# USE OF PULSE PROTEOLYSIS TO INVESTIGATE THE STABILITY DETERMINANTS OF THE MUMPS NUCLEOCAPSID-BINDING DOMAIN

By

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In Partial Fulfillment of the Bachelors degree
With Honors in

Biochemistry

THE UNIVERSITY OF ARIZONA

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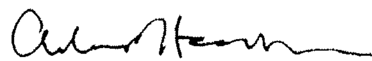
Approved by:



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## Abstract:

The mumps Nucleocapsid-Binding Domain (NBD) protein is a small protein that mediates attachment of the viral polymerase to the nucleocapsid. Mumps NBD lacks tertiary structure in isolation; however, it folds into a specific three-dimensional structure when interacting with the N protein. The lack of structure is thought to weaken this binding, facilitating forward motion of the polymerase. Since the closely related NBD from measles is extremely stable without binding, we hypothesize that the mumps NBD may have accumulated mutations that reduce its stability. The mumps NBD is an attractive protein to study the determinants of stability due to the latent capacity to be stabilized.

One approach to investigating stability determinants is to create multiple variants, and screen for those which have decreased proteolytic susceptibility indicating increased stability. In this work, we investigated the applicability of pulse proteolysis to screen variants of the mumps NBD. The mumps NBD was mutated using Error Prone PCR. Three mutated mumps NBD variants were then compared to the wild-type in order to determine information about the relative proteolytic susceptibility of the mutants. The use of different concentrations of urea during the proteolysis was investigated as a means to increase the observed differences between variants.

## Introduction:

The Mumps and Measles viruses are members of the paramyxovirus virus which cause illnesses in humans and animals. Paramyxoviruses use a multisubunit replication complex, containing an RNA-dependent RNA polymerase, in order to carry out transcription and replication of their genetic material. The paramyxovirus replication complex utilizes an adaptor protein to mediate attachment between the viral polymerase and the nucleoprotein complex containing the viral genome. The attachment point is a small helical bundle domain, the Nucleocapsid-Binding Domain (NBD), which acts as a tether for the polymerase during replication. The Mumps NBD protein is 49 residues long and has a molecular weight of 11,346 kDa[1]. The NBD proteins of various paramyxoviruses have been found to be very similar in amino acid sequence and have been found to have very similar triple helix bundles during crystallization. However, in previous experiments, results have shown that the Mumps NBD is found to be partially disordered in solution, lacking a tertiary structure and mainly in a molten globular state, while the Measles NBD is very stable in solution and in a tight three alpha-helical bundle (like that seen of both domains during crystallization as seen in *Figures 1, 2 and 3*). When the Mumps NBD is in solution it is disordered but becomes ordered once bound to its binding site on the nucleocapsid. When the Mumps NBD protein is bound to the Nucleocapsid-Binding Site, it has a similar structure to the Measles NBD protein regardless of whether or not the Measles NBD.

*Figure 1: Three Dimensional Structure of the Nucleocapsid-binding domain from the mumps virus polymerase*

*Figure* 1 shows a representation of the three alpha-helical bundle that is formed by the Mumps Nucleocapsid-Binding Domain of the polymerase through crystallization under highly specific stabilizing conditions. These conditions are not the same conditions that the mumps virus is in during the normal lifecycle. However, it can be seen in this figure (as well as in *Figure 2*) that there is a tertiary structure that can be induced under certain conditions used for crystallization and therefore the protein has the potential to either be structured or unstructured. The tertiary structure represented in this figure is believed to be similar, if not the same, to the structure of the Mumps Nucleocapsid-Binding Domain when bound to the Nucleocapsid-Binding Site.

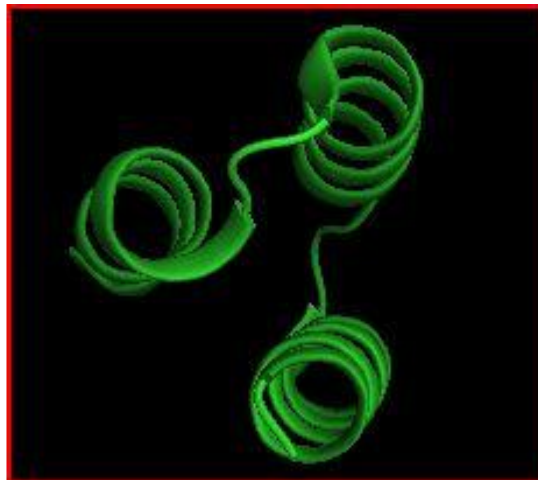


*Figure 2: Alternative view of the three Dimensional Structure of the Nucleocapsid-binding domain from the mumps virus polymerase.*

This is an alternative view of the same protein seen in *Figure 1*. Again, the protein is stable in the three alpha-helical bundle.

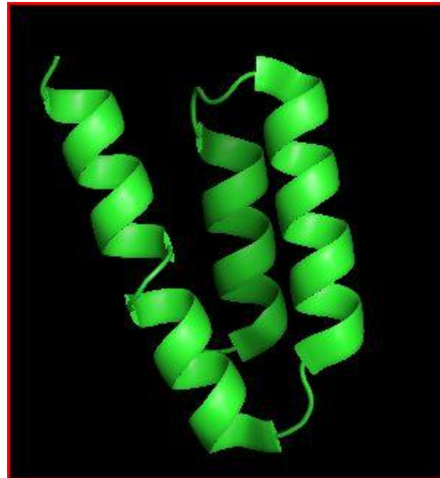


*Figure 3: Three Dimensional Structure of the Nucleocapsid-binding domain from the measles virus polymerase.*

Like, the Mumps NBD structure seen in *Figure 1* and *Figure 2*, the Measles Nucleocapsid-Binding Domain can be seen to have a three alpha-helical bundle. From previous research, it has been determined that the measles Nucleocapsid-Binding Domain is commonly in this tertiary structure regardless of whether it is bound to the Nucleocapsid-Binding Site or not. It is from this structure that it has been determined that despite the similar tertiary structures and the sequence similarities of the Mumps and Measles NBD proteins, the proteins are significantly different when compared with respect to stability.

The objectives of the experiments that were carried out and that are described in this paper were to design a practical test for analyzing the stability of the NBD proteins and to produce and categorize mutants of the Mumps NBD proteins based on their respective stabilities. Because the mumps and the measles Nucleocapsid-Binding Domain proteins have been found to have significantly different properties in isolation, but have very similar sequences and structures, they provide a good system for studying the determinants of structural specificity. In particular, the mumps protein appears to be destabilized relative e to the measles protein, suggesting that it may be possible to restore some stability to it. Finding mutations that stabilize the mumps protein may reveal strategies for engineering other proteins.

On way to find stabilizing mutations is to make a collection of variants of the mumps protein and screen them for alterations in stability. To do so, a procedure is needed to quantify the level of stability for the molecules. By producing a method that can give relative stabilities, mutated NBD proteins can

then be compared to each other as well as the wild type protein. Having a technique for measuring the relative stabilities will aid in understanding what aspects of the protein structure gives rise to stabilization or destabilization. The approach taken in this work is to develop the method of pulse proteolysis for use with the mumps NBD (Park and Marqusee). In this method, proteins are exposed to protease for a brief time, and then the amount of remaining protein provides a simple measure of structural stability, as resistance to proteolysis is conferred by structural stability. This method is also useful since it can be applied to proteins in cell lysate, without need to purify each variant.

Error-prone Polymerase Chain Reaction (PCR) was used to induce random mutagenesis in the genetic sequence that coded for the Mumps NBD protein in order to produce several different mutants of the Mumps NBD protein. Pulse proteolysis with different concentrations of Urea was used to affect the stability of the proteins that were produced from the mutated genes from the Error-Prone PCR products as well as the wild-type Mumps Nucleocapsid-Binding Domain. Then, the destabilized proteins were subjected to the presence of Thermolysin. Thermolysin is a protease that cleaves and degrades proteins based on relatively non-specific hydrophobic amino acids and will degrade the protein more when there is less tertiary structure present. Theoretically, the higher the concentration of Urea present, the less tertiary structure there is on the protein. Therefore, there should be more cleavage due to Thermolysin after the protein sample is allowed to equilibrate with a high concentration of Urea. SDS-PAGE gels are then used to separate out the protein from the other materials in the mixture (namely Thermolysin and Urea) since the mumps NBD protein has a significantly different molecular weight than the other molecules present. A MATLAB code was produced and used to analyze the gels produced during the SDS-PAGE in order to have specific data that could be compared to calibration curves as well as the data for the other concentrations of Urea in order to give a quantitative measure of stability (or lack of stability) for the mutated Mumps NBD protein variants compared to the wild-type mumps NBD. Theoretically there should be a larger change in the ratio of the band intensities (with

Thermolysin present/without Thermolysin present) after equilibrated with a higher Urea concentration since the presence of Urea destabilizes the alpha-helical bundle and favors the molten globular form of the protein which is more susceptible to cleave by Thermolysin.

Ideally, at the end of these experiments, multiple mutants will be produced and sequenced. These mutants would be analyzed for stability and once multiple mutants are analyzed, they could be compared to the original structure of the Mumps NBD protein (and the stable Measles NBD protein) so that a range of stabilities could be compared. If these mutants can be produced for a range of stabilities, their structures could be analyzed so that protein stability as a whole could be better understood. These experiments would ideally lead to a more general understanding of protein stability. Eventually, the method produced by this experiment could be used to analyze the role of specific residues on tertiary structure and the effect of the environment on larger proteins in order to understand protein stability dynamics in general.

## Methods:

### Error-Prone PCR

This technique is used to create different DNA sequences that have been mutated slightly from the original DNA sequence coding for the Mumps virus NBD protein. 50 µL of each of the pre-made primers for both the upper strand and the lower strand were added to 100µL of 10X Error-Prone PCR buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 70 mM $MgCl_2$, 0.1% gelatin) and 100µL DMSO along with 6 µL of 10mM $MnCl_{2.}$ 50 µL of the template plasmid (2ng/µL) and 10 µL of each dCTP(100mM), dTTP (100mM), dATP (20mM) and DGTP (20mM). 10 µL of Taq Polymerase was added and the mixture was brought up to 1000 µL with Molecular Biology grade water. This mixture was used for a20 reaction pool. The pool was separated into 20 different 50µL aliquots and then ran in the PCR machine. After the PCR reaction

was completed the 20 aliquots were removed and mixed together, separated into 20 new aliquots and ran in the PCR machine again. Again, after the second round of the PCR reaction was completed, the 20 aliquots were removed from the machine and mixed together then separated into 20 different aliquots and placed in the PCR machine for a third PCR reaction. After the third round of the PCR reaction, the 20 samples were removed and combined. The PCR product was then gel-purified by using a low-melt agarose gel. Multiple rounds of the PCR reaction were carried out so that there would be a higher rate of mutation because the mutated sequences were subjected to mutation multiple times.

**Ligation-Independent Cloning**

This technique is necessary in order to clone the PCR product into petHSUL. 30 µL of the gel-purified PCR product were mixed with 5 µL 10 NEB buffer 2, 0.5 µL 100mM dGTP, 0.5 µL 100X BSA, 13.5 µL DNAse free $H_20$ and 1 µLt4 DNA polymerase. The T4 DNA polymerase treatment was allowed to be carried out and then the T4 polymerase was deactivated by heating. 1 µL of the LIC-ready petHSUL vector and 1 µL of the T4DNA polymerase treated insert were mixed and allowed to anneal. Equal volume of 10 mM EDTA was combined with the insert and vector mixture. 50µL of thawed DH5α cells were added to the mixture and transformed then plated on LB/amp plates. The cultures were grown overnight at 37°C.The colonies were picked out and allowed to grow up using PCR by adding 2 µL of each culture to the corresponding PCR tube. The PCR products were checked using an agarose gel and then glycerol stocks were made and stored.

**Pulse Proteolysis**

Pulse Proteolysis is used to destabilize the coded mumps so that the stability of the protein can be analyzed in comparison to the original protein and the mutated proteins. Thermolysin is a protease that cleaves and degrades proteins based on relatively non-specific hydrophobic amino acids and will degrade the protein more when there is less tertiary structure present.

BL21/DE3 cells harboring the expression plasmid pAH2018 (mumps NBD/sumo fusion in petHSUL) were grown overnight in 5 mL of SOB broth and 5 µL Amp. 1 mL of the overnight culture was transferred into a new test tube with 15 mL of SOB broth and 15 µL Amp. The culture was allowed to grow in the shaker at 250 rpm at 37°C for 30 minutes. The optical density was checked to be between 0.6 and 0.8 to insure adequate growth. The culture was then induced with 15 µL IPTG for 4 hours in order to produce the production of the Mumps NBD protein. The culture was spun down. A post-induction sample was stored after being resuspended in 33µL of Buffer A (5mM Tris pH 7.0, 0.25 M NaCl, 1mM CaCl). The rest of the culture was spun down and resuspended in 300 µL of Buffer A + 5 M Urea (5mM Tris pH 7.0, 0.25 M NaCl, 1mM CaCl, 5M Urea) and allowed to lyse. Tubes A, B and C were designated and 50 µL of the lysed cells were separated into each the tubes. Buffer A + 5 M Urea was added to make 0.5M, 2.5M and 5.0 M solutions in tubes A, B, and C. The samples were brought to equilibrium by waiting for 60 minutes. The samples were spun down and the separated into tubes A+, A-, B+, B-, C+ and C- with 195 µL of the corresponding sample in each tube. Tubes A+, B+, and C+ had 5µL Thermolysin added, while tubes A-, B-, and C- had 5µL Buffer A added. After 2 minutes, 50 µL of 0.5 EDTA were added to all 6 tubes in order to stop all Thermolysin activity. Samples were flash frozen and stored. The remainder of the lysed cells was used to create the calibration curve samples. Seven tubes S1, S2, S3, S4, S5, S6, and S7 were designated and the relative urea concentrations were 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 respectively where S1 contained 30µL of Lysed Cells and 0µL of Buffer A + 5M Urea. All tubes were allowed to come to equilibrium for 1 hour and then stored until the SDS-PAGE gel was run.

### SDS-PAGE Gel

Two gels were run at the same time in the SDS-PAGE gel apparatus. A 10% separating gel was used and a 4.25% stacking gel was used. The gels were sealed by agarose. 10µL of 5X loading dye was added to each tube containing 30µL of the samples (both calibration samples and proteolysis samples). 30µL of the sample/dye mixture was added into the respective wells. The gels were run for 4 hours at ~15 milliamps.

The gels were removed from the apparatus and stained overnight in the same container. They were then destained the next morning for 24 hours. The gels were photographed and analyzed using MATLAB. When photographed, areas on the gel which had bands (indicating the presence of a molecule of specific molecular weight compared to the protein molecular weight ladder) showed up darker than the regions where molecules were not present.

**MATLAB Analysis**

Each of the gels photographed after the Pulse Proteolysis and the SDS-PAGE Gel, the photographs are analyzed using a MATLAB program. As directed, the program distinguished between separate bands on the SDS-PAGE gel. The program integrated the darkness of band in order to find the relative density when compared to the different concentrations of the sample loaded into the specific lanes.

To begin the analysis, the photograph of the calibration curve gel is uploaded through the program. The number of bands associated with the calibration curve is indicated and the bands are selected. Volume of cells and volume of buffer for each band in the Calibration curve gel is entered into the program. The program then plots the relative density versus volume of cells. Next, the photograph of the pulse proteolysis gel is uploaded through the program. The six different bands are selected (0.5M Urea with Thermolysin, 0.5 M Urea without Thermolysin, 2.5 Urea M with Thermolysin, 2.5 M Urea without Thermolysin, 5.0 M Urea with Thermolysin and 5.0 M Urea without Thermolysin). The value of the relative density of each band is then plotted onto the calibration curve graph. Another graph is also produced by the band – a bar graph relating the relative density of each band with respect to the specific molarity. The program can also produce a bar graph with three values – one bar for each of the molarities from the pulse proteolysis – for the relative change in concentration of protein due to the presence of Thermolysin.

## Results:

The following figures *(Figure 4 through Figure 11)* are the raw data collected from the Pulse Proteolysis. *Figures 4 and 5* depict the calibration curve gel and the Pulse Proteolysis gel for the wild-type Mumps NBD protein. *Figures 6 through 11* are the calibration curve gels and the pulse proteolysis gels for the three mutated mumps NBD protein variants.

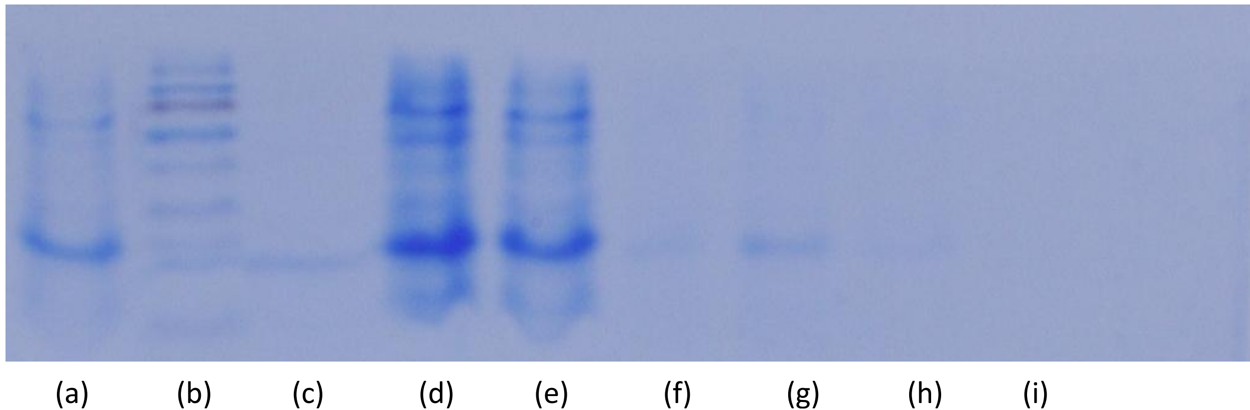



*Figure 4: Wild-type mumps Nucleocapsid Binding Domain protein Calibration Curve SDS-PAGE gel.*
In lane (a), the standard 0.1X load was loaded, which is the same standard 0.1X in *Figure 5* lane (a). Lane (b) is the protein molecular weight ladder. Lane (c) was skipped. Lane (d) was the 1X sample, Lane (e) was the 0.5X sample. Lane (f) was the 0.2X sample. Lane (g) was the 0.1X sample. Lane (h) was the 0.05X sample. Lane (i), which cannot be significantly seen in the picture, was the 0.02X sample. For each of the sample lanes, 10µL of 5X protein loading dye was mixed with 30 µL of the sample and then 30µL of the sample/dye mixture was loaded into the respective SDS-PAGE gel well. MATLAB analysis can be found in *Figure 13.*

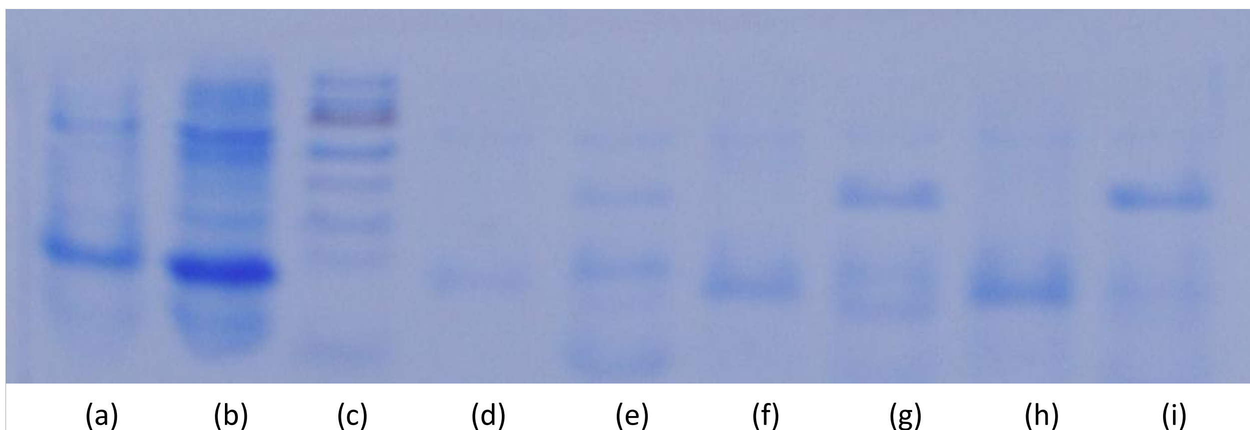
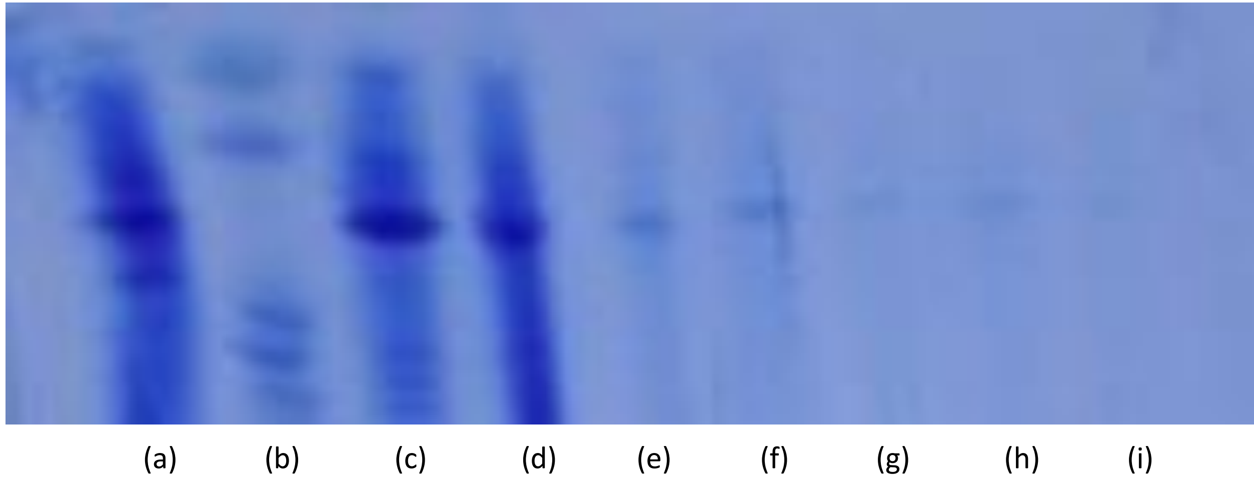



*Figure 5: Wild-type mumps Nucleocapsid Binding Domain protein Pulse Proteolysis SDS-PAGE gel.*

In lane (a) the standard 0.1X load was loaded with is the same standard 0.1X in *Figure 4* lane (a). Lane (b) was the post-induction sample. Lane (c) is the protein molecular weight ladder. Lane (d) was the 0.5M Urea sample without Thermolysin. Lane (e) was the 0.5M Urea sample with Thermolysin. Lane (f) was the 2.5M Urea sample without Thermolysin. Lane (g) was the 2.65M Urea sample with Thermolysin. Lane (h) was the 5.0M Urea concentration without Thermolysin present. And lane (i) was the 5.0 M uea concentration with Thermolysin. Each sample SDS-PAGE gel lane was loaded with 30µL of 75% sample/ 25% 5X protein loading dye. MATLAB analysis can be found in *Figure 12.*



(a) (b) (c) (d) (e) (f) (g) (h) (i)

*Figure 6: First mutated mumps Nucleocapsid Binding Domain protein variant Calibration Curve SDS-PAGE gel.*

In lane (a), the standard 1X load was loaded, which is the same standard 1X in *Figure 7* lane (a). Lane (b) is the protein molecular weight ladder. Lane (c) was the 1X sample, Lane (d) was the 0.5X sample. Lane (e) was the 0.2X sample. Lane (f) was the 0.1X sample. Lane (g) was the 0.05X sample. Lane (h) was the 0.02X sample. Lane (i), which cannot be significantly seen in the picture, was the 0.01X sample. For each of the sample lanes, 10µL of 5X protein loading dye was mixed with 30 µL of the sample and then 30µL of the sample/dye mixture was loaded into the respective SDS-PAGE gel well. MATLAB analysis can be found in *Figure 15.*

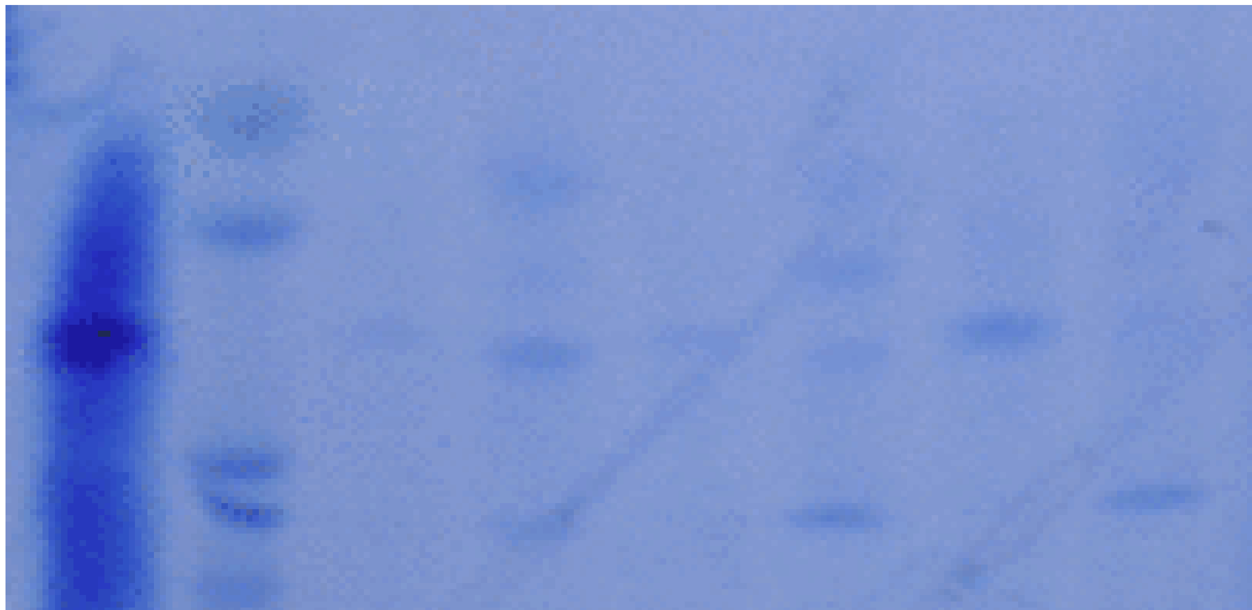



*Figure 7: First mutated mumps Nucleocapsid Binding Domain protein variant Pulse Proteolysis SDS-PAGE gel.*

In lane (a) the standard 1X load was loaded with is the same standard 1X in *Figure 6* lane (a). Lane (b) is the protein molecular weight ladder. Lane (c) was the 5.0M Urea sample with Thermolysin. Lane (d) was the 5.0M Urea sample without Thermolysin. Lane (e) was the 2.5M Urea sample with Thermolysin. Lane (f) was the 2.5M Urea sample without Thermolysin, Lane (g) was the 0.5M Urea concentration with Thermolysin present. And lane (h) was the 0.5 M uea concentration without Thermolysin. Each sample SDS-PAGE gel lane was loaded with 30µL of 75% sample/ 25%5X protein loading dye. MATLAB analysis can be found in *Figure 14.*

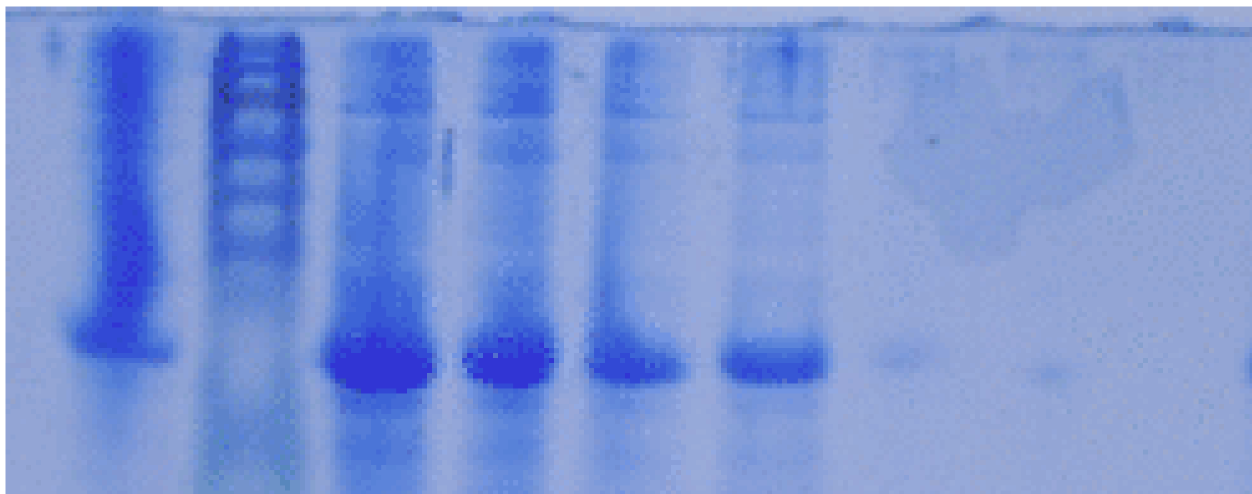



*Figure 8: Second mutated mumps Nucleocapsid Binding Domain protein variant Calibration Curve SDS-PAGE gel.*

In lane (a), the standard 0.1X load was loaded, which is the same standard 0.1X in *Figure 9* lane (a). Lane (b) is the protein molecular weight ladder. Lane (c) was the 1X sample. Lane (d) was the 0.5X sample. Lane (e) was the 0.2X sample. Lane (f) was the 0.1X sample. Lane (g) was the 0.05X sample. Lane (h) was the 0.02X sample. Lane (i), which cannot be significantly seen in the picture, was the 0.01X sample. For each of the sample lanes, 10µL of 5X protein loading dye was mixed with 30 µL of the sample and then 30µL of the sample/dye mixture was loaded into the respective SDS-PAGE gel well. MATLAB analysis can be found in *Figure 17.*

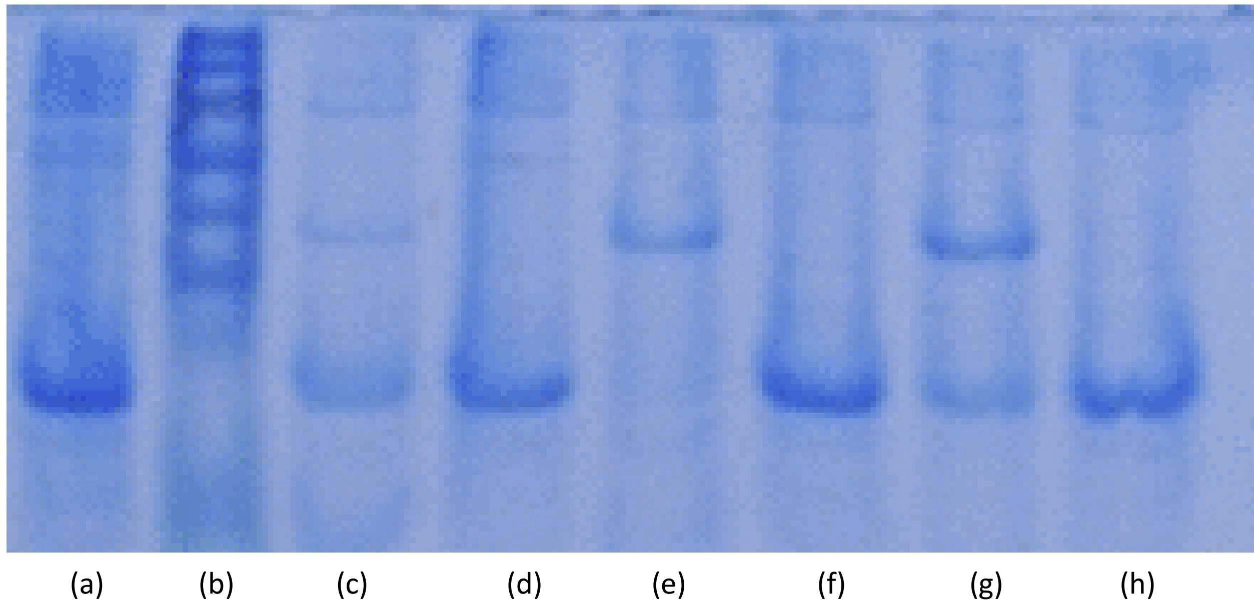



*Figure 9: Second mutated mumps Nucleocapsid Binding Domain protein variant Pulse Proteolysis SDS-PAGE gel.*

In lane (a) the standard 0.1X load was loaded with is the same standard 0.1X in *Figure 8* lane (a). Lane (b) is the protein molecular weight ladder. Lane (c) was the 0.5M Urea sample with Thermolysin. Lane (d) was the 0.5M Urea sample without Thermolysin. Lane (e) was the 2.5M Urea sample with Thermolysin. Lane (f) was the 2.65M Urea sample without Thermolysin. Lane (g) was the 5.0M Urea concentration with Thermolysin present. And lane (h) was the 5.0 M uea concentration without Thermolysin. Each sample SDS-PAGE gel lane was loaded with 30µL of 75% sample/ 25% 5X protein loading dye. MATLAB analysis can be found in *Figure 16.*

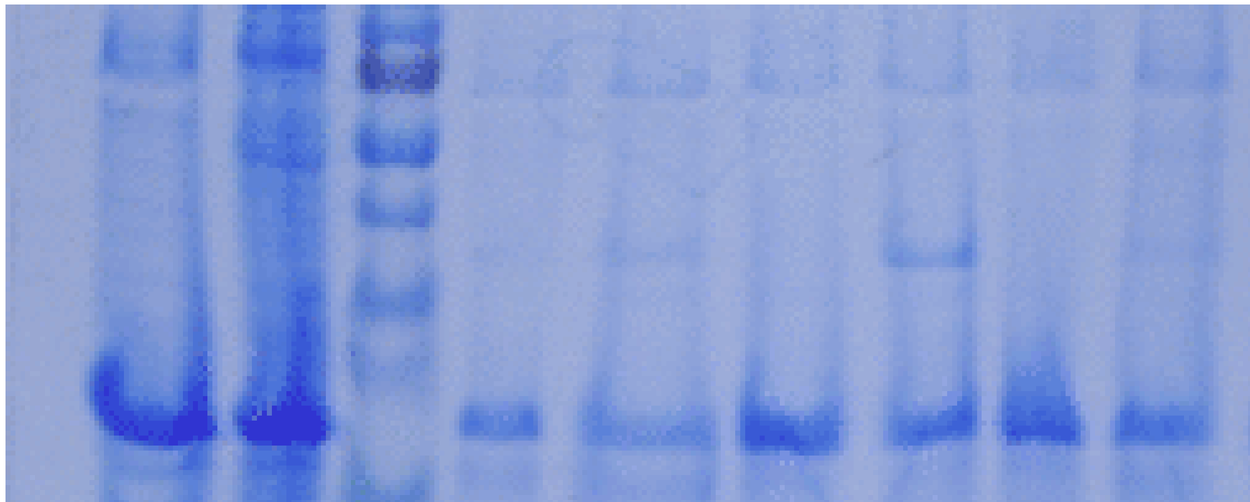



*Figure 10: Third mutated mumps Nucleocapsid Binding Domain protein variant Calibration Curve SDS-PAGE gel.*

In lane (a), the standard 1X load was loaded, which is the same standard 1X in *Figure 11* lane (a). Lane (b) is the post-induction sample. Lane (c) is the protein molecular weight ladder. Lane (d) was the 0.02X sample. Lane (e) was the 0.05X sample. Lane (f) was the 0.1X sample. Lane (g) was the 0.2X sample. Lane (h) was the 0.5X sample. Lane (i) was the 1X sample. Lane (i) was the 0.01X sample. For each of the sample lanes, 10µL of 5X protein loading dye was mixed with 30 µL of the sample and then 30µL of the sample/dye mixture was loaded into the respective SDS-PAGE gel well. MATLAB analysis can be found in *Figure 19.*

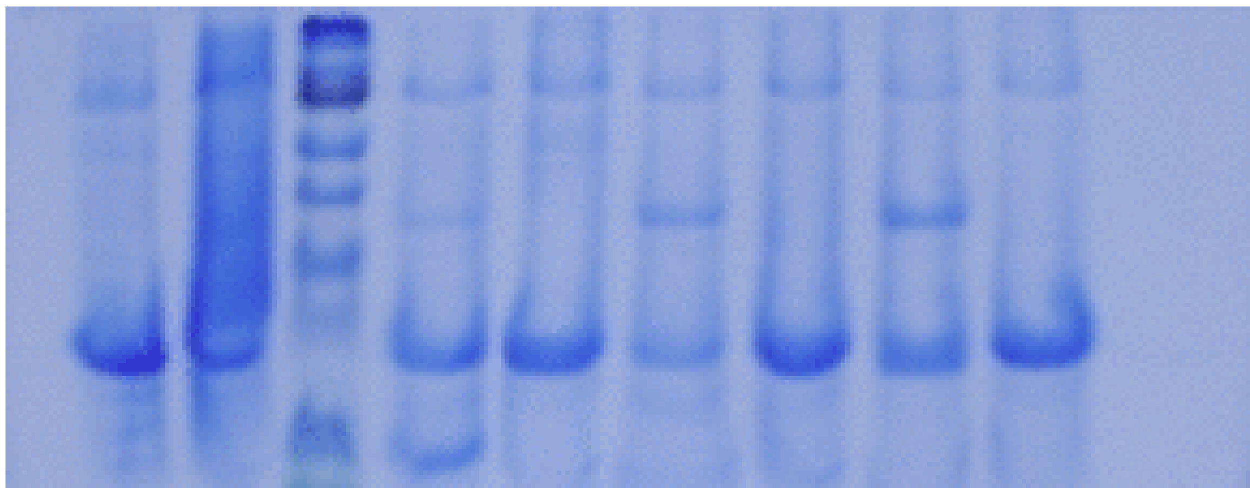



*Figure 11: Third mutated mumps Nucleocapsid Binding Domain protein variant Pulse Proteolysis SDS-PAGE gel.*

In lane (a) the standard 1X load was loaded with is the same standard 1X in *Figure 10* lane (a). Lane (b) is the post-induction sampele. Lane (c) is the protein molecular weight ladder. Lane (d) was the 0.5M Urea sample with Thermolysin. Lane (e) was the 0.5M Urea sample without Thermolysin. Lane (f) was the

2.5M Urea sample with Thermolysin. Lane (g) was the 2.65M Urea sample without Thermolysin. Lane (h) was the 5.0M Urea concentration with Thermolysin present. And lane (i) was the 5.0 M uea concentration without Thermolysin. Each sample SDS-PAGE gel lane was loaded with 30µL of 75% sample/ 25% 5X protein loading dye. MATLAB analysis can be found in *Figure 18.*

The wild-type mumps NBD protein underwent pulse proteolysis. When subjected to 0.5 M Urea without Thermolysin, the intensity of the band on the SDS-PAGE gel was 1908; however when subjected to 0.5 M Urea and Thermolysin for 2 minutes, the intensity of the band on the SDS-PAGE gel increased to 3531. The sample subjected to 2.5 M Urea but not Thermolysin yielded a band with an intensity of 4759 and when Thermolysin was present the intensity was 4283. For the last set of bands, when the protein was equilibrated with 5.0 M Urea, the band has an intensity of 9316 while the band with Thermolysin had an intensity of 2998. This data is represented in *Figure 12a.* In *Figure 12b*, it can be seen that when the Mumps NBD wild-type protein was equilibrated with 0.5 M Urea, the concentration of the protein after cleavage by Thermolysin was nearly 1.85 times larger than the original concentration without Thermolysin. When subjected to 2.5 M Urea and Thermolysin, the concentration was only 0.90 the concentration without Thermolysin. When in conditions of 5.0 M Urea and Thermolysin, the concentration was 0.32 times the concentration compared to when the Thermolysin was not present.

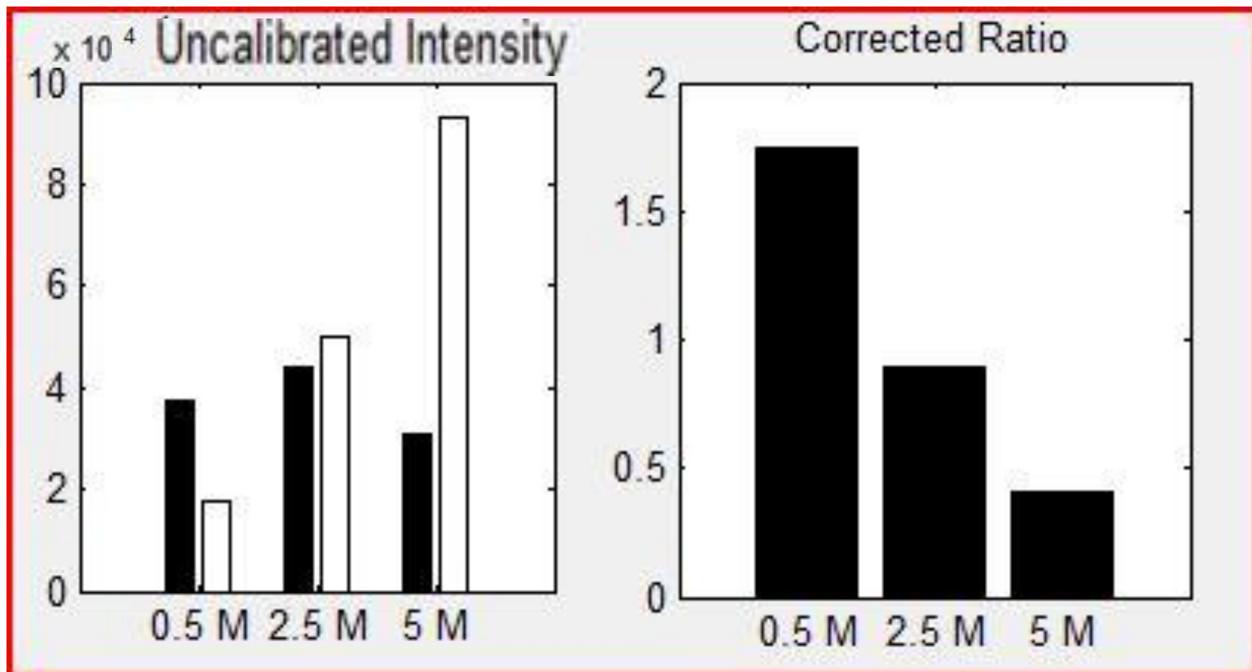



*Figure 12: Uncalibrated Band Intensities with respect to Urea Concentration and Thermolysin Presence after Pulse Proteolysis and separation by SDS-PAGE and the corrected Ratio with respect to Urea Concentration for bands with Thermolysin divided by the correlated band without Thermolysin for the wild-type mumps Nucleocapsid-Binding Domain.*

(a) In the graph on the left of *Figure 12*, the uncalibrated band intensities are given. The bars that are shaded in represent the sample that had been lysed with Thermolysin while the unfilled bars represent the band intensities for the samples that did not have Thermolysin present. These values were calculated by integrating the total density of protein dye in the specific band. The MATLAB code integrated the density and removed the density due to the overall background density to result in the relative intensities for each selected band of the SDS-PAGE gel. Each band on the SDS-PAGE gel was from a different lane. The wells leading to each lane were filled with 30 µL of a 75% sample/25% protein dye mixture and then allowed to run for approximately 90 minutes.

(b) In the graph on the right of *Figure 12,* the ratios of the band intensities represented in *Figure 12a* are seen. The ratios were calculated by dividing the band intensity of the sample with Thermolysin by the band intensity for the sample without Thermolysin. It is evident that as the concentration of the Urea present in the equilibrated state directly correlates to the relative change in protein concentration. The higher the concentration of the Urea, the smaller the ratio and therefore the lower the amount of mumps NBD present after lysing.

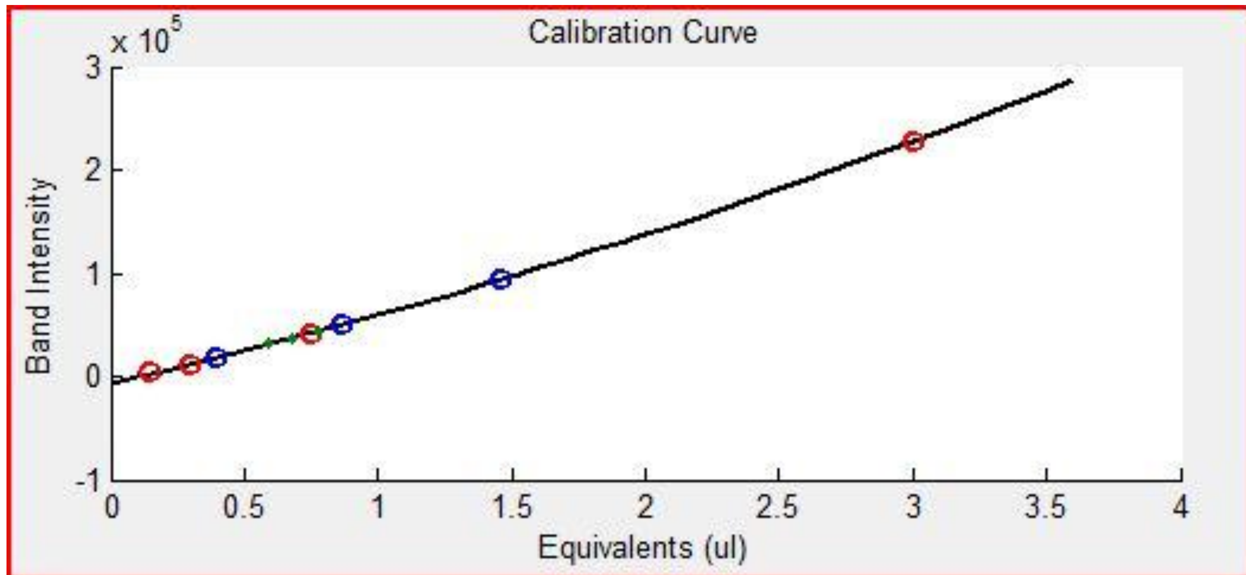



*Figure 13: Calibration Curve from the Calibration SDS-PAGE gel along with the data points from the Pulse Proteolysis Gel.*

The red open circles indicate the values of the band intensities from the Calibration SDS-PAGE gel. The blue open circles represent the band intensities from the Pulse Proteolysis SDS-PAGE gel that correlate to the samples that did not have Thermolysin present while the green dots refer to the band intensities for the samples that did have Thermolysin. The values for the data represented by the green dots and the blue open circles are the same values from the graph in *Figure 12a*. Equivalents of the specific bands were entered into the program and then associated with specific selected bands. Each band area was integrated to determine the intensity (after removing the density attributed to the overall background of the SDS-PAGE gel). The black curve was generated by the MATLAB code and was determined by the data presented by the Calibration curve, not the data from the Pulse Proteolysis.

The following figures, *Figure 14 through Figure 19*, are representative of the Calibration Curves and the Pulse Proteolysis data from the three Mumps Nucleocapsid-Binding Domain variants produced from the Error-Prone PCR technique. Each of the Mumps NBD variants underwent the same Pulse Proteolysis as the wild-type NBD. Using the MATLAB code, the band intensities for all of the samples were calculated and then compared to the calibration curve gels that were produced without Pulse Proteolysis.

The first mutant that was used yielded results which aligned with what was theoretically anticipated. The band intensity of the sample without Thermolysin in the 0.5 M Urea was 984 and the band intensity of the sample after treated with Thermolysin in the 0.5 M Urea was 421. This indicates a

concentration of the Mumps NBD protein that was about 0.43 times smaller than without the Thermolysin. For the 2.5 M samples, the band intensity without Thermolysin was 1130 and with Thermolysin was 244. The concentration decreased by a factor of 0.22 due to the presence of Thermolysin. The band intensities for the samples equilibrated with the 5.0 M Urea were 1057 without Thermolysin and 190 without Thermolysin which was a change in concentration by a factor of 0.18 due to the presence of Thermolysin at such high Urea concentration. These results suggest that the Thermolysin was most affective when the sample was first subjected to the 5.0 M Urea.

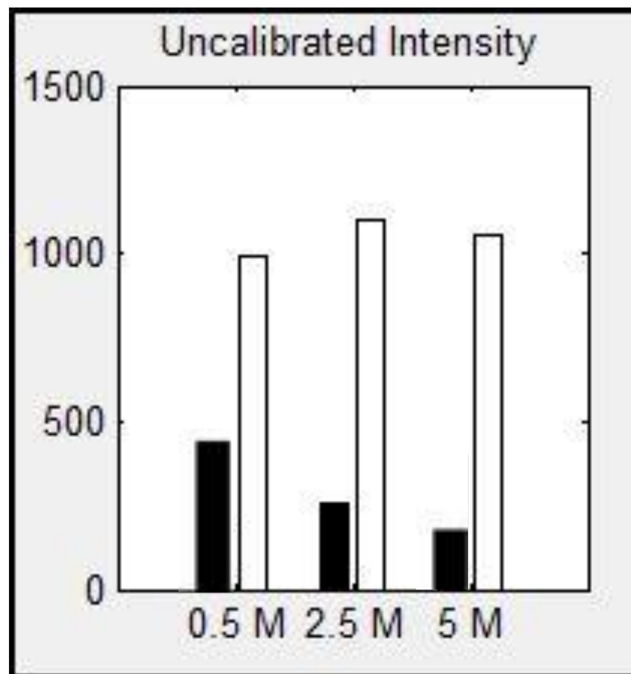



*Figure 14: Uncalibrated Band Intensities with respect to Urea Concentration and Thermolysin Presence for the first mutated Mumps Nucleocapsid-Binding Domain variant after Pulse Proteolysis and separation by SDS-PAGE.*

The bars that are shaded in represent the sample that had been lysed with Thermolysin while the unfilled bars represent the band intensities for the samples that did not have Thermolysin present. It is evident that the concentrations of the protein without Thermolysin regardless of the urea concentration is reasonably consistent while the concentration of the protein, as determined by the level of band intensity, has decreased due to the presence of Thermolysin. Also, it can be seen that the presence of the Thermolysin decreases the concentration of the mumps NBD Protein more at higher concentrations of Urea than at lower concentrations. These values were calculated by integrating the total density of protein dye in the specific band. Each band on the SDS-PAGE gel was from a different lane. The wells leading to each lane were filled with 30 µL of a 75% sample/25% protein dye mixture and then allowed

to run for approximately 90 minutes. The MATLAB code integrated the density and removed the density due to the overall background density to result in the relative intensities for each selected band of the SDS-PAGE gel.

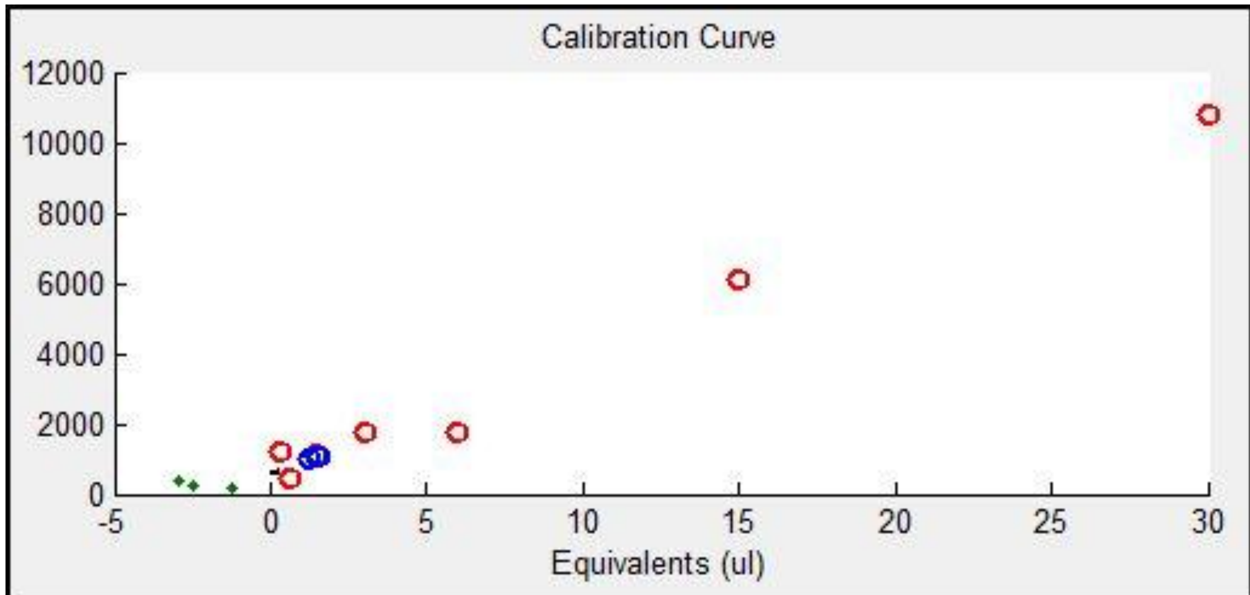



*Figure 15: Calibration Curve from the Calibration SDS-PAGE gel along with the data points from the Pulse Proteolysis Gel for the first mutated mumps Nucleocapsid-Binding Domain variant*
The red open circles indicate the values of the band intensities from the Calibration SDS-PAGE gel. The blue open circles represent the band intensities from the Pulse Proteolysis SDS-PAGE gel that correlate to the samples that did not have Thermolysin present while the green dots refer to the band intensities for the samples that did have Thermolysin. The values for the data represented by the green dots and the blue open circles are the same values from the graph in *Figure 14* and are relative to the amount of protein present in each of the bands of the calibration curve gel. According to the MATLAB coding, the values of the band intensities after subjected to Thermolysin relate to negative values of the volume of the mumps NBD protein. This however, is with respect and cannot be accepted. There was not a specific calibration curve equation that could be generated from the data on the calibration curve gel.

The second mutation made in the mumps Nucleocapsid-Binding Domain was subjected to Pulse Proteolysis and compared to the calibration curve SDS-PAGE gel to yield the data depicted in *Figure 16* and *Figure 17*. The band densities of the samples equilibrated with 0.5 M Urea are 8005 without Thermolysin and 5817 after being in the presence of activated Thermolysin for 2 minutes. This relates to a decrease in the protein concentration by a factor of 0.72 due to the presence of Thermolysin. For the 2.5 M Urea samples, the band intensities were 7644 without Thermolysin and 2107 with Thermolysin.

The ratio of the band intensity from the sample with Thermolysin to the sample without Thermolysin was 0.27. Without Thermolysin in the 5.0 M Urea, the band intensity was 8601 and with Thermolysin in the 5.0 M Urea, the band intensity representative of the sample was 4034. This is a decrease by 0.47 in protein concentration. It can be seen from these results that the sample that was affected the most by the presence of the Thermolysin was the sample that was equilibrated with the 2.5 M Urea.

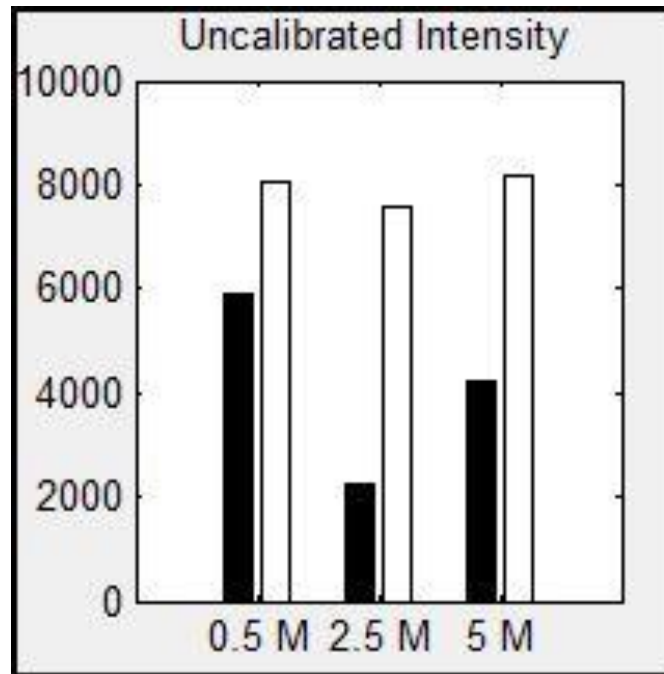



*Figure 16: Uncalibrated Band Intensities with respect to Urea Concentration and Thermolysin Presence for the second mutated Mumps Nucleocapsid-Binding Domain variant after Pulse Proteolysis and separation by SDS-PAGE.*

The bars that are shaded in represent the sample that had been lysed with Thermolysin while the unfilled bars represent the band intensities for the samples that did not have Thermolysin present. It is evident that the concentrations of the protein without Thermolysin regardless of the urea concentration is reasonably consistent while the concentration of the protein, as determined by the level of band intensity, has decreased due to the presence of Thermolysin. The largest decrease in Mumps NBD concentration is seen when Thermolysin is present in the 2.5 M Urea sample whereas the smallest decrease in protein concentration is found in the sample with only 0.5 M urea. These values were calculated by integrating the total density of protein dye in the specific band. Each band on the SDS-PAGE gel was from a different lane. The wells leading to each lane were filled with 30 µL of a 75% sample/25% protein dye mixture and then allowed to run for approximately 90 minutes. The MATLAB code integrated the density and removed the density due to the overall background density to result in the relative intensities for each selected band of the SDS-PAGE gel.

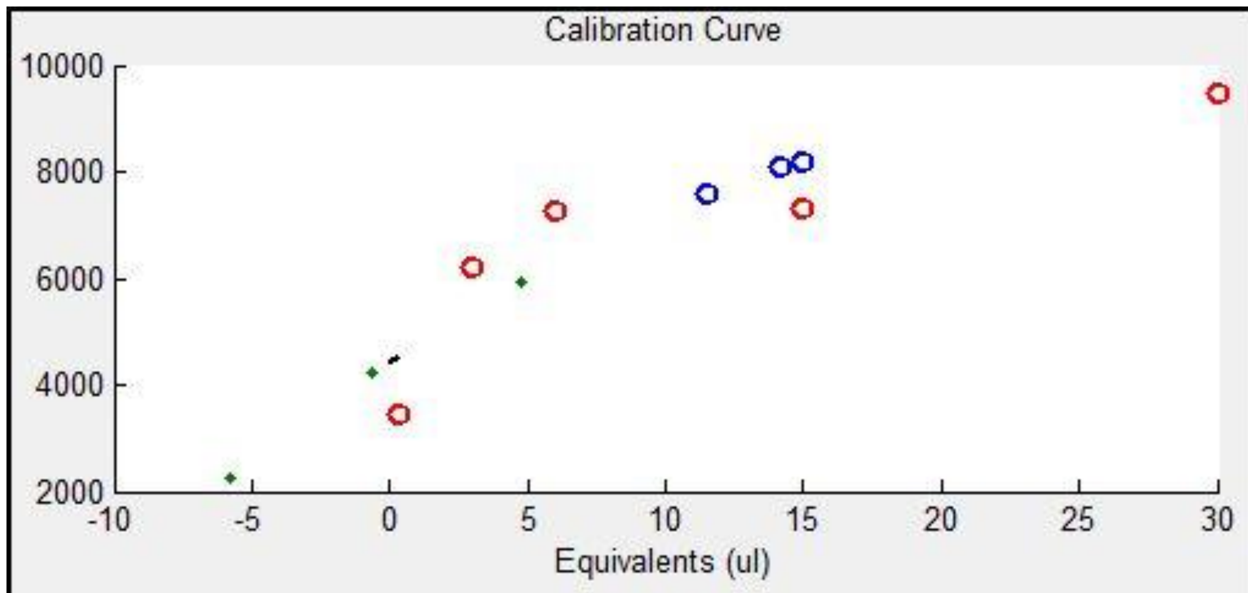



*Figure 17: Calibration Curve from the Calibration SDS-PAGE gel along with the data points from the Pulse Proteolysis Gel for the second mutated mumps Nucleocapsid-Binding Domain variant*

The red open circles indicate the values of the band intensities from the Calibration SDS-PAGE gel. The blue open circles represent the band intensities from the Pulse Proteolysis SDS-PAGE gel that correlate to the samples that did not have Thermolysin present while the green dots refer to the band intensities for the samples that did have Thermolysin. The values for the data represented by the green dots and the blue open circles are the same values from the graph in *Figure 16* and are relative to the amount of protein present in each of the bands of the calibration curve gel. MATLAB calculated that the equivalents of the mumps NBD protein needed to produce the band intensities found with Thermolysin present are negative. This will be assessed later and cannot be accepted. An equation for the calibration curve could not be generated for this data; however, the pulse proteolysis data can be compared to the relative changes in protein concentration.

For the third mutated mumps Nucleocapsid-Binding Domain, the band intensity for the sample in the 0.5 M Urea without Thermolysin was 11206 and with the Thermolysin was 8981. This gives a relative change in concentration of 0.80. For the samples equilibrated with the 2.5 M Urea, the band intensity for the sample without Thermolysin present was 12593 and the band intensity for the sample with Thermolysin present for 2 minutes was 6329. This data indicates that the concentration of the mutant mumps NBD decreased by a factor of 0.50 due to the presence of the Thermolysin. The band intensity without Thermolysin and in 5.0 M Urea was 11477 and with Thermolysin was 9862 which is a

decrease with a factor of 0.86. It can be seen that the sample that was most negatively affected by the presence of the Thermolysin was the mutant that was equilibrated with the 2.5 M Urea.

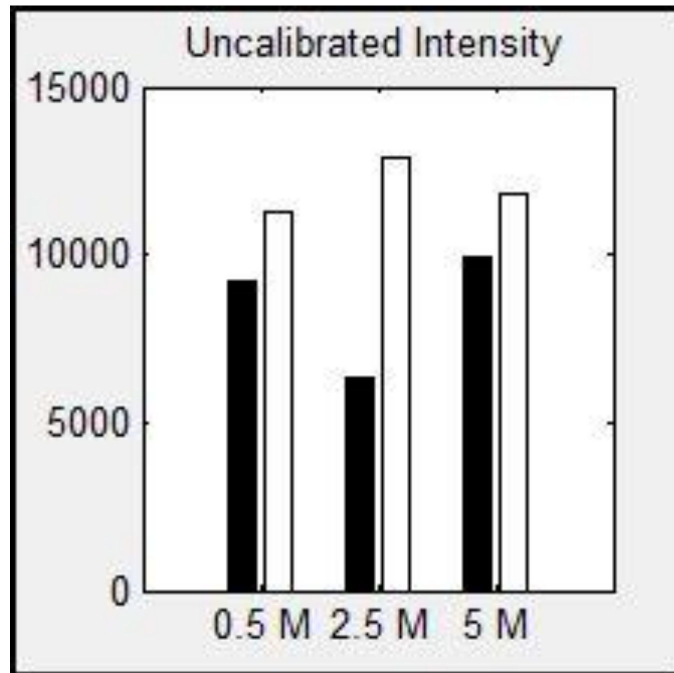



*Figure 18: Uncalibrated Band Intensities with respect to Urea Concentration and Thermolysin Presence for the third mutated Mumps Nucleocapsid-Binding Domain variant after Pulse Proteolysis and separation by SDS-PAGE.*

The bars that are shaded in represent the sample that had been lysed with Thermolysin while the unfilled bars represent the band intensities for the samples that did not have Thermolysin present. It is evident that the concentrations of the protein without Thermolysin regardless of the urea concentration is reasonably consistent while the concentration of the protein, as determined by the level of band intensity, has decreased due to the presence of Thermolysin. Thermolysin affected the sample that equilibrated with 2.5 M Urea the most while the least affected was the sample that was equilibrated with the 5.0 M Urea. These values were calculated by integrating the total density of protein dye in the specific band. Each band on the SDS-PAGE gel was from a different lane. The wells leading to each lane were filled with 30 µL of a 75% sample/25% protein dye mixture and then allowed to run for approximately 90 minutes. The MATLAB code integrated the density and removed the density due to the overall background density to result in the relative intensities for each selected band of the SDS-PAGE gel.

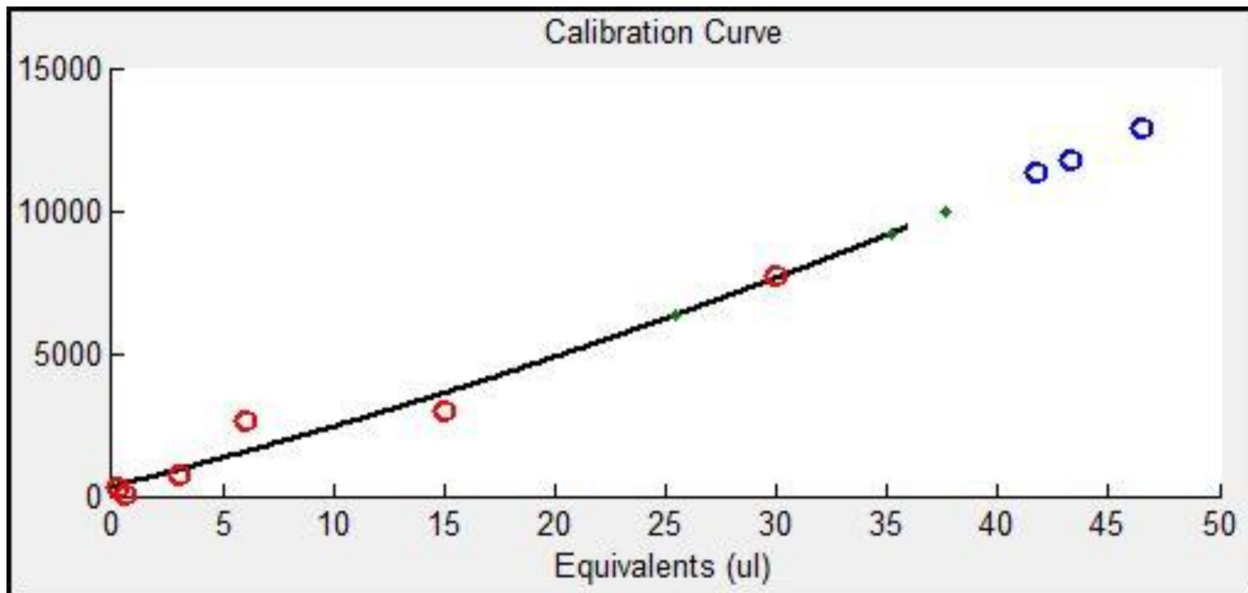



*Figure 19: Calibration Curve from the Calibration SDS-PAGE gel along with the data points from the Pulse Proteolysis Gel for the third mutated mumps Nucleocapsid-Binding Domain variant*
The red open circles indicate the values of the band intensities from the Calibration SDS-PAGE gel. The blue open circles represent the band intensities from the Pulse Proteolysis SDS-PAGE gel that correlate to the samples that did not have Thermolysin present while the green dots refer to the band intensities for the samples that did have Thermolysin. The values for the data represented by the green dots and the blue open circles are the same values from the graph in *Figure 18* and are relative to the amount of protein present in each of the bands of the calibration curve.

## Discussion:

The Error-Prone Polymerase Chain Reaction used in this experiment was slightly altered from the common technique used for an Error-Prone Polymerase Chain Reaction. The genetic code for the Mumps NBD protein is relatively short and thus has to be handled carefully. The commonly used error-prone PCR method did not provide enough mutations in the genetic sequence in the mumps NBD protein to be effective for the overall objective of this experiment. Therefore, the procedure was slightly altered by increasing the concentration of Manganese and repeated three times. This allowed for more mutations to occur in the first round and then in the subsequent rounds of PCR, there were more mutations added to the previously mutated genetic sequences. This produced genetic sequences with

only 1 to 4 mutations that could be placed into expression lines and then utilized for the remainder of the overall experiment. Once the mutated genetic sequences were developed and placed in bacterial expression lines, the genetic sequences were “turned on” and expressed allowing the production of these mutated mumps NBD protein variants to be produced and then isolated.

The presence of Urea was expected to cause destabilization in the mumps NBD protein. When a protein is destabilized, the level of tertiary protein structure is decreased. The decrease in tertiary structure theoretically allows Thermolysin to cleave more off of the proteins. When the sample is then analyzed using an SDS-PAGE gel, the band that represents the sample that had contained Thermolysin should decrease in intensity compared to the band that represents the sample under the same conditions except without the presence of Thermolysin. This is because as the Thermolysin cleaves the protein, the parts of the protein will run through the gel faster than the whole protein. The lane with the sample including the Thermolysin should have a lighter band at the molecular weight of the mumps NBD protein but will also have a band for the Thermolysin present with a molecular weight significantly greater than the mumps NBD protein band.

The wild-type mumps Nucleocapsid-Binding Domain, when subjected to Pulse Proteolysis was most stable when equilibrated with 0.5 M Urea and least stable in the 5.0 M Urea, as expected. It was found, however, that the concentration of the wild-type protein increased due to the presence of the Thermolysin in the 0.5 M Urea sample. This result was not expected and indicates that some additional work is needed in making the method reproducible, reliable and quantitative. The increase in the concentration of the protein as the concentration of Urea increased is indicative of an error. Since the assay was set up to have to same concentration of the mumps NBD protein at the start of the pulse proteolysis something in the methods were wrong. Although this method was attempted multiple times none of the raw data gels turned out exactly as expected theoretically. On the other hand, the overall trend in the change in the ratio of the concentration of the protein in the Thermolysin present sample

compared to the concentration of the protein without Thermolysin is the trend that was expected. The higher the concentration of Urea, the larger the decrease in concentration was due to Thermolysin. This finding signifies that the increase in Urea concentration lead to less tertiary structure allowing Thermolysin to cleave more of the wild-type mumps NBD protein.

Through the course of the work done in this experiment, there were three different mutated Mumps NBD proteins that were produced and analyzed. These three Mumps NBD variants underwent the same Pulse proteolysis and analysis that the wild type Mumps NBD protein underwent. The genetic sequence variations have not been sequenced yet. Once sequenced, this information could lead to more possible analysis.

The first mutated mumps NBD protein variant, like the wild-type, was destabilized as the concentration of Urea increased. In fact, as seen in *Figure 6* even at a concentration of 0.5 M Urea, the concentration of protein decreased by a factor of 0.43. At a concentration of 2.5 M Urea the ratio was down to 0.22 and then for the 5.0 M urea sample the ratio was 0.18. This suggests that the mutation that was caused by the Error-Prone PCR was destabilizing because it made the protein more susceptible to cleavage by Thermolysin. One possible mutation (but certainly not the only mutation) that might have caused this change in stability is the change of a hydrophilic residue to a hydrophobic residue that has a neutral $pK_a$ and is destabilizing at most basic conditions. When compared to the calibration curve produced for the first mutated mumps NBD protein variant, it can be seen that there were very low amounts of the protein without the Thermolysin present and even lower amounts once the Thermolysin was added. However, the equivalents suggested by the graph produced by the MATLAB coding in deceiving as it suggests that there are less than 0 µL of protein in the samples with the Thermolysin. This strongly indicates that the MATLAB code is not accurate but the trends in the ratios of concentrations of protein with Thermolysin present compared to protein concentration without Thermolysin can still be analyzed for a general understanding of the affect the mutation had on the stability.

On the other hand, the second mutated mumps NBD protein variant decreased in concentration due to the Thermolysin in the 0.5M Urea by a factor of 0.72, decreased by a factor of 0.27 in the 2.5 M Urea and only by 0.47 in the 5.0 M Urea. The third mutated mumps NBD protein variant decreased in concentration due to the Thermolysin in the 0.5 M Urea by a factor of 0.80, decreased by a factor of 0.50 in the 2.5 M Urea and by 0.86 in the 5.0 M Urea. Both of these mutated mumps NBD protein variants were most destabilized in the 2.5 M Urea. In the second mutated variant, the most stable environment was in the 0.5 M Urea but the third mutated variant was most stable in the 5.0 M Urea. This suggests that the 5.0 M concentration of Urea actually caused the mutated variants to be stabilized. This indicates that the mutations, while they destabilized the mumps NBD protein under mild conditions the mutations become increasingly stable under harsh conditions.

Like the graph produced for the first mutated variant, the graph for the second mutated variant suggests that there are negative equivalents for some of the Pulse Proteolysis samples with Thermolysin present. However, again, it can be seen that there was degradation due to the Thermolysin and the specific equivalents of the protein according to the MATLAB code are not accurate. On the other hand, the MATLAB code suggests a reasonable amount of proteins for each of the samples for the third mumps NBD protein variant.

A mutation that could cause this change in stability is the change of an amino acid from a hydrophobic residue to a residue that has a side chain with a high $pK_a$. Although Thermolysin cleaves at hydrophobic residues, there are multiple other hydrophobic residues that Thermolysin could still cleave at. However, the change of a hydrophobic amino acid to a hydrophilic residue can often destabilize an alpha-helical bundle which would be apparent in the decrease in protein concentration in the 0.5 M Urea and 2.5 M Urea samples. However, since an increase in Urea concentration often leads to an increase of pH, if the mutation caused a hydrophobic residue to change to a hydrophilic residue with a high $pK_a$, the stability of the protein would change as the Urea concentration increased. Consider if the

Leucine residue (residue 27) found near the middle of the second alpha helix for the mumps NBD protein was changed to a Tyrosine due to a genetic alteration in the coding during the Error-Prone PCR technique. As the pH increased in the mutated protein the Tyrosine could become deprotonated and gain a negative charge, which could allow it to interact with the Lysine which in a separate alpha helix but is directly across from the mutated residue. Since this Lysine is only the third residue in the mumps protein, if this Leucine to Tyrosine mutation had occurred it would pull the alpha-helix bundle much closer making the protein less susceptible to degradation by Thermolysin.

This is only an example of how the structure of the protein could strongly affect the stability of the protein under different conditions. However, this experiment did not allow for the genetic sequences of the mutated mumps NBD variants to be determined. In following experiments, it would be beneficial to determine the genetic sequences produced from the Error-Prone PCR in order to determine the residues for the coded protein. Once the residues are determined, the effect of the mutations could be directly correlated to the observed change in stability found in the Pulse Proteolysis analysis.

The Pulse Proteolysis technique developed through these experiments gives a direct way to test relative stabilities. Each of the three mutations has been suggested to be destabilizing under certain conditions, but two of these mutations can be stabilizing at high pH values. The Pulse proteolysis technique does need more work done. This is not a specifically quantitative measurement yet. However, as the MATLAB code and the experimental procedure are perfected, the level of stability analysis will increase. The Calibration curve calculations especially need to be addressed in later work.

Another avenue that needs to be addressed in later work is the relative stabilities of the mumps Nucleocapsid-Binding Domain protein to the Measles Nucleocapsid-Binding Domain protein. Pulse Proteolysis techniques are being developed for the Measles NBD protein currently. This technique however, is significantly different because of the pre-existing stability of the Measles NBD protein in

solution. In this technique, the Thermolysin is left active for almost an hour, while in this experiment, Thermolysin was only used to cleave the mumps NBD protein for 2 minutes. Similar stability analysis can be carried out for the measles NBD Pulse Proteolysis technique; however, the findings cannot yet be directly related because of the difference in Thermolysin presence.

Overall, the experimental procedure that was developed is successful in providing a way to rank the relative stability of the variants of the mumps protein. Most significantly, the ratios of the protein concentration present after treated with Thermolysin compared to the sample without Thermolysin equilibrated at a given Urea concentration. The Pulse Proteolysis method using Thermolysin for 2 minutes was effective and allowed for a difference in protein concentration to be seen. The MATLAB code was useful in finding the relative ratios for the density of the band intensities but still requires more work in order to better correlate the Pulse Proteolysis data to the Calibration Curve gel data. As this method continues to become more refined, the stability analysis will be more effective in comparing the stability of different mutated mumps NBD proteins to the mumps NBD wild-type and eventually the measles NBD wild-type so that a broader range of protein stability can be understood.

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