amyloidogenic, while lysozyme is amyloidogenic. The primary sequences of lysozyme and RNase are compared and contrasted in Figure 1.7.

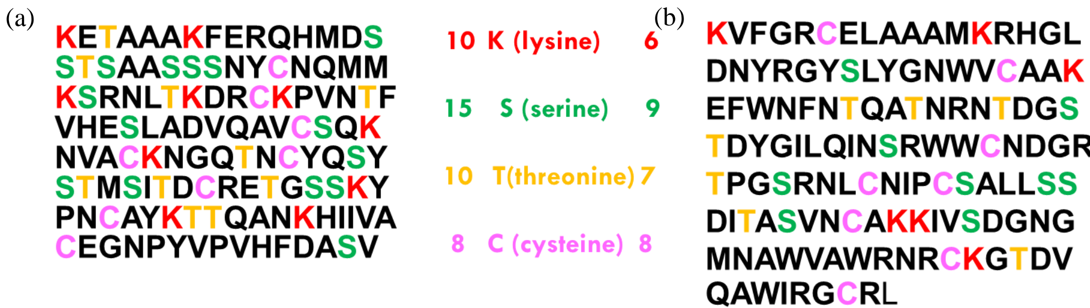

Figure 1.7: Sequence comparison of RNase (a) and lysozyme (b) with the residues lysine, serine, threonine, and cysteine highlighted [61, 62].

Three PAH quinones are chosen to obtain a substituent gradient to monitor how various substituents affect protein cross-linking of lysozyme. The three chosen quinones are PBQ, 2-chloro-1,4-benzoquinone (CBQ), and 2-methyl-1,4-benzoquinone (MBQ). The quinones, the PAHs from which they are derived, and their chemical effects are shown in Table 1.2. The experiments consist of *in vitro* incubations of lysozyme with a benzoquinone in physiological conditions, in order to obtain samples in a time- and concentration- dependent manner. These samples will then be studied via SDS-PAGE to determine if cross-linking is occurring, and if so, to what extent.

Table 1.2: PAHs, PAH Quinones, and Relative Effects

PAH	PAH Quinone	Effect
Benzene	PBQ	Unsubstituted
CH_3 Methylbenzene	CH_3 MBQ	Electron Donating
Cl Chlorobenzene	Cl CBQ	Electron Withdrawing

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Chapter II

Materials and Methods

2.1 Introduction to Experimentation

This thesis attempts to understand the reaction between three benzoquinones and the protein lysozyme. To do so, an experimental protocol had to be created to be able to limit the variables affecting the results, and it had to be replicable and reasonable, as well. To observe the effects of this reaction, *in vitro* incubations at physiological conditions were used. All solutions for experimentation were created in lab. For this thesis, the primary method of analysis was SDS-PAGE, which is very common technique for separating proteins by molecular mass [1]. All chemical components of SDS-PAGE were created in lab.

It is necessary to discuss these materials and methods in detail because errors in these procedures and steps propagate into errors in the finalized data. To obtain the most accurate results possible, great care must be taken during every step of the experimental process. It is for these reasons that the materials and methods are of great importance. Without the knowledge and understanding of the processes necessary for experimentation, data cannot be obtained or interpreted properly.

2.2 Reagents and Solutions

General:

For this project, all solutions were created from basic reagents. All chemicals have been purchased from Fisher Scientific™ and are of reagent quality unless specified otherwise. All water used in either the creation of solutions or in experiments was deionized water (dI-water). The water was purified by a Millipore™ system (Milli-Q).

2.2.1 Solutions for Incubations

Several solutions had to be created in order to run the incubations of lysozyme and quinone. These include phosphate buffer (50 mM, pH 7.0), quinone stock solutions (PBQ, CBQ, and MBQ, respectively), phosphate-methanol buffer, and lysozyme stock solution.

Phosphate buffer was made by first using the Henderson-Hasselbalch equation (shown in equation 1) to calculate the weights of monosodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4) needed to produce a 50 mM buffer at pH 7.0.

$$pH = pKa + log\frac{[Base]}{[Acid]} \tag{1}$$

NaH_2PO_4 and its conjugate base, Na_2HPO_4, were chosen because phosphoric acid has multiple dissociation constants with a pK_a around 7.2 for NaH_2PO_4 [2]. By creating an equilibrium between NaH_2PO_4 and Na_2HPO_4, an effective buffer range can be obtained at pH 7.0. Phosphate buffer was used as the buffer for all of the reactions

because of its buffer range and because phosphates are commonly found in biological systems and used in biological experimentation [3]. Phosphate buffer was stored at 4 °C in glass media bottles.

To make quinone stocks, the quinone of interest (PBQ, CBQ, or MBQ) was weighed and placed into a volumetric flask. Because of issues with solubility (mechanical agitation was not enough to solubilize the quinone in the phosphate buffer), the quinones were dissolved in 10% MeOH/ 90% phosphate buffer.

Lysozyme stock solutions were prepared by careful weighing and transfer to a volumetric flask. The lysozyme was diluted to volume with phosphate buffer. The stock was then sealed in tubes with Ar gas and kept at -80 °C until use. The Ar gas and cold temperatures were utilized to prevent the degradation and oxidation of the lysozyme stock solutions.

2.2.2 Solutions for SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a complicated technique that requires many solutions. Since SDS-PAGE experimentation is well established in protein studies, and protocols can easily be obtained. Only a brief overview of important solutions will be given. The protocol followed was from Kim and coworkers [4].

The electrode buffering system was comprised of Tris(hydroxymethyl)aminomethane (Tris) base. The loading dye contained bromophenol blue as the dye and mercaptoethanol as a denaturing agent. The staining solution for the gels contained Coomassie Brilliant Blue G250.

2.3 SDS-PAGE

SDS-PAGE is an analytical technique that employs the anionic detergent SDS to cause the separation of macromolecules by molecular weight through electrophoretic methods [3, 5]. It is an extremely valuable and effective tool used in biochemistry and cellular biology today. What separates SDS-PAGE from other polyacrylamide techniques is its use of the detergent SDS, which plays two important factors. First, SDS works to denature the tertiary structure of a protein [1, 3, 5]. Secondly, SDS applies a uniformly distributed negative charge across the protein [5].

SDS is an amphiphilic molecule, meaning that it has both hydrophilic (coming from the sulfate head of the molecule) and hydrophobic properties (coming from the hydrocarbon tail) [6]. These properties of SDS allow it to disrupt the hydrophobic interactions that stabilize the tertiary structure of a protein [5]. The molecular structure of SDS is given in Figure 2.3.1.

Figure 2.3.1. Chemical Structure of SDS

SDS alone cannot fully break down and linearize the tertiary structure of many proteins because many proteins contain disulfide bonds, which are covalent bonds, making them

much stronger than hydrophobic interactions [1, 5]. To overcome the disulfide bonds, the chemical mercaptoethanol is used to break them. The molecular structure of mercaptoethanol is given in Figure 2.3.2.

SH OH

Figure 2.3.2. The chemical structure of mercaptoethanol

By using a combination of SDS, heat denaturing, and mercaptoethanol, the tertiary structure of a protein can be reduced to a linearized chain.

The second important aspect of SDS is its negative charge. SDS binds to proteins in a ratio of about 1.4 grams of SDS per 1 gram of protein, which works out to be approximately one SDS molecule for every two amino acid residues on a protein [1, 5, 7-9]. Because of this binding property, SDS applies an extremely large negative charge that masks the intrinsic charge of the protein being studied [5]. Because of the denaturing and electronic properties of SDS, the result is that proteins are all similar in shape and charge-to-mass ratios, which means that SDS-PAGE separates proteins purely by molecular mass [1, 5].

The mobility of a protein through the gel matrix should vary linearly with the logarithm of their masses, so the mass of a protein can be determined within 5 to 10% accuracy [5]. To obtain a comparison of molecular weights, a molecular marker is typically used. A molecular marker contains several proteins of known molecular mass that can be used to compare against unknowns [1].

The principle of the SDS-PAGE apparatus is primarily derived from the attraction of charges. In a typical setup (an example is displayed in Figure 2.3.3), a vertical gel is placed in a housing that contains electrically conductive buffer [1].

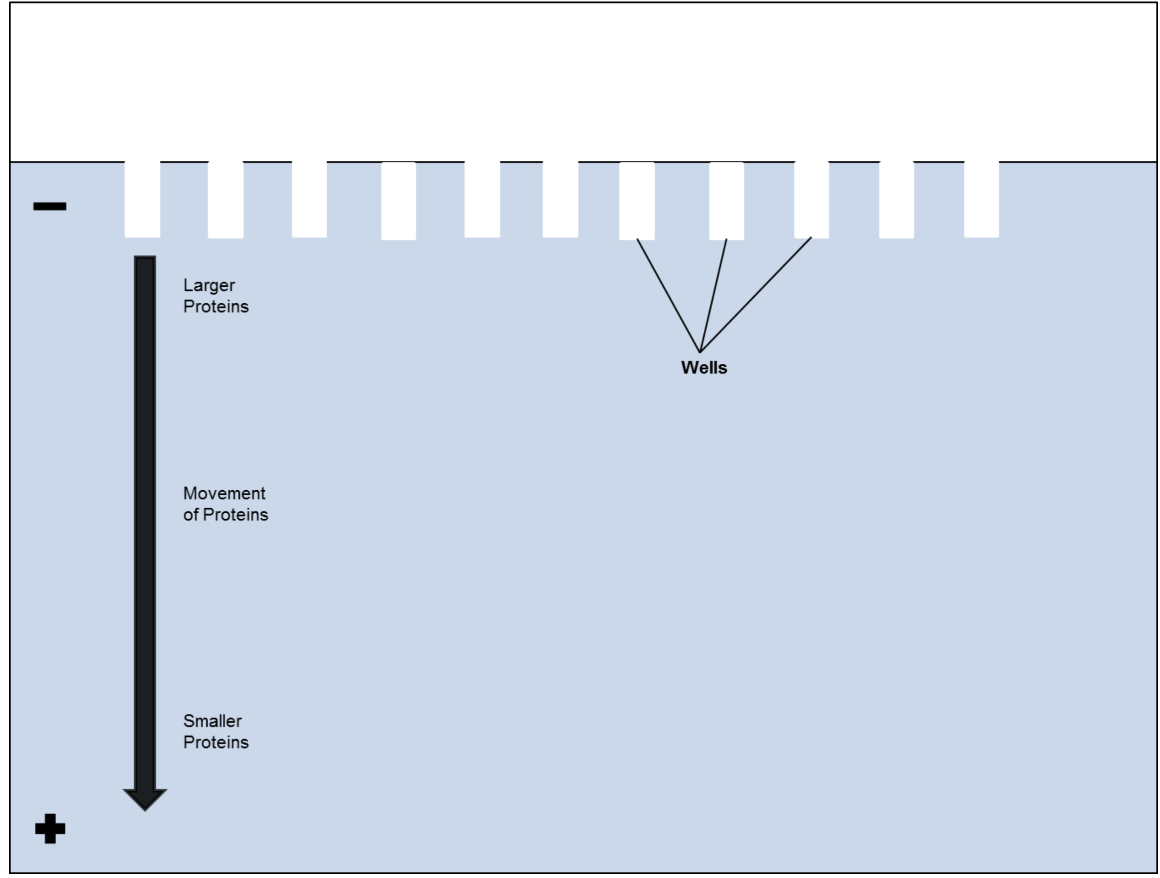

Figure 2.3.3. Schematic illustration of an SDS-PAGE Gel [5, 10].

There is a negative electrode (cathode) at the top, where the samples are loaded, and there is a positive electrode (anode) at the bottom. The samples are highly negative due to the SDS, which first dissociates from Na^+, and then interacts with the linearized polypeptide chain [1]. The negatively charged proteins are attracted to the positive electrode at the bottom of the gel [1]. There are two gels in SDS-PAGE: there is the stacking gel and the

resolving or running gel. The stacking gel is extremely porous and allows all of the proteins (regardless of size) to quickly reach the bottom of it before entering the resolving gel. This is important so that all of the sample molecules start at the same height on the gel at the same time. The resolving gel allows for the separation of the proteins by their mass [1]. The analysis of representative gels was quantified using ImageJ software [11]. ImageJ analyzes lanes of equal size to be able quantitatively compare bands of an SDS-PAGE gel [11]. The primary bands can be compared by creating an area histogram of the intensities of each SDS-PAGE lane. The areas can then be normalized and compared against each other to observe the rate of formation. Representative gels from each quinone were analyzed using ImageJ software.

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Chapter III

Results and Conclusions

3.1 Introduction

This chapter covers the results and conclusions of the experiments on the protein-crosslinking effects of PBQ, MBQ, and CBQ on lysozyme. The experiments show the effects of the quinones on lysozyme over a time- and concentration-dependent manner as compared with a control. They are then compared against each other to gain a greater understanding of the possible results of a substituent effect of the groups on MBQ and CBQ when reacted with lysozyme. This will help elucidate how electron donating and electron withdrawing groups may affect their reactivity. All of the results in this chapter are presented in the form of SDS-PAGE gels that analyze these reactions.

3.2 Results

3.2.1 SDS-PAGE of PBQ and Lysozyme

Figure 3.4.1 shows the SDS-PAGE gel of the reaction of 0.10 mM lysozyme with 3.0 mM PBQ. In the 10 (L2) and 30 (L3) min wells, dimer formation is seen at approximately 27 kDa with slight trimer formation at approximately 42 kDa. In the 60 (L4), 120 (L5), and 180 (L6) min wells, oligomerization and aggregation are seen in addition to trimers, with the aggregation being most substantial at 120 min (L5). After 120 min, the bands begin to fade because there is a solubility issue of the formed protein aggregate that was not able to reach the resolving gel. This is further confirmed by the solid dark brown plaques seen in the wells past 240 min (L7) for PBQ at this concentration. This gel was analyzed using the ImageJ software, and the results of this analysis are in Table 3.4.1.

Figure 3.4.2 shows the gel for the reaction of lysozyme (0.10 mM) with PBQ (1.0 mM). There is a substantial decrease in aggregate formation as compared to the PBQ (3.0 mM) gel. The 10 (L2) and 30 min (L3) wells only display mild formation of lysozyme dimers, whereas in the PBQ (3.0 mM) run, they were already forming trimers. Noticeable aggregation does not occur until the 180 (L6) and 240 min (L7) wells, and it is never as striking as seen in the PBQ (3.0 mM) runs. The trimer formation is not as drastic as seen in the PBQ (3.0 mM) run either. There was a small amount of plaque in the 300 min (L8) well and a large amount in the 24 hr (L9) well at PBQ (1.0 mM).

Figure 3.4.3 shows the SDS-PAGE results for the reaction of lysozyme (0.10 mM) with PBQ (0.50 mM). This concentration of PBQ is only a five times that of the

protein itself. As would be expected with the trends described above, the PBQ (0.50 mM) run has significantly less oligomerization and aggregation. True dimer formation does not occur until one hr (L4) into the reaction. Polymeric aggregation is not seen until the 24 hr (L9) reaction. At this concentration of PBQ, there was no precipitate in any of the wells, including the 24 hr (L9) well.

Figure 3.4.4 shows lysozyme reacted with PBQ (0.10 mM), which is the lowest experimental concentration of quinone. At this concentration, the concentration of quinone is equal to the concentration of lysozyme. Dimer formation still occurred fairly readily at the 60 min (L4) well; however, no other significant product was ascertained until the 24 hr (L9) well. The dimer bands slightly increased in intensity after each hr. No aggregation was displayed at this low of a concentration, not even in the 24 hr (L9) well.

3.2.3 SDS-PAGE of CBQ and Lysozyme

Figure 3.4.5 shows the results of lysozyme (0.10 mM) reacted with CBQ (3.0 mM). During the incubation, red-brown plaque formation occurred in as early as 30 min (L3), and the same trend was observed in the wells of the SDS-PAGE gels. Dimer formation occurred readily in 10 min (L2) for this concentration. At 30 min (L3), and all the way to 24 hr (L9), trimer formation and further aggregation was seen on the gel, along with red-brown plaques resting in the wells of the gel. The aggregation for CBQ is much more severe than that of PBQ, and the bands are much more defined. CBQ's reactivity with lysozyme is most likely more intense than that of PBQ because the chloride group is electron withdrawing, enhancing its reactivity. This gel was analyzed using the ImageJ software, and the results of this analysis are in Table 3.4.2.

Figure 3.4.6 shows the results of lysozyme (0.10 mM) reacted with CBQ (1.0 mM). Dimer formation still occurs at 10 min (L2), although it is much less than that of the CBQ (3.0 mM) reaction. Trimer formation does resolve until the 60 min (L4) well, whereas aggregate bands do not appear until 120 min (L5) and onward. Visible plaques in the wells of the CBQ (1.0 mM) reaction were not seen until the 240 min (L7) sample of CBQ. This is a drastic reduction from the CBQ (3.0 mM) experiment, which had plaque formation as early as 30 min (L3) into the experiment, but this is to be expected with the drop in concentration from 30 to 1 (CBQ to lysozyme) to a 10 to 1 reaction. The most intense banding of the CBQ (1.0 mM) reaction is at the 3 (L6) to 5 hr (L8) range, with 24 hr (L9) being slightly faded in comparison. This is most likely indicative of extensive polymeric aggregation occurring in the time frame between 5 to 24 hr (L8 and L9).

Figure 3.4.7 shows the gel resulting from the analysis of lysozyme with CBQ (0.50 mM). The formation of oligomeric products has decreased drastically from the CBQ (1.0 mM) concentration. Dimer formation is still present in as early as 10 min (L2), although it is only a very light band. Very faint trimer bands can be seen at the 180 min (L6) and onward wells. There are some light bands at 180 min (L6) and past, indicating a small amount of aggregate formation. There were no visible plaques seen in any of the wells of this experiment. By analyzing this gel, it appears that CBQ readily induces lysozyme to undergo oligomerization into dimers, but further polymerization is not as easily achieved.

Figure 3.4.8 displays the results of SDS-PAGE for the reaction between lysozyme (0.10 mM) and CBQ (0.10 mM). The 1 to 1 reaction of CBQ and lysozyme shows a

much faster formation of dimers when compared to similar concentrations of PBQ. In fact, dimer formation still occurs at the 10 min (L2) well, although the band is faint. A very faint trimer band can be seen in the 24 hr (L9) well, but the rest of the wells only have the formation of dimers. This further substantiates that CBQ can readily induce lysozyme to cross-link into a dimer, but addition of further units does not readily occur until much higher concentrations of CBQ are achieved.

3.2.4 SDS-PAGE of MBQ and Lysozyme

Figure 3.4.9 shows the results of SDS-PAGE of lysozyme (0.10 mM) reacted with MBQ (3.0 mM). Even at the high concentration of 30 to 1 MBQ to lysozyme, the electron donating effect is very apparent in the lack of reactivity leading to protein crosslinking. Dimer formation occurs minimally at the 120 min (L5) mark, with no oligomerization in the wells before it. There is a slight band at approximately 42 kDa that may be a trimer in the 24 hr (L9) well, but there is no polymeric aggregation or further oligomerization induced by MBQ. The reactivity is substantially lower than both PBQ and CBQ, and even at this high concentration, almost no protein crosslinking occurs. There was no plaque formation during the incubation or SDS-PAGE experiments. This gel was analyzed using the ImageJ software, and the results of this analysis are in Table 3.4.3.

Figure 3.4.10 displays the SDS-PAGE results of lysozyme (0.10 mM) reacted with MBQ (1.0 mM). Dimer formation does not occur until the 5 hr (L8) mark, which is in stark contrast to CBQ which had heavy dimerization at 10 min (L2) in similar concentrations of lysozyme and CBQ. The SDS-PAGE experiments do not show if other

pathways of reactivity are taken by the MBQ, but as far as protein cross-linking, only minimal amounts occur for this benzoquinone.

Figure 3.4.11 shows the resulting gel of lysozyme (0.10 mM) incubated with MBQ (0.50 mM). There is a faint dimer band in the 24 hr (L9) well. Other than that, no other significant findings are marked.

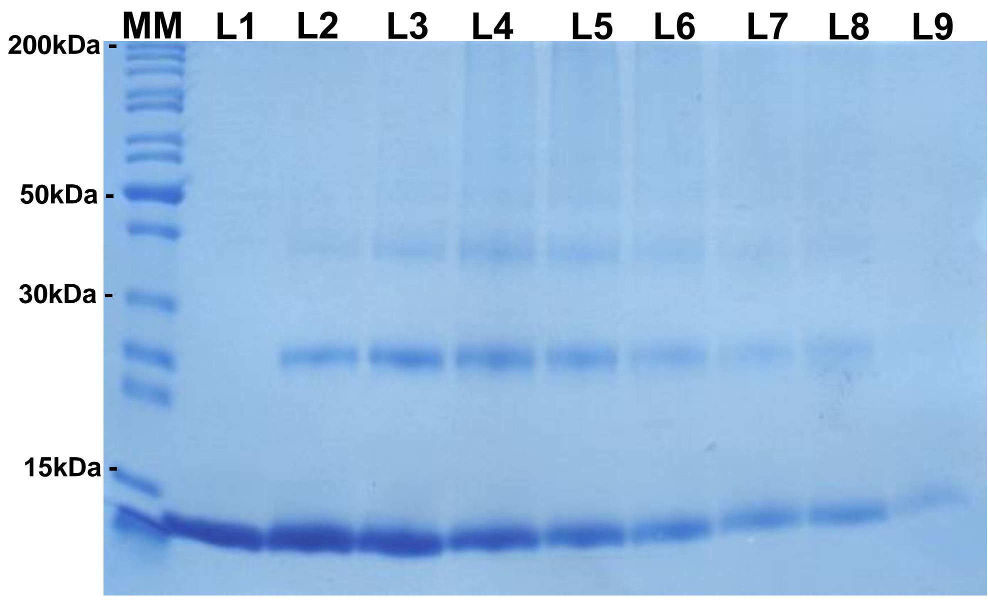
Figure 3.4.12 shows the resulting gel of lysozyme (0.10 mM) reacted with MBQ (0.10 mM). There is a possible faint dimer band at 24 hr (L9), but the 1 to 1 reaction has very little reactivity with lysozyme. There are no other significant findings from this gel.

3.3 Conclusions

In conclusion, lysozyme is readily modified by the three benzoquinones of interest (PBQ, CBQ, and MBQ) under physiological conditions (37 °C and pH=7.0). These results are confirmed by SDS-PAGE experiments which show dimer, trimer, and polymeric aggregate formation from reactions between lysozyme and quinone when compared to controls with just lysozyme. Quantitative analysis of the gels by ImageJ software leads to the same results. Furthermore, this reaction of protein cross-linking is shown to be dependent on the concentration of quinone, along with a direct relationship between the length of the reaction and the extent of modification of lysozyme. The SDS-PAGE experiments also display the implication of the substituent effect on lysozyme modification by benzoquinones. CBQ, with an electron withdrawing chloride, was by far the most reactive of the benzoquinones tested. CBQ readily induced formation of dimers and polymeric aggregates, even at lower concentrations. It also formed amyloidogenic plaques in as little as 30 min at a 3.0 mM concentration of CBQ. MBQ, on the other hand, had an electron donating methyl group which was shown to cause a substantial decrease in the reactivity of the benzoquinone. MBQ only formed products at high concentrations and longer incubation times. PBQ, with no substituent, showed an intermediate level of reactivity when compared to CBQ and MBQ. It readily formed aggregates at high concentration and long incubation times, but did not at lower concentrations and shorter incubation times.

3.4 Figures

Figure 3.4.1. SDS-PAGE PBQ (3.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



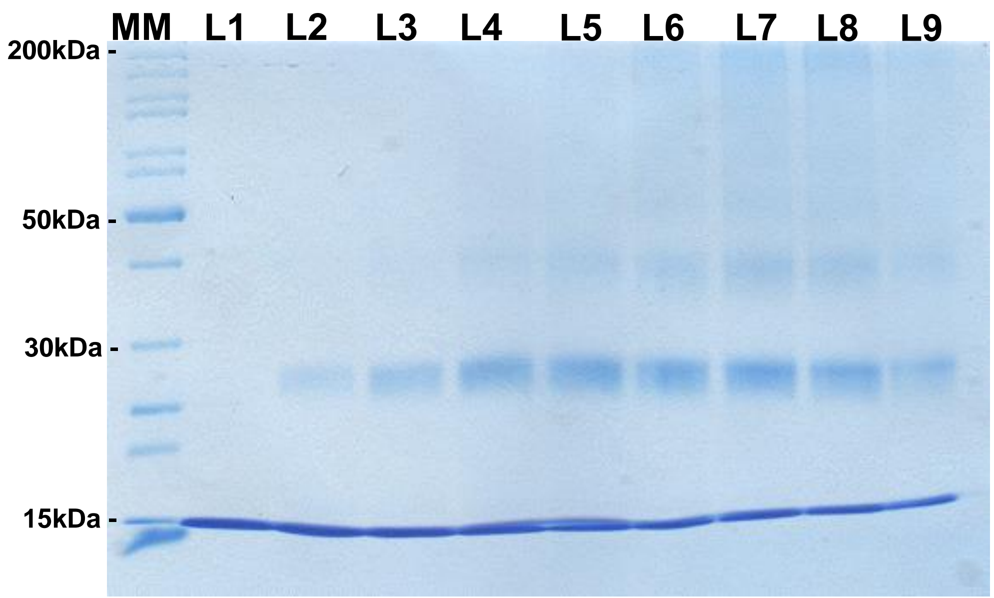
SDS-PAGE gel of reaction between PBQ (3.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with PBQ
L3: 30 Min Reaction with PBQ
L4: 60 Min Reaction with PBQ
L5: 120 Min Reaction with PBQ
L6: 180 Min Reaction with PBQ
L7: 240 Min Reaction with PBQ
L8: 300 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

Table 3.4.1: Normalized Ratios of Band Intensities for PBQ 3.0 mM

	Control	10 min	30 min	60 min	120 min	180 min	240 min	300 min	24 hr
Initial	1	0.74997	0.61051	0.52979	0.55584	0.59859	0.65932	0.71015	0.89591
Dimer	0	0.20445	0.29983	0.32470	0.31025	0.26166	0.21432	0.22828	0.10409
Trimer	0	0.04559	0.08966	0.14550	0.13392	0.13975	0.12636	0.06157	0.00000

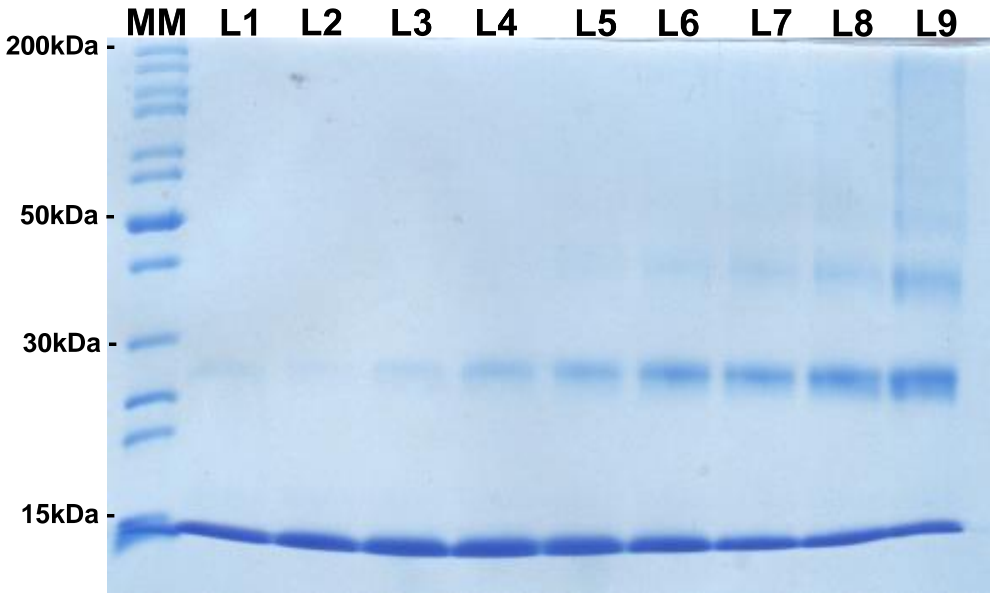
Figure 3.4.2. SDS-PAGE PBQ (1.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between PBQ (1.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with PBQ
L3: 30 Min Reaction with PBQ
L4: 60 Min Reaction with PBQ
L5: 120 Min Reaction with PBQ
L6: 180 Min Reaction with PBQ
L7: 240 Min Reaction with PBQ
L8: 300 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

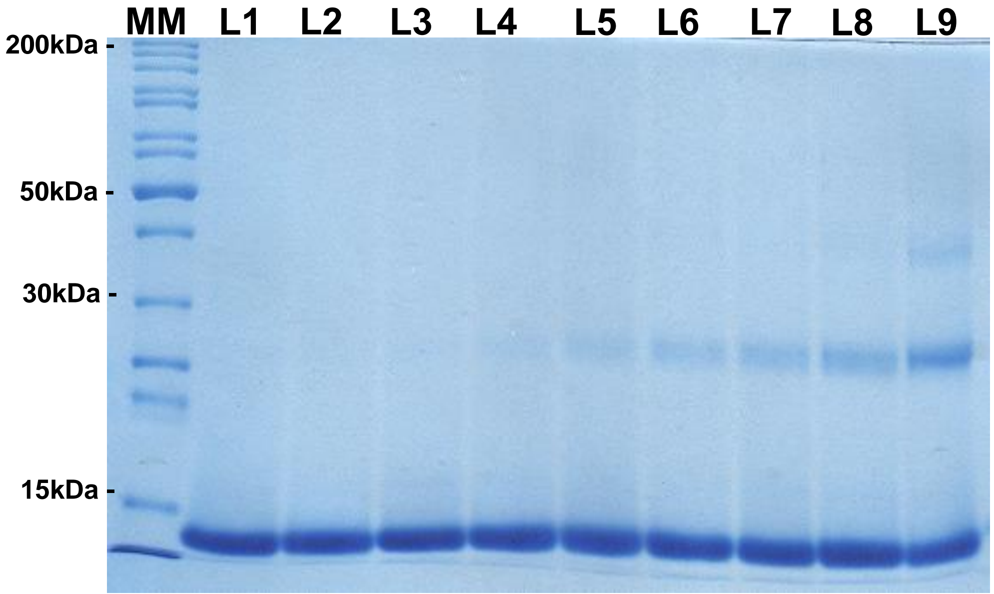
Figure 3.4.3. SDS-PAGE PBQ (0.50 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between PBQ (0.50 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with PBQ
L3: 30 Min Reaction with PBQ
L4: 60 Min Reaction with PBQ
L5: 120 Min Reaction with PBQ
L6: 180 Min Reaction with PBQ
L7: 240 Min Reaction with PBQ
L8: 300 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

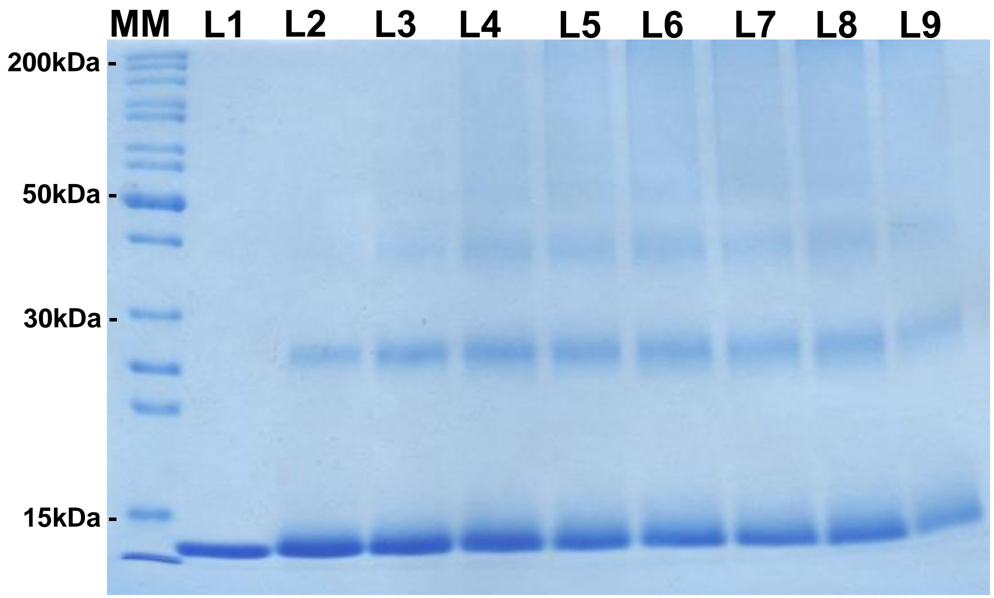
Figure 3.4.4. SDS-PAGE PBQ (0.10 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between PBQ (0.10 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with PBQ
L3: 30 Min Reaction with PBQ
L4: 60 Min Reaction with PBQ
L5: 120 Min Reaction with PBQ
L6: 180 Min Reaction with PBQ
L7: 240 Min Reaction with PBQ
L8: 300 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

Figure 3.4.5. SDS-PAGE CBQ (3.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



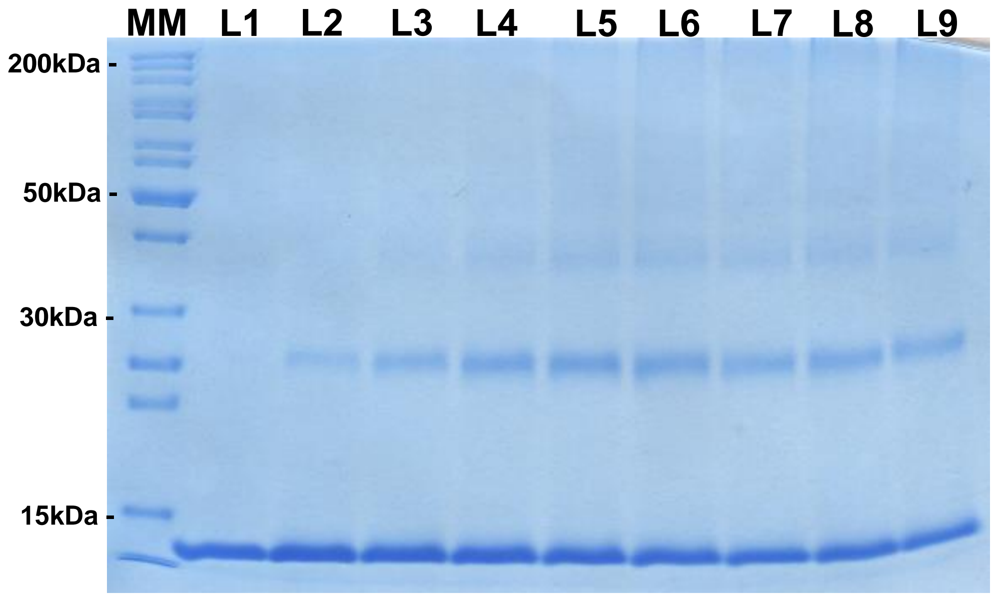
SDS-PAGE gel of reaction between CBQ (3.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with CBQ
L3: 30 Min Reaction with CBQ
L4: 60 Min Reaction with CBQ
L5: 120 Min Reaction with CBQ
L6: 180 Min Reaction with CBQ
L7: 240 Min Reaction with CBQ
L8: 300 Min Reaction with CBQ
L9: 24 hr Reaction with CBQ

Table 3.4.2: Normalized Ratios of Band Intensities for CBQ 3.0 mM

	Control	10 min	30 min	60 min	120 min	180 min	240 min	300 min	24 hr
Initial	1	0.73480	0.67876	0.59273	0.58289	0.57302	0.59153	0.58705	0.76589
Dimer	0	0.20559	0.24332	0.30044	0.33213	0.29858	0.30677	0.27210	0.15595
Trimer	0	0.05961	0.07792	0.10683	0.08497	0.12840	0.10169	0.14085	0.07816

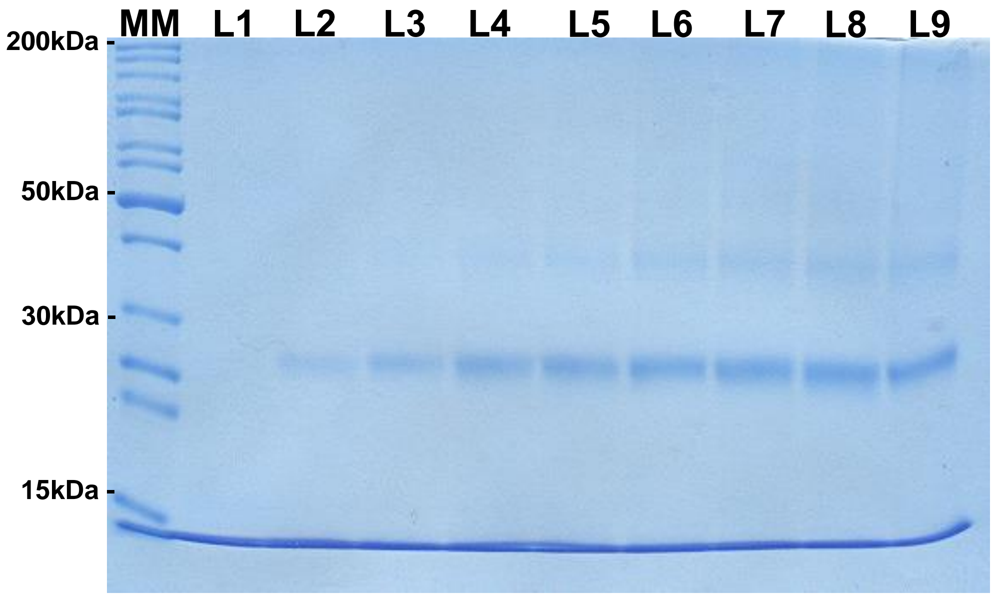
Figure 3.4.6. SDS-PAGE CBQ (1.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between CBQ (1.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with CBQ
L3: 30 Min Reaction with CBQ
L4: 60 Min Reaction with CBQ
L5: 120 Min Reaction with CBQ
L6: 180 Min Reaction with CBQ
L7: 240 Min Reaction with CBQ
L8: 300 Min Reaction with CBQ
L9: 24 hr Reaction with CBQ

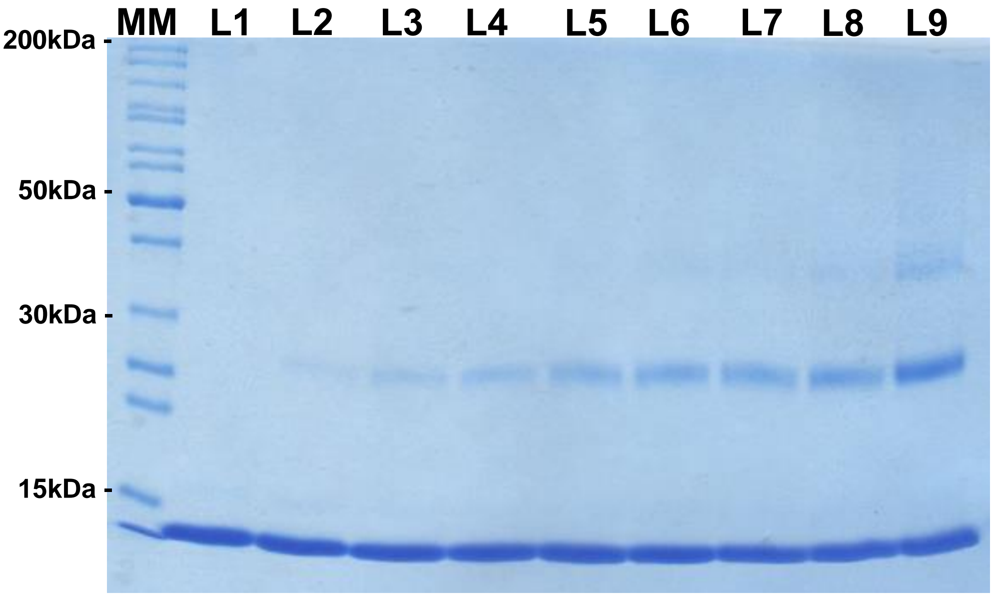
Figure 3.4.7. SDS-PAGE CBQ (0.50 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between CBQ (0.50 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with CBQ
L3: 30 Min Reaction with CBQ
L4: 60 Min Reaction with CBQ
L5: 120 Min Reaction with CBQ
L6: 180 Min Reaction with CBQ
L7: 240 Min Reaction with CBQ
L8: 300 Min Reaction with CBQ
L9: 24 hr Reaction with CBQ

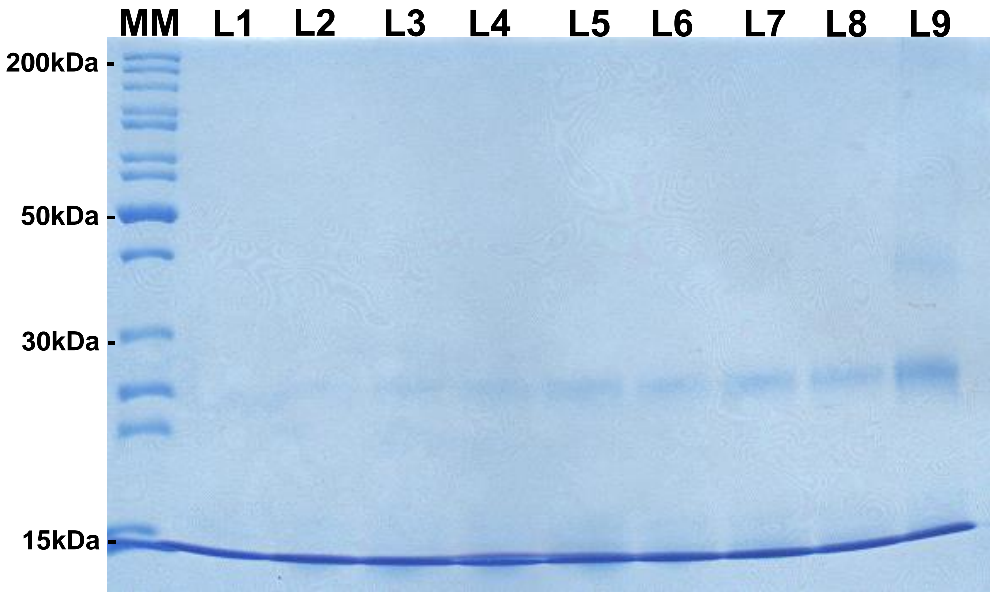
Figure 3.4.8. SDS-PAGE CBQ (0.10 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between CBQ (0.10 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with CBQ
L3: 30 Min Reaction with CBQ
L4: 60 Min Reaction with CBQ
L5: 120 Min Reaction with CBQ
L6: 180 Min Reaction with CBQ
L7: 240 Min Reaction with CBQ
L8: 300 Min Reaction with CBQ
L9: 24 hr Reaction with CBQ

Figure 3.4.9. SDS-PAGE MBQ (3.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



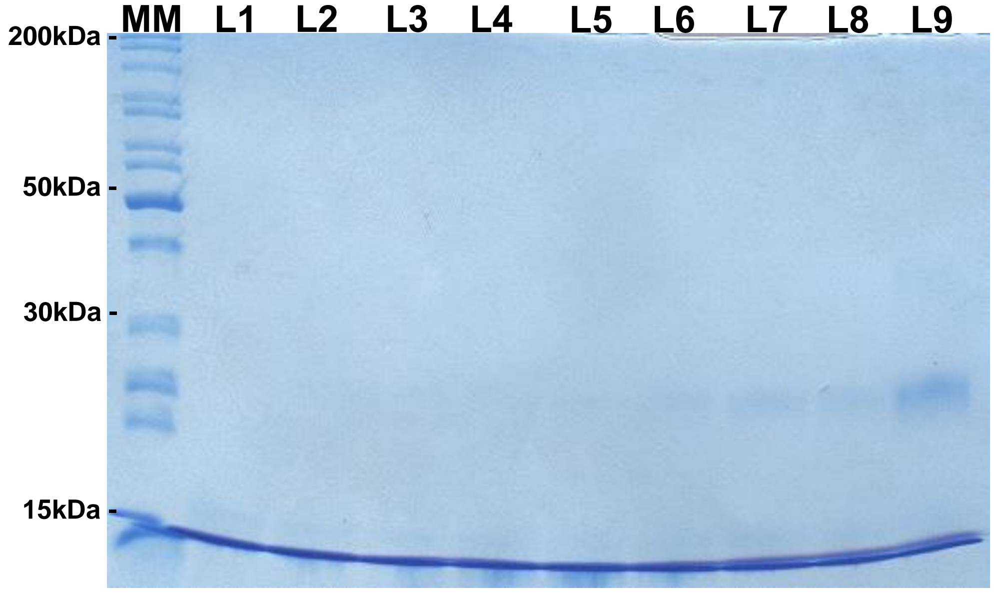
SDS-PAGE gel of reaction between MBQ (3.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with MBQ
L3: 30 Min Reaction with MBQ
L4: 60 Min Reaction with MBQ
L5: 120 Min Reaction with MBQ
L6: 180 Min Reaction with MBQ
L7: 240 Min Reaction with MBQ
L8: 300 Min Reaction with MBQ
L9: 24 hr Reaction with MBQ

Table 3.4.3: Normalized Ratios of Band Intensities for MBQ 3.0 mM

	Control	10 min	30 min	60 min	120 min	180 min	240 min	300 min	24 hr
Initial	1.00000	0.90926	0.80546	0.88535	0.70443	0.73124	0.67725	0.75168	0.44026
Dimer	0.00000	0.09074	0.19454	0.11465	0.29557	0.26876	0.32275	0.24832	0.47248
Trimer	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.08726

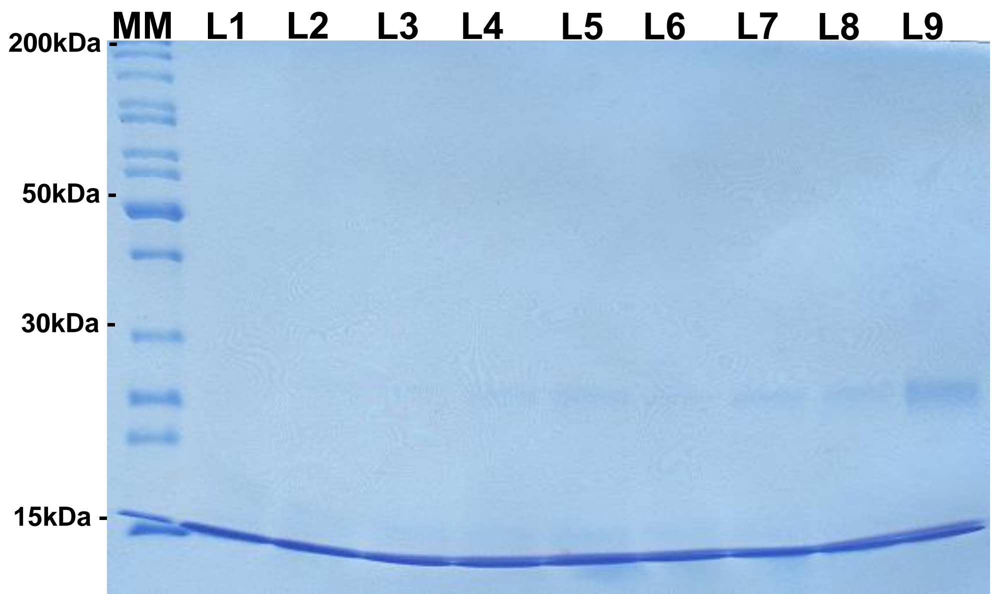
Figure 3.4.10. SDS-PAGE MBQ (1.0 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between MBQ (1.0 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with MBQ
L3: 30 Min Reaction with MBQ
L4: 60 Min Reaction with MBQ
L5: 120 Min Reaction with MBQ
L6: 180 Min Reaction with MBQ
L7: 240 Min Reaction with MBQ
L8: 300 Min Reaction with MBQ
L9: 24 hr Reaction with MBQ

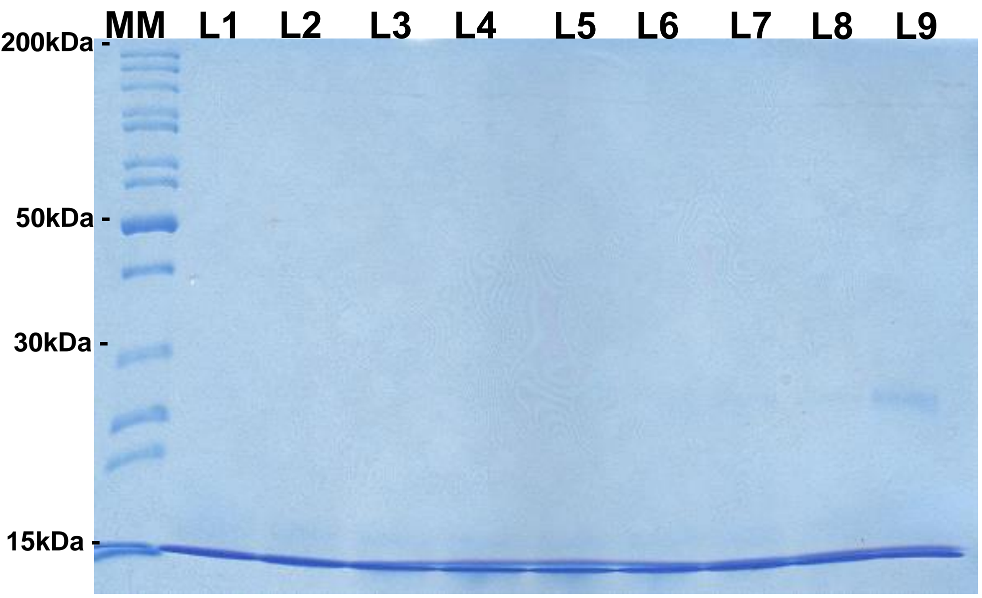
Figure 3.4.11. SDS-PAGE MBQ (0.50 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between MBQ (0.50 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with MBQ
L3: 30 Min Reaction with MBQ
L4: 60 Min Reaction with MBQ
L5: 120 Min Reaction with MBQ
L6: 180 Min Reaction with MBQ
L7: 240 Min Reaction with MBQ
L8: 300 Min Reaction with MBQ
L9: 24 hr Reaction with MBQ

Figure 3.4.12. SDS-PAGE MBQ (0.10 mM) with Lysozyme (0.10 mM) (Data Duplicated)



SDS-PAGE gel of reaction between MBQ (0.10 mM) with Lysozyme (0.10 mM) at 37 °C in a time-dependent manner at pH 7.0. The 24 hr control contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 24 hr Control Lysozyme
L2: 10 Min Reaction with MBQ
L3: 30 Min Reaction with MBQ
L4: 60 Min Reaction with MBQ
L5: 120 Min Reaction with MBQ
L6: 180 Min Reaction with MBQ
L7: 240 Min Reaction with MBQ
L8: 300 Min Reaction with MBQ
L9: 24 hr Reaction with MBQ

Appendix A

Optimization of SDS-PAGE Experiments

SDS-PAGE experiments are a long and arduous process that cannot be mastered the first time. Appendix A covers initial gels that were the results of optimization experiments to obtain the best parameters and to refine experimental technique. Some of the parameters that were experimented with were the times that the gels ran, the concentration of electrode buffer, the loading volumes, and the dyes used to stain the gels. Figure A.1 shows the first SDS-PAGE experiment with lysozyme and PBQ at concentrations of 0.145 and 5.0 mM, respectively. The intent of this experiment was to optimize the conditions to detect protein modifications utilizing SDS-PAGE. If the concentration of lysozyme is too high or too low, the data will not be useful or be able to resolve the effective oligomerization that was under scrutiny. Similar results would be seen with excessive amounts of quinone reactant. Lysozyme controls were ran for both 0 and 24 hr, to observe any change that occurred to lysozyme on its own while *in vitro*.

The 10 min (L3) well shows heavy dimer formation and slight trimer formation. The wells of 30 min (L4) and 60 min (L5) show dimer, trimer, and polymeric aggregate formation. The aggregate formation is seen in the striking bands in the higher kDa ranges. The oligomer bands have almost completely faded for 180 min (L6) and 24 hr (L7) reaction wells due to the heavy aggregation, leading to polymers of lysozyme with high molecular mass. The lysozyme aggregates formed at 180 min (L6) and 24 hr (L7) are so massive, they do not even reach the 200 kDa cut off range. This indicates that with the current conditions, the reaction is proceeding too fast to observe an even time gradient. Figures 3.4.2 and 3.4.3 show SDS-PAGE of lysozyme (0.145mM) after treatment of PBQ (1.5 mM). Both of these gels have defects (the 60 min well was skipped for Figure 3.4.2 and physical damage was sustained by the gel in Figure 3.4.3), however, and a stark jump

between the first and second hr is still seen. Figure 3.4.4 shows lysozyme at a very low concentration of 0.050 mM reacted with PBQ (1.5 mM), but only for a total of 5 hr. The bands on this gel are faint, so an increase in lysozyme concentration is desired.

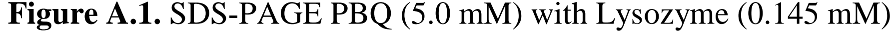
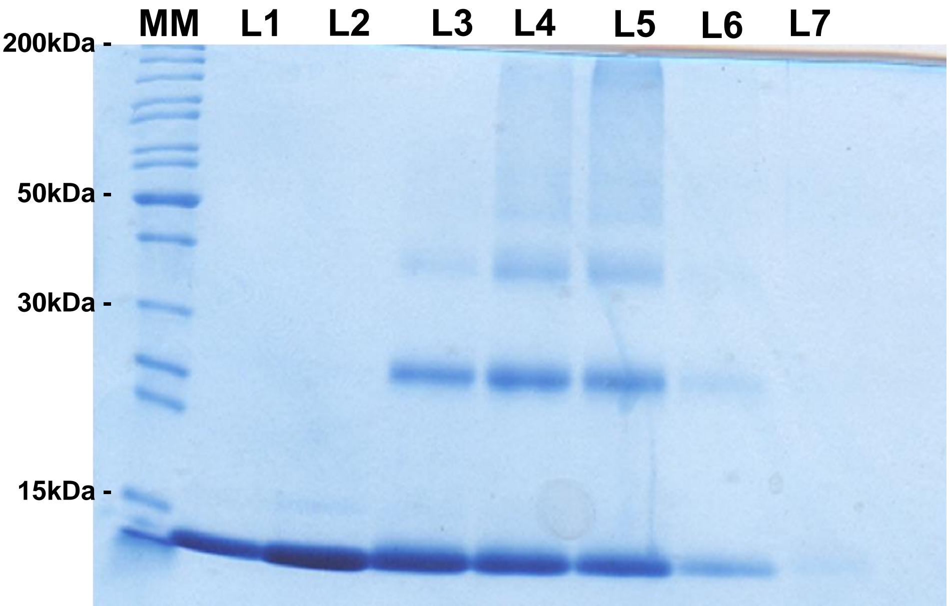


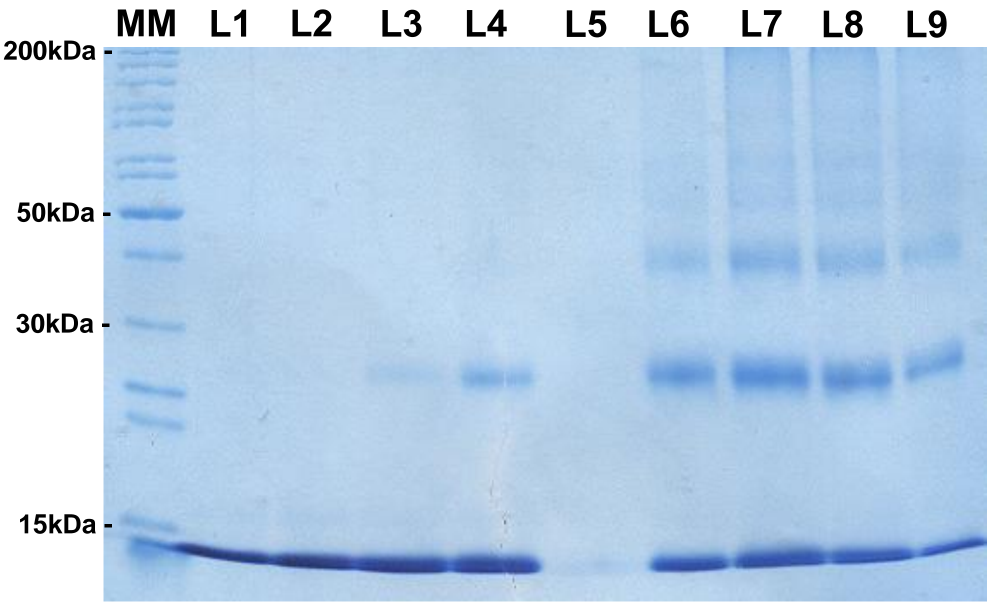
Figure A.1. SDS-PAGE PBQ (5.0 mM) with Lysozyme (0.145 mM)



SDS-PAGE gel of reaction between PBQ (5.0 mM) with Lysozyme (0.145 mM) at 37 °C in a time-dependent manner at pH 7.0. The 0 and 24 hr controls contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 0 hr Control Lysozyme
L2: 24 hr Control Lysozyme
L3: 10 Min Reaction with PBQ
L4: 30 Min Reaction with PBQ
L5: 60 Min Reaction with PBQ
L6: 180 Min Reaction with PBQ
L7: 24 hr Reaction with PBQ

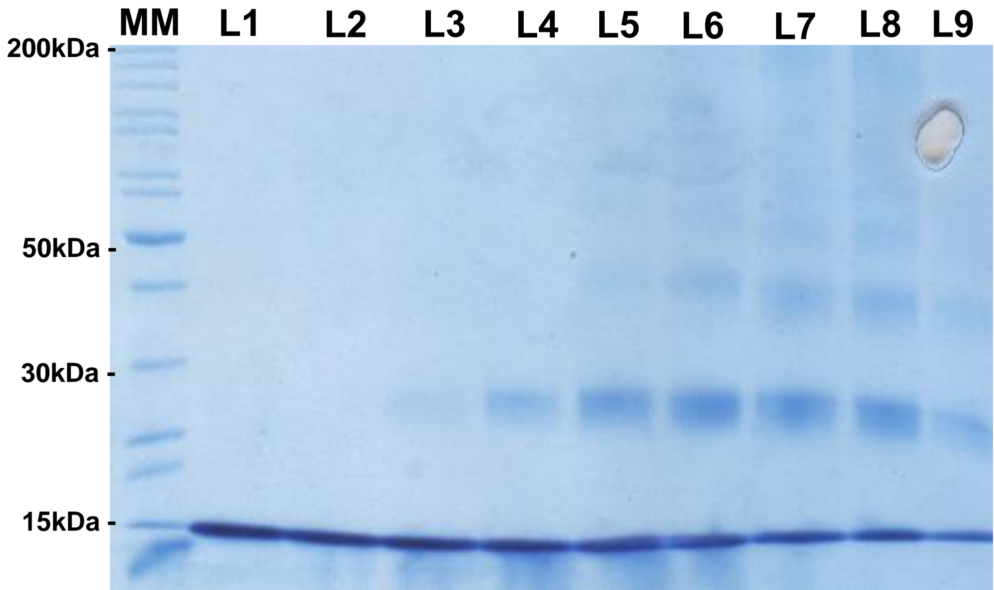
Figure A.2. SDS-PAGE PBQ (1.5 mM) with Lysozyme (0.145 mM) (Data Duplicated)



SDS-PAGE gel of reaction between PBQ (1.5 mM) with Lysozyme (0.145 mM) at 37 °C in a time-dependent manner at pH 7.0. The 0 and 24 hr controls contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time. L5 was not loaded.

MM (Molecular Marker)
L1: 0 hr Control Lysozyme
L2: 24 hr Control Lysozyme
L3: 10 Min Reaction with PBQ
L4: 30 Min Reaction with PBQ
L5: 60 Min Reaction with PBQ
L6: 120 Min Reaction with PBQ
L7: 180 Min Reaction with PBQ
L8: 240 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

Figure A.3. SDS-PAGE PBQ (1.5 mM) with Lysozyme (0.145 mM) (Data Duplicated)



SDS-PAGE gel of reaction between PBQ (1.5 mM) with Lysozyme (0.145 mM) at 37 °C in a time-dependent manner at pH 7.0. The 0 and 24 hr controls contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time. Note defect over L8/L9.

MM (Molecular Marker)
L1: 0 hr Control Lysozyme
L2: 24 hr Control Lysozyme
L3: 10 Min Reaction with PBQ
L4: 30 Min Reaction with PBQ
L5: 60 Min Reaction with PBQ
L6: 120 Min Reaction with PBQ
L7: 180 Min Reaction with PBQ
L8: 240 Min Reaction with PBQ
L9: 24 hr Reaction with PBQ

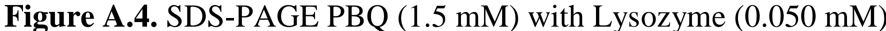
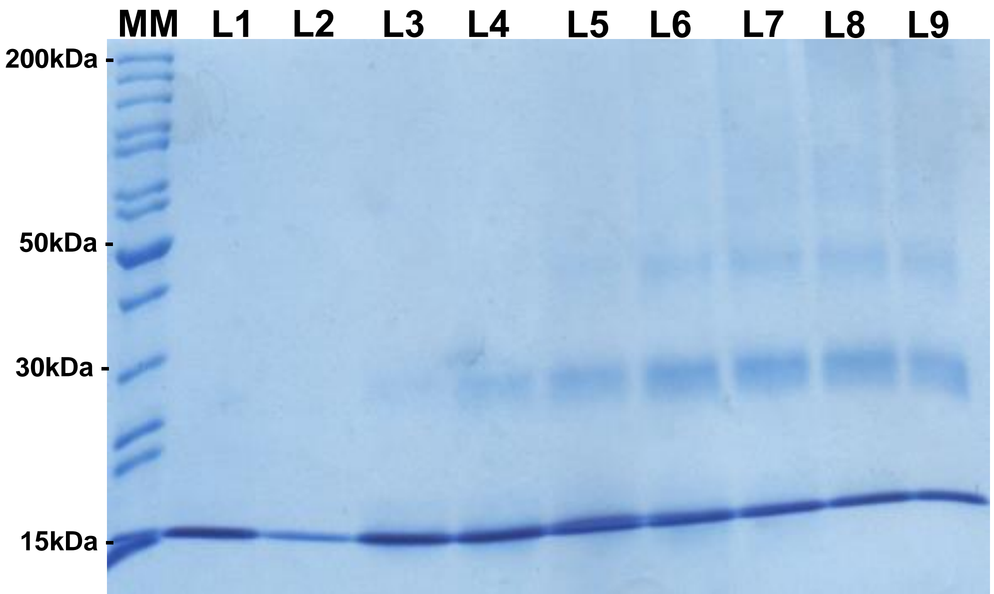


Figure A.4. SDS-PAGE PBQ (1.5 mM) with Lysozyme (0.050 mM)



SDS-PAGE gel of reaction between PBQ (1.5 mM) with Lysozyme (0.050 mM) at 37 °C in a time-dependent manner at pH 7.0. The 0 and 5 hr controls contained only lysozyme and phosphate buffer (50 mM, pH 7.0). Samples were transferred immediately to -20 °C freezer to halt the reaction at the specified time.

MM (Molecular Marker)
L1: 0 hr Control Lysozyme
L2: 5 hr Control Lysozyme
L3: 10 Min Reaction with PBQ
L4: 30 Min Reaction with PBQ
L5: 60 Min Reaction with PBQ
L6: 120 Min Reaction with PBQ
L7: 180 Min Reaction with PBQ
L8: 240 Min Reaction with PBQ
L9: 300 Min Reaction with PBQ

Appendix B

Incubation Tables

The tables provided in this appendix give the experimental details of each experiment. They contain information such as the volumes of solutions used, times, and loading volumes. These tables serve as an effective guide book of each experiment. They display the time- and concentration-dependent manner of the experiments, as well as give the details of the SDS-PAGE experiment.

Table B.1. Reaction details of PBQ (5.0 mM) with Lysozyme (0.145 mM)

0514 2013, [Q] 5.0 mM	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol		
20 mM/50ml stock, PBQ	108.10	1.00		108.10	in 10%MeOH/buffer		20	5	180	45		
Actual PBQ												
(uL)Final Lys->0.145 mM									Lys vol	37.4		
				mg	ml							
Stock Lys	0.698			20	2							
Freezing time												
	Total	MM	Ctr 0	Ctr 24h	10	30	1h		3h		24h	
0.698 mM St Lys (uL)	37.4		6.2	6.2								
Protein (ug)	375.5		62.6	62.6	62.6	62.6	62.6		62.6		62.6	
Phosphate buffer (uL)	97.6		23.8	23.8								
Incub Time			0 min	5hr	10 min	30 min	1h		3h		24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 m	45		0.0	0.0								
Final Incubate Vol (uL)	180		30	30								
Aliquot Taken (uL) X 4					30	30	30		30		30	Tot FIN vol
			3 x 10	3 x 10	3 x 10	3 x 10	3 x 10		3 x 10		3 x 10	150.0
LANE		L1	L2	L3	L4	L5	L6		L8		L10	
		MM	Ctr 0	Ctr 24h	10	30	1h		3h		24h	
Loading Dye		0	10	10	10	10	10		10		10	
Incub Aliquot of Total		0	10	10	10	10	10		10		10	
Loading Vol (uL)		12	10	10	10	10	10		10		10	
Protein loaded (ug)			7	10	10	10	10		10		10	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.2. Reaction details of PBQ (1.5 mM) with Lysozyme (0.145 mM)

0521 2013, [Q] 1.5 mM	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol		
5 mM/50ml stock, PBQ	108.10	0.25		27.03	in 10%MeOH/buffer		5	1.5	252	75.6		
Actual PBQ												
(uL)Final Lys->0.145 mM									Lys vol	52.3		
				mg	ml							
Stock Lys	0.698			20	2							
Freezing time												
	Total	MM	Ctr 0	Ctr 24h	10	30	1h	2h	3h	4h	24h	
0.698 mM St Lys (uL)	52.3		6.2	6.2								
Protein (ug)	525.7		62.6	62.6	62.6	62.6	62.6	62.6	62.6	62.6	62.6	
Phosphate buffer (uL)	124.1		23.8	23.8								
Incub Time			0 min	5hr	10 min	30 min	1h	2h	3h	4h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 mM	75.6		0.0	0.0								
Final Incubate Vol (uL)	252		30	30								
Aliquot Taken (uL) X 4					30	30	30	30	30	30	30	Tot FIN vol
			3 x 10	3 x 10	3 x 10	3 x 10	3 x 10	3 x 10	3 x 10	3 x 10	3 x 10	210.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8		L10	
		MM	Ctr 0	Ctr 24h	10	30	1h	2h	3h	4h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	10	10	10	10	10	10	10	10	10	
Loading Vol (uL)		12	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			10	10	10	10	10	10	10	10	10	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.3. Reaction details of PBQ (1.5 mM) with Lysozyme (0.050 mM)

0522 2013, [Q] 1.5 mM	FW	mmol		mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, PBQ	108.10	1.00		108.10	in 10%MeOH/buffer		20	1.5	540	40.5			
Actual PBQ													
(uL)Final Lys->0.05 mM				mg	ml				Lys vol	38.7			
Stock Lys	0.698			20	2								
Freezing time													
	Total	MM	Ctr 0	Ctr 5h	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	38.7		4.3	4.3									
Protein (ug)	388.5		43.2	43.2	32.4	32.4	32.4	32.4	32.4	32.4	32.4	32.4	
Phosphate buffer (uL)	460.8		55.7	55.7									
Incub Time			0 min	5hr	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C												
20 mM Q stock, [Q]f 0.5 mM	40.5		0.0	0.0									
Final Incubate Vol (uL)	540		60	60									
Aliquot Taken (uL) X 4					45	45	45	45	45	45	45	45	Tot FIN vol
			3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	270.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8		L9	L10	
		MM	Ctr 0	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		12	16	16	16	16	16	16	16	16	16	16	
Protein loaded (ug)			7	7	7	7	7	7	7	7	7	7	
0.15 mM Lys(ug),25ul	32												
0.10 mM Lys(ug),25ul	22												
0.05 mM Lys(ug),25ul	11												

Table B.4. Reaction details of PBQ (3.0 mM) with Lysozyme (0.10 mM)

0603 2013, [Q] 3.0 mM	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, PBQ	108.10	1.00	108.10	in 10%MeOH/buffer		20	3	540	81			
Actual PBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	77.3			
Stock Lys	0.698		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	77.3		14.3									
Protein (ug)	777.0		143.9	64.7	64.7	64.7	64.7	64.7	64.7	64.7	64.7	
Phosphate buffer (uL)	381.7		85.7									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 mM	81		0.0									
Final Incubate Vol (uL)	540		100									
Aliquot Taken (uL) X 4				45	45	45	45	45	45	45	45	Tot FIN vol
			3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	270.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	23	10	10	10	10	10	10	10	10	
Protein loaded (ug)			20	9	9	9	9	9	9	9	9	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.5. Reaction details of PBQ (1.0 mM) with Lysozyme (0.10 mM)

0603 2013, [Q] 1.0 mM	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, PBQ	108.10	1.00	108.10	in 10%MeOH/buffer		20	1	540	27			
Actual PBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	77.3			
Stock Lys	0.698		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	77.3		14.3									
Protein (ug)	777.0		143.9	64.7	64.7	64.7	64.7	64.7	64.7	64.7	64.7	
Phosphate buffer (uL)	435.7		85.7									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 mM	27		0.0									
Final Incubate Vol (uL)	540		100									
Aliquot Taken (uL) X 4				45	45	45	45	45	45	45	45	Tot FIN vol
			3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	3 x 15	270.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.6. Reaction details of PBQ (0.50 mM) with Lysozyme (0.10 mM)

0606 2013, [Q] 0.5 mM	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
5 mM/50ml stock, PBQ	108.10	0.25	27.03	in 10%MeOH/buffer		5	0.5	720	72			
Actual PBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1		14.3									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	544.9		85.7									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 mM	72		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL) X 4				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.7. Reaction details of PBQ (0.10 mM) with Lysozyme (0.10 mM)

0606 2013, [Q] 0.1 mM	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
5 mM/50ml stock, PBQ	108.10	0.25	27.03	in 10%MeOH/buffer		5	0.1	720	14.4			
Actual PBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1		14.3									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	602.5		85.7									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 0.5 mM	14.4		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL) X 4				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	
0.15 mM Lys(ug),25ul	32											
0.10 mM Lys(ug),25ul	22											
0.05 mM Lys(ug),25ul	11											

Table B.8. Reaction details of CBQ (3.0 mM) with Lysozyme (0.10 mM)

0612 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, CBQ	142.52	1.00	142.52	in 10%MeOH/buffer		20	3	720	108			
Actual CBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	Ctr 24h	MM	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1	8.6										
Protein (ug)	1035.9	86.3		86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	508.9	51.4										
Incub Time		0 min		10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	108	0.0										
Final Incubate Vol (uL)	720	60										
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
		3 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.9. Reaction details of CBQ (1.0 mM) with Lysozyme (0.10 mM)

0612 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, CBQ	142.52	1.00	142.52	in 10%MeOH/buffer		20	1	720	36			
Actual CBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	Ctr 24h	MM	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1	8.6										
Protein (ug)	1035.9	86.3		86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	580.9	51.4										
Incub Time		0 min		10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	36	0.0										
Final Incubate Vol (uL)	720	60										
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
		3 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.10. Reaction details of CBQ (0.50 mM) with Lysozyme (0.10 mM)

0612 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, CBQ	142.52	0.25	35.63	in 10%MeOH/buffer		5	0.5	720	72			
Actual CBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	Ctr 24h	MM	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1	8.6										
Protein (ug)	1035.9	86.3		86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	544.9	51.4										
Incub Time		0 min		10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	72	0.0										
Final Incubate Vol (uL)	720	60										
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
		3 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.11. Reaction details of CBQ (0.10 mM) with Lysozyme (0.10 mM)

0612 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, CBQ	142.52	0.25	35.63	in 10%MeOH/buffer		5	0.1	720	14.4			
Actual CBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	103.1			
Stock Lys	0.698		20	2								
Freezing time												
	Total	Ctr 24h	MM	10	30	1h	2h	3h	4h	5h	24h	
0.698 mM St Lys (uL)	103.1	8.6										
Protein (ug)	1035.9	86.3		86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	602.5	51.4										
Incub Time		0 min		10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	14.4	0.0										
Final Incubate Vol (uL)	720	60										
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
		3 x 15		4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.12. Reaction details of MBQ (3.0 mM) with Lysozyme (0.10 mM)

0619 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, MBQ	122.12	1.00	122.12	in 10%MeOH/buffer		20	3	720	108			
Actual MBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	102.1			
Stock Lys	0.705		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.705 mM St Lys (uL)	102.1		14.2									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	509.9		85.8									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	108		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.13. Reaction details of MBQ (1.0 mM) with Lysozyme (0.10 mM)

0619 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, MBQ	122.12	1.00	122.12	in 10%MeOH/buffer		20	1	720	36			
Actual MBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	102.1			
Stock Lys	0.705		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.705 mM St Lys (uL)	102.1		14.2									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	581.9		85.8									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	36		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.14. Reaction details of MBQ (0.50 mM) with Lysozyme (0.10 mM)

0619 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
20 mM/50ml stock, MBQ	122.12	0.25	30.53	in 10%MeOH/buffer		5	0.5	720	72			
Actual MBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	102.1			
Stock Lys	0.705		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.705 mM St Lys (uL)	102.1		14.2									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	545.9		85.8									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	72		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Table B.15. Reaction details of MBQ (0.10 mM) with Lysozyme (0.10 mM)

0619 2013	FW	mmol	mg	solution		[Q]i	[Q]f	Incub Vol	Q vol			
5 mM/50ml stock, MBQ	122.12	0.25	30.53	in 10%MeOH/buffer		5	0.1	720	14.4			
Actual MBQ												
(uL)Final Lys->0.1 mM			mg	ml				Lys vol	102.1			
Stock Lys	0.705		20	2								
Freezing time												
	Total	MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
0.705 mM St Lys (uL)	102.1		14.2									
Protein (ug)	1035.9		143.9	86.3	86.3	86.3	86.3	86.3	86.3	86.3	86.3	
Phosphate buffer (uL)	603.5		85.8									
Incub Time			0 min	10 min	30 min	1h	2h	3h	4h	5h	24h	
Incub Temp	37°C											
20 mM Q stock, [Q]f 3 mM	14.4		0.0									
Final Incubate Vol (uL)	720		100									
Aliquot Taken (uL)				60	60	60	60	60	60	60	60	Tot FIN vol
			4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	4 x 15	360.0
LANE		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	
		MM	Ctr 24h	10	30	1h	2h	3h	4h	5h	24h	
Loading Dye		0	10	10	10	10	10	10	10	10	10	
Incub Aliquot of Total		0	15	15	15	15	15	15	15	15	15	
Loading Vol (uL)		15	10	10	10	10	10	10	10	10	10	
Protein loaded (ug)			9	9	9	9	9	9	9	9	9	

Acknowledgements

Department:

UTC Department of Chemistry

Thank you for all of the time, patience, knowledge, and lessons (both in and out of the classroom). My time here has been invaluable and shaped me as scholar and chemist for life.

Individuals:

Advisor — Dr. Jisook Kim

Thank you for your patience with me throughout my time working for you. This would have never happened without your guidance and your faith in me. I wish you the best with your family and all of your new students!

Fellow Researcher: Michelle Smith

DHON Examiner: Dr. Titus Albu

DHON Examiner: Dr. John P. Lee

DHON Liason: Dr. Timothy Gaudin

Funding:

Grote Chemistry Fund

Provost Student Research Award

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