ABSTRACT

Overexpression and Characterization of Metallo-β-lactamase from *Klebsilla pneumoniae*: NDM-1

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Since bacterial pathogens are able to develop resistance after exposure to antibiotics, antibiotic resistance is a serious problem in medicine. Resistance to antibiotics occurs by the production of β-lactamases, which cleave the β-lactam bond present in common antibiotics such as penicillin. This study focuses on the overexpression and characterization of the New Delhi metallo-β-lactamase enzyme by conducting studies on the dependence of ionic strength, buffer concentration, and pH on the β-lactamase-catalyzed hydrolysis of Penicillin G. Results showed that NDM-1 was successfully purified. The effect of ionic strength on MOPS buffer showed an initial decrease and then an increase in the k_{cat}/K_m, while phosphate buffer showed an increase and then decrease in the k_{cat}/K_m value. In the case of buffer effect, MOPS and phosphate buffer showed a strong decrease in k_{cat}/K_m as buffer concentration increased. The pH studies showed some scattering data points, suggesting that the protein is unable to endure some acid/base conditions.

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OVEREXPRESSION AND CHARACTERIZATION OF METALLO-B-LACTAMASE FROM *KLEBSILLA PNEUMONIAE*: NDM-1

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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Waco, Texas

May 2014

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for allowing me to finish this thesis. I thank Him for the gift of life, health, and the opportunity to participate in research.

It is with my deepest gratitude that I acknowledge the support of my committee chair and thesis advisor, Dr. Sung-Kun Kim, who directed this research project. Dr. Kim helped me to stay motivated and organized, while fostering a spirit of exploration and intellectual curiosity in regards to both research and scholarship. Thank you for your patience and guidance. Without your assistance, this thesis would not have been possible.

I would also like to thank my committee members, Dr. Sung-Joon Kim and Dr. Cheolho Sim for taking time to review my thesis and offer valuable feedback.

Additionally, I am indebted to my many colleagues who supported me. I would also like to extend a special thank you to Nicole Solida, who first introduced me to the biochemistry lab techniques needed for my project. Thank you for your time and guidance through this experience. I would also like to thank Dr. Mieke Lahousse for her immeasurable help in guiding me through biochemical concepts and Sara Schlesinger for her help and support.

I would also like to thank my parents, Mr. Young Hwan Park and Mrs. You Soon Park, for their support and for encouraging me to perform at the best of my ability.

Finally, I would like to thank the rest of my family and friends for their endless support and words of inspiration.

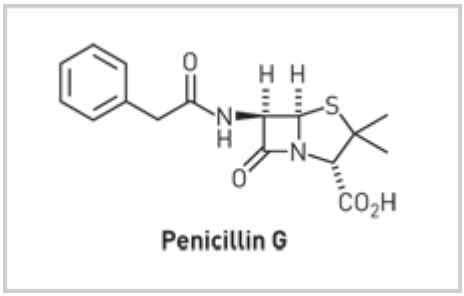
CHAPTER ONE

Introduction

Before the utilization of antibiotics in clinical medicine, even small cuts could easily get infected and prove to be fatal. Alexander Fleming, a microbiologist at St. Mary's Hospital in London, was the first to observe the antibiotic properties of penicillin when he discovered a secretion of mold that actively killed bacteria in 1928 (Henry 2005). Additional experiments by Chain and Florey determined that penicillin could be harvested as a drug to combat bacterial infections. After the discovery and development of penicillin, it was hailed as a miracle drug— a panacea for bacterial infections.

At the University of Oxford in 1945, Dorothy Hodgkin solved the chemical structure of penicillin. Hodgkin's research revealed that penicillin, originally assumed to be an enzyme, was actually a small molecule (Henry 2005). Penicillin is now known to contain a β-lactam group, which functions by preventing the formation of peptidoglycan cross-links in bacterial cell walls (Henry 2005). This disruption in the bacterial cell wall prevents the cell from undergoing division and usually results in cell death. Therefore, the antibiotic works by making the bacteria unable to survive and reproduce. Although this study focuses on penicillin, other β-lactam antibiotics that are structurally similar include cephalosporins, monobactams and carbapenems. The structure of these beta-lactam antibiotics are shown in Figure 1 (a), (b), (c), and (d).

(a)



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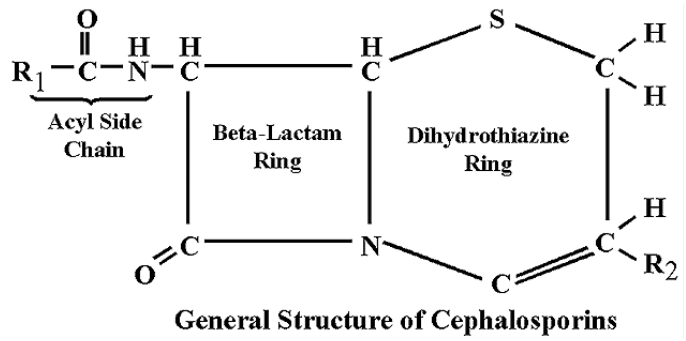

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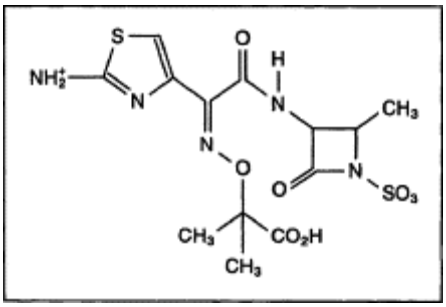

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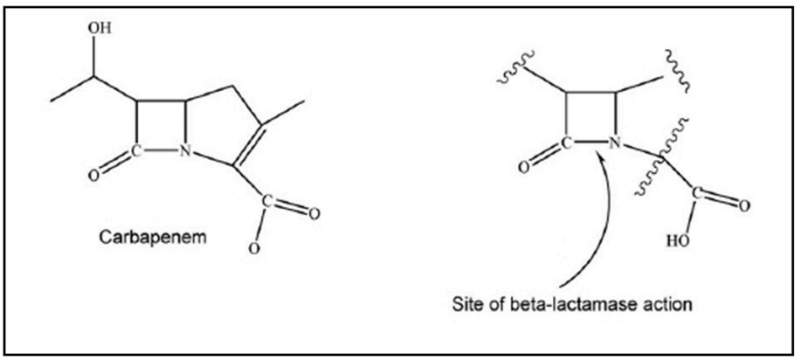

Figure 1. The structures of beta-lactams. (a) Penicillin G (Henry 2005) (b) Cephalosporins (Lindgren 2013) (c) Monobactams, Azetreonam (Hellinger 1999) (d) Carbapenems (Drug 2013)

After his discovery of the uses for penicillin, Fleming predicted that one day in the near future, "penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and, by exposing his microbes to nonlethal quantities of the drug, make them resistant" (Henry, 2005). Alexander Fleming was correct in his predictions, as antibiotic resistance is now one of the most preeminent public health concerns of the 21st century.

Resistance to antibiotics increases when people take sub-lethal quantities or misuse antibiotics for viral infections. The resistance to β-lactam antibiotics arises due to the production of enzymes known as β-lactamases, which hydrolyze the β-lactam bonds and inactivate, in this case, the antibiotic penicillin. Typically, in the case of a bacterial infection, first-line antibiotics are used, which are selected for their safety, availability, and cost. β-lactam antibiotics are often included in this category because they are effective in targeting the necessary infection, easier to synthesize or harvest, are locally available, and less expensive than second or third line antibiotics. In the case of first-line drug-resistant organisms, second or third-line antibiotics are administered. Unfortunately, second-line and third-line drugs are often much more costly, target a broader spectrum, and are often locally unavailable. Because of the rising resistance to first-line antibiotics by bacterial organisms, more costly and less effective drugs need to be developed and administered to meet immediate medical needs.

Resistance is partially attributed to the relative accessibility to antibiotics. Since people have a larger access to antibiotics, many only take sub-lethal quantities, which may only kill a fraction of the bacterial organisms in the body. Some of the remaining

bacteria can have a mutation that codes for resistance to the antibiotic. These organisms multiply and become a daunting force that is resistant to many of the commonly prescribed antibiotics. If another type of treatment, such as a second-line antibiotic, is either unavailable or has not yet been developed, the infection can prove as lethal as it was before the invention of penicillin.

The research on antibiotic resistance has shifted from developing novel antibiotics or modifying current antibiotics to designing drugs to inhibit the mechanism of β-lactamases. Since the resistance to β-lactam antibiotics occurs from the production of β-lactamases, finding a mechanism to inhibit the formation of β-lactamase enzymes would render the β-lactam antibiotics to be effective against bacterial infections.

Many kinds of beta-lactamases have been found worldwide. The beta-lactamases have been categorized to classes A-D: class A, C, and D are serine β-lactamases while class B are metallo-β-lactamases, which require at least one zinc ion at the enzyme active site for catalytic activity (Brebrone 2007). Zhongjle Liang's research reveals the molecular models of NDM-1 and its complex of antibiotics in Figure 2.

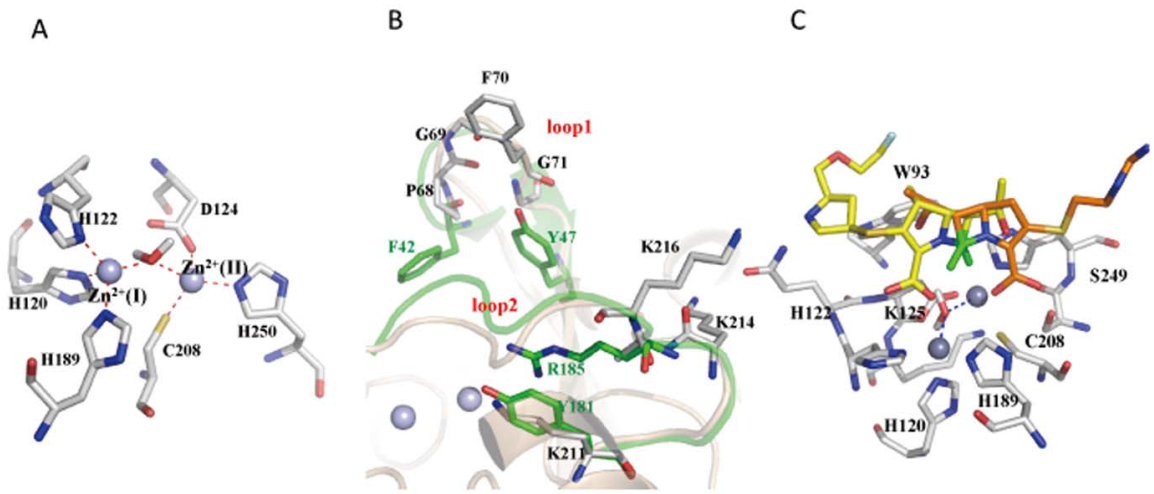

Figure 2. Molecular models of NDM-1 and its complex with antibiotics (Liang 2011) (a) shows two zinc ions in the active site of NDM-1 (b) shows the structure of NDM-1 and VIM-2. The structure of NDM-1 is illustrated in grey and the structure of VIM-2 is illustrated in green. (c) shows the binding mechanism of both imipenem and carbapenems in the active site of NDM-1. Imipenem and carbapenems are shown in orange and yellow, respectively (Liang 2011).

These figures are shown to help illustrate the interaction of NDM-1 with penicillin. Additionally, Figure 2 (b) is a comparison of the loop structure for NDM-1 and VIM-1. Both structures have some overlapping structures, which could potentially predict similarities in how they function mechanistically by having similar chemical characteristics. Correspondingly, they may also have similar mechanisms for inhibition.

New Delhi metallo-β-lactamase (NDM-1) is a relatively new metallo-β-lactamase that is resistant to almost all β-lactam antibiotics. NDM-1 was first detected in a *Klebsiella pneuominae* isolate from a Swedish patient of Indian origin in 2008 (Walsh 2011). Since then, it has been identified in a diverse range of bacteria in India, Pakistan, United Kingdom, United States, Canada and Japan. Because NDM-1 is resistant to the antibiotics of the carbapenem family, a common line of treatment for antibiotic resistant

bacterial infections, it only responds to colistin and tigecyline. The ability of this enzyme to transfer between different bacterial species and the relative ineffectiveness again β-lactam bonds poses a massive threat for public health. To aid in the study of the inhibition of this β-lactamase, a study of the dependence on the ionic strength, buffer concentration, and pH for the β-lactamase catalyzed hydrolysis of Penicillin G was conducted. For reference, the structure of Penicillin G was shown in Figure 1 (a). HongMin Zhang's research discovered the x-ray structures of NDM-1 and other metallo-beta-lactamases, which are included in Figure 3 and Figure 4. Overall the NDM-1 has many overlapping areas with VIM-2 and IMP-1, which may be helpful when determining patterns between different molecule. The slight differences, however, may cause differences in the mechanism of inhibition.

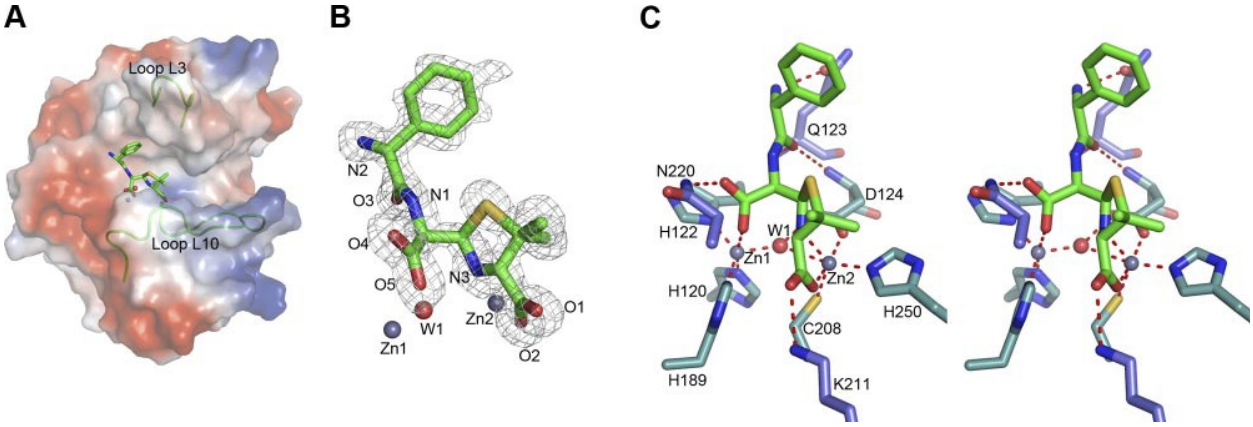

Figure 4. X-ray crystallography of NDM-1 and the active sites. In the stick model, hydroxide ions are shown as red spheres and the zinc ions are shown purple spheres. (a) shows the grooves on the surface of NDM-1. (b) shows a F_o-F_c map. (c) shows all the residues on the active site in a stick model. The blue carbons represent molecules that are attached to zinc ions and purple carbons represent molecules that undergo hydrogen bonding with the ampicillin (Zhang 2005).

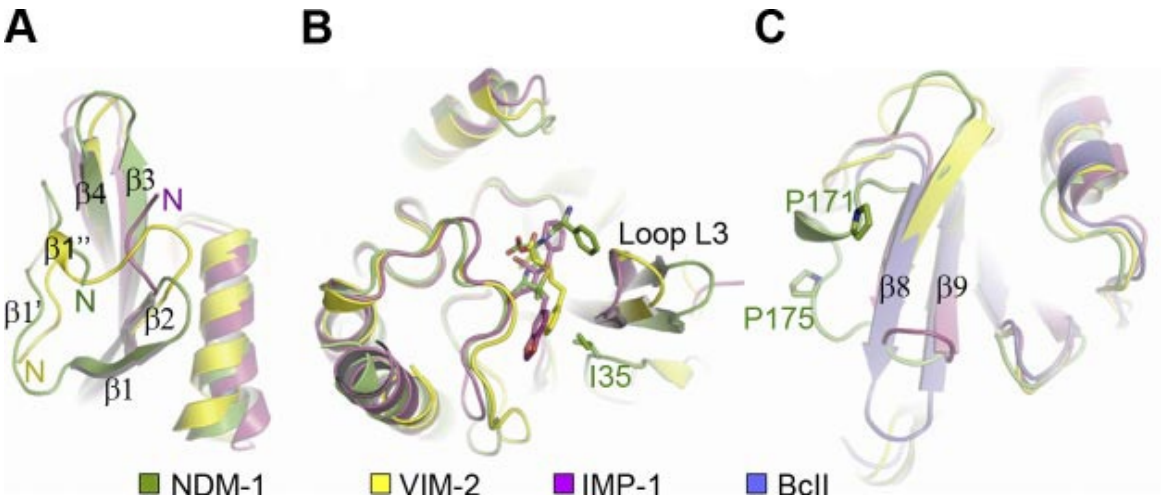

Figure 3. The three dimensional structures of various metallo-beta-lactamases, including NDM-1 by X-ray crystallography, with a ribbon representation of VIM-2, IMP-1, and BcII overlapped onto NDM-1. NDM-1, VIM-2, IMP-1, and BcII are depicted by the colors green, yellow, pink, and blue, respectively. (a) is a representation of the sideways orientation at the N terminus. (b) shows the top view of the 3 (a). Both the inhibitors on VIM-2 and IMP-1 and hydrolyzed ampicillin molecules in NDM-1 are shown. The residue on I35 makes the interior of the loop more hydrophobic when compared to VIM-2 and IMP-1. (c) shows the side view of the overlap at β8 in VIM-2, IMP-1, and BcII. Two proline residues disrupted the β8 strand in NDM-1 (Zhang 2011).

Interestingly, despite the recent emergence of NDM-1, the study of NDM-1 is still not sufficiently extensive. In an effort to characterize the enzyme, the overexpression and purification were performed. After the successful purification, we characterized the enzyme with an emphasis on the ionic strength and buffer effect in order to obtain further information on its kinetic properties. Additionally, pH-dependent studies were conducted to understand the acid/base mechanism of the enzyme.

CHAPTER TWO

Materials and Methods

Expressing the Protein of Interest

The glass tubes containing 10 mL Luria Broth (LB) medium, 10 μL ampicillin, and the desired BL21 (DE3) culture were prepared and allowed to grow overnight at 37°C in the shaker to begin the process of expressing the protein. The overnight culture was poured into the flask containing 1 liter LB medium with 1 mL of ampicillin. It was grown at 37°C in the shaker for 2 to 3 additional hours. Then 1 mL of 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and allowed to grow at 28°C for approximately 8 hours. The cell pellet was collected via centrifugation at 4,750 rpm at 4.0°C for 15 minutes. The cells were stored at -80°C for subsequent experiments.

The addition of IPTG allowed for expression of the protein by mimicking allolactose, a lactose metabolite that activated transcription of the lac operon. The lacZ gene was replaced with the gene of interest, and IPTG was used to induce gene expression. This slowed down growth and also induced a greater expression of proteins and thus a larger expression of enzymes.

French Pressure Cell Press

The pellet was thawed and resuspended in the appropriate buffer. Then French Pressure Cell was performed. The French Pressure Cell press disrupted the cell membrane to isolate the protein by passing it through a narrow valve with high pressure.

Centrifugation

Another step of purification was centrifugation. The ultracentrifugation was performed on the solution, and the supernatant was filtered with a 0.8 μm syringe filter and then a 0.4 μm syringe filter to get the crude extract. Centrifugation works by the sedimentation principle, where applying centripetal acceleration allows the denser substances to separate out to the bottom of the tube. The object rotates around a fixed axis, and the force is perpendicular to the axis, which allows the heavier items to pellet at the bottom. To lessen the denaturing effects from heat by friction, most centrifuges work in refrigeration. The pellet formed from centrifugation was quickly removed from the tube so that the pellet would not be disturbed by pipetting or decanting. The rate of centrifugation was measured in rpm (rate per minute). For ultracentrifugation, the concept was the same; the only difference is that the rate of rotation around the fixed axis is much higher. This higher speed allows a larger majority of the molecules to be in the pellet.

Ni^{2+} -affinity column

The next step of purifying protein was running the filtered supernatant, or crude extract, through Ni^{2+} column. The chromatography column contains nickel beads. The beads allow electron-rich molecules like histidine to bind to the column, since nickel is a transition metal. For this column, the greater amount of histidine on the protein allowed it to bind with more affinity to the nickel beads. The protein was bound to the nickel beads on the nickel column because of the poly-His tag. Additionally, any undesired

protein was removed by extensive washing process. Imidizole was utilized to release the column-bound protein. The desired protein was collected using the fractionator with the appropriate buffers.

Wizard Plus SV Minipreps DNA Purification Systems

Production of Clear Lysate

This procedure was written in numbered format for convenience.

1. Use the centrifuge for 5 minutes to pellet the 10 mL overnight culture.
2. Resuspend the pellet completely with 250 µL of Cell Resuspension Solution.
3. Pour 250 µL of Cell Lysis Solution to each culture.
4. Mix the two solutions by inverting the sample four times.
5. Pour 10 µL of Alkaline Protease Solution to each culture.
6. Mix the solutions by inverting the sample four times.
7. Incubate the cultures at room temperature, around 20 °C, for 5 minutes.
8. Pour 350 µL of Neutralization Solution to each culture.
9. Mix the solutions by inverting the sample four times.
10. Use the centrifuge for 10 minutes at the highest speed.

Blending of Plasmid DNA

11. To blend the plasmid DNA, place the spin column inside the collection tube.
12. Pour off the clear lysate into the spin column.
13. Use the centrifuge for one minute at the highest speed.

14. Discard what has passed through the spin column, and replace the column into the collection tube.

Washing

15. Mix with 750 µL of Wash Solution.
16. Use the centrifuge for one minute at the highest speed.
17. Discard what has passed through the spin column, and replace the column into the collection tube.
18. Repeat Step 15 with an additional 250 µL of Wash Solution.
19. Use the centrifuge for one minute at the highest speed.

Elution

20. Pour the remaining solution in the Spin Column to a sterile 1.5 mL microcentrifuge tube.
21. Do not pour any leftover Wash Solution into microcentrifuge tube. If there is any remaining Wash Solution in the Spin Column, centrifuge the Spin Column for one minute at the highest speed and then pour the remaining solution in the Spin Column to a sterile 1.5 mL microcentrifuge tube.
22. Pour 100 µL of Nuclease-Free Water into the Spin Column.
23. Use the centrifuge for one minute at the highest speed.
24. Remove the column.
25. Freeze the remaining, eluted DNA at -20°C or below for storage.

Bacterial strain and plasmid

For cloning and expression, E. coli strain BL21-DE3 was used. The gene coding for NDM-1 from *K. pneumoniae* was cloned into pET100D using the Champion™ pET Directional TOPO® Expression Kit from Invitrogen, with the first 24 amino acids removed due to the presence of a signal peptide.

Bacterial Transformation

This procedure was written in numbered format for convenience.

1. 10 mL of autoclaved LB medium was transferred into a 15 mL sterile culture tube.
2. 10 µL of E. coli BL21-DE3 was added to LB media and grown for 18 hours in a benchtop shaker at 37°C and 200 rpm.
3. 1 mL of the overnight culture was added to 30 mL of LB medium in an autoclaved 150 ML Erlenmeyer flask.
4. The flask was shaken for 2 hours at 2000 rpm at 37°C.
5. Inoculation stopped once the optical density measured at 600 nm was between 0.2 – 0.5 absorbance units.
6. The solution was poured into a 50 mL conical tube that was centrifuged for 10 minutes at 4°C and 3000 rpm.
7. After centrifuging, the supernatant was discarded and the pellet was suspended with 30mL of ice cold 80 mM $MgCl_2$ and 20 mM $CaCl_2$.
8. The pellet was centrifuged again for 10 minutes at 4°C and 3000 rpm.

9. The supernatant was dumped and the pellet was resuspended with 2 mL of ice cold 100 mM $CaCl_2$.
10. The 200 µL aliquots were placed into 1.5 mL microcentrifuge tubes and were stored at -80°C.
11. 1.2% agar plates were prepared by transferring 1.2 g of Bacto-Agar into 100 mL of LB medium. The solution was autoclaved using the preset liquid setting.
12. After autoclaving, the solution was allowed to cool to approximately 55°C before adding 100 mg/mL ampicillin. Equal amounts of agar solution were poured into 5 Petri dishes.
13. The rim of the flask was sterilized prior to each pour by running the flask through the open flame of a Bunsen burner.
14. The gel was allowed to sit for 15 to 20 minutes, or until solidified.
15. The bottom of the Petri dish was labeled with the date and the antibiotic name, and the dish was wrapped in parafilm to seal it.
16. The plates were stored upside down in the refrigerator.
17. Before plating cells, the gels were placed in an incubator for at least 30 minutes to bring up to temperature.
18. One of the 1.5 mL microcentrifuge tubes with the competence cells was obtained.
19. Then 1.0 µL of 100ng/µL plasmid were added to the competent cells and placed on an ice bath for 30 minutes.
20. For the heat shock, the Isotemp was set up at 42°C and the tubes with the competent cells were heated for exactly 90 seconds after the ice bath.

21. The tubes were placed in the ice bath for 2 minutes.
22. 800 µL of LB medium were added, and the tube was incubated at 37 °C at 100 rpm for 65 minutes.
23. The tube was centrifuged at 6000 rpm for 4 minutes.
24. The supernatant was decanted, and the cells were resuspended with the remaining 100 µL of solution.
25. 100 µL of solution was plated on the agar plate.
26. After plating, the plate was placed in an incubator, upside down for 12 hours.

Overexpression, purification, assay

This procedure was written in numbered format for convenience.

1. Inoculation of the clone began with a single colony picked from the agar plate with a sterilized pipet tip and transferred to a 10 mL culture tube with 10 mL of autoclaved LB medium and 10 µL 50mg/mL ampicillin.
2. The culture was placed in a desktop shaker at 37 °C and 215 rpm for 16 hours.
3. The E. coli BL21 (DE3) cells harboring pET100/NDM-1 plasmid were grown at 37° C in 1 L of LB medium with 1 mL of 50 mg/mL.
4. The culture was grown until OD_{600} 0.5 or approximately 4 hours.
5. When the culture is greater that OD_{600} of 0.5, 1 mL of isopropyl β-D-1-thiogalactopyranodisde (IPTG) was added to the culture, and the temperature was turned down to 26°C.
6. The culture was then grown for another 15 hours.

7. The culture was split into 4 Beckman centrifuge bottles.
8. The bottles were centrifuged at 4750 rpm for 20 minutes.
9. The cells were collected and resuspended in 5 mL of Buffer A (30 mM Tris/HCl (pH 8.0) and 100 mM NaCl and were lysed by passing them through a French press four times at 12,000 psi.
10. The cell lysate was centrifuged at 20,000 rpm for 20 min at 4 °C and the supernatant was filtered with 0.8 and 0.45 filters (Millipore).
11. The protein was loaded onto a Ni^{2+} affinity column previously equilibrated with resuspension buffer (Buffer A).
12. The column was washed with 3% 30 mM Tris/HCl (pH 8.0), 100 mM NaCl and 500 mM imidazole (Buffer B) for 60 minutes followed by protein elution with a gradient of 3-50% Buffer B over 60 minutes.
13. The protein eluted with 100 mM imidazole and collected fractions were concentrated using a Macrosep® centrifugal filter while exchanging the buffer with Buffer A to remove imidazole from the protein sample.
14. The concentrators were centrifuged at 4000 rpm for 20 minutes. After the first centrifugation, the filter was loaded with Buffer A and centrifuged again. This was repeated three times.
15. After three buffer exchange steps, 100% glycerol was added to make the final concentration 10% glycerol, and the protein was stored at -20 °C.
16. The concentration of the protein was measured using a Bradford assay.

17. The protein was also checked for purity with a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V. The gel was stained with Coomassie Brilliant Blue and was viewed under an ultraviolet (UV) transiluminator.

Dependence on ionic strength for beta-lactamase catalyzed hydrolysis of Penicillin G

This procedure was written in numbered format for convenience.

1. This experiment was conducted to determine the dependence on the second order rate constant k_{cat}/K_m for the beta-lactamase catalyzed hydrolysis of penicillin G on the ionic strength of the medium, adjusted with NaCl at room temperature, pH 7.0 with MOPS and phosphate buffer at a constant buffer concentration.
2. The ionic strength was calculated from 0.5 CZ for all ionic species present, including the buffer.
3. Ionic strength was measured at 0.2 M, 0.5 M, 1.0 M, and 1.5 M, adjusted by NaCl.
4. The UV machine was first blanked by 1 mL of the MOPS buffer.
5. The samples were prepared by adding 1 mL of MOPS into a sterile quartz cuvette.
6. 1 uL of the enzyme and varying amounts of Penicillin G were added.
7. The solution was mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
8. The sample was immediately loaded into the UV machine and the NDM-1 was assayed for penicillinase activity in 50 mM MOPS (pH 7.0) with varying volumes of penicillin G.

9. This procedure was repeated for the four different ionic strength concentrations for both MOPS and phosphate buffer at an absorbance of 232 nm.

Dependence on buffer concentration of the beta--lactamase catalyzed hydrolysis of Penicillin G

This procedure was written in numbered format for convenience.

1. This procedure was conducted to determine the dependence on buffer strength on the second order rate constant k_{cat}/K_m for the beta-lactamase catalyzed hydrolysis of penicillin G on the ionic strength of the medium, adjusted with NaCl, room temperature, pH 7.0 with MOPS and phosphate buffer at a constant buffer concentration.
2. The ionic strength was kept constant throughout at 1.0 M by varying the concentration of NaCl.
3. Buffer concentration was measured at 0.025 M, 0.05 M, 0.075 M, 0.1 M, 0.15 M, 0.2 M, adjusted by NaCl for both MOPS (pH 7.0) and phosphate buffer (pH 7.0).
4. The UV machine was first blanked by 1 mL of the MOPS buffer.
5. The samples were prepared by first adding 1 mL of MOPS into a sterile quartz cuvette.
6. 1 uL of the enzyme and varying amounts of Penicillin G were added.
7. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
8. The sample was immediately loaded into the UV machine, and the NDM-1 was assayed for penicillinase activity with varying volumes of penicillin G.

9. The steps 1-8 in this procedure were repeated for the six different buffer concentrations MOPS at an absorbance of 232 nm.

10. The procedure was also repeated for phosphate buffer, as shown below.

11. The UV machine was first blanked by 1 mL of the phosphate buffer.

12. The samples were prepared by first adding 1 mL of the phosphate buffer into a sterile quartz cuvette.

13. 1 uL of the enzyme and varying amounts of Penicillin G were added.

14. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.

15. The sample was immediately loaded into the UV machine, and the NDM-1 was assayed for penicillinase activity with varying volumes of penicillin G.

16. The steps 11-14 in this procedure were repeated for the six different buffer concentrations of phosphate buffer at an absorbance of 232 nm.

Dependence on pH for the Beta-lactamase-catalyzed hydrolysis of Penicillin G

This procedure was written in numbered format for convenience.

1. This procedure was conducted to determine the dependence on pH for k_{cat}/K_m for beta-lactamase-catalyzed hydrolysis of Penicillin G at 25 degrees Celsius and constant ionic strength of 1.0 NaCl.
2. The ionic strength was kept constant at 1.0M by varying the concentration of NaCl.
3. The absorbency was measured at pH 6.5, pH 7.0, pH 7.5 for MOPS.

4. The samples were prepared by first adding 1 mL of the MOPS buffer into a sterile quartz cuvette.
5. 1 uL of the enzyme and varying amounts of Penicillin G were added.
6. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
7. The sample was immediately loaded into the UV machine and the NDM-1 was assayed for penicillinase activity in 50mM MOPS (pH 7.0) with varying volumes of penicillin G.

As outlined below, this procedure was repeated for the MES pH 5.0, pH 5.5, pH 6.0 and pH 6.5, TAPS buffer pH 7.5, pH 8.0 and pH 8.5 and Acetate buffer at pH 5.0 and pH 5.5 at an absorbance of 232 nm.

This procedure was written in numbered format for convenience.

1. The absorbency was measured at pH 5.5, pH 6.0, and pH 6.5 for MES.
2. The samples were prepared by first adding 1 mL of the MES buffer into a sterile quartz cuvette.
3. 1 uL of the enzyme and varying amounts of Penicillin G were added.
4. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
5. The sample was immediately loaded into the UV machine, and the NDM-1 was assayed for penicillinase activity in 50mM MES (pH 7.0) with varying volumes of penicillin G.

This procedure was written in numbered format for convenience.

1. The absorbency was measured at pH 7.5, pH 8.0, and pH 8.5 for TAPS.
2. The samples were prepared by first adding 1 mL of the TAPS buffer into a sterile quartz cuvette.
3. 1 uL of the enzyme and varying amounts of Penicillin G were added.
4. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
5. The sample was immediately loaded into the UV machine and the NDM-1 was assayed for penicillinase activity in 50mM TAPS (pH 7.0) with varying volumes of penicillin G.

This procedure was written in numbered format for convenience.

1. The absorbency was measured at pH 5.0 and pH 5.5 for Acetate buffer.
2. The samples were prepared by first adding 1 mL of the Acetate buffer into a sterile quartz cuvette.
3. 1 uL of the enzyme and varying amounts of Penicillin G were added.
4. The solutions were mixed by turning the cuvette upside down three times, with the opening covered by parafilm.
5. The sample was immediately loaded into the UV machine and the NDM-1 was assayed for penicillinase activity in 50mM TAPS (pH 7.0) with varying volumes of penicillin G.

CHAPTER THREE

Results

SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to separate proteins based on size. This was done after the desired protein had been isolated to verify the purity, using the nickel column. SDS is a detergent, which denatures and separates proteins with more than one subunit into individual polypeptides. As SDS denatures the secondary and tertiary structures, this process applies a negative charge to each protein. SDS gives the proteins the same charge to mass ratio, allowing them to be differentiated based purely on mass and not polarity, since all molecules have a uniform negative charge. To check the purity after the purification process, the proteins were loaded into one well at the end of the gel submerged in an appropriate buffer. To conclude the molecular weight of the desired protein, a molecular marker was placed in a well next to our protein and was run in a separate lane of the gel. An electric current was used, causing the proteins, all with a negative charge, to move towards the positive end of the gel. The proteins migrated based on their size. Theoretically, smaller proteins move the farthest from their original location. Since we viewed the proteins on a gel, Coomassie Brilliant Blue R-250, a staining solution, was used to stain the gel. Then, the separated proteins were seen as distinct bands on the gel separated according to molecular weight. Lower percentage gels separate higher molecular weight proteins, and higher percentage gels are more suitable for smaller molecular weight proteins. Thus, we

used 12% polyacrylamide gel to accommodate our target protein, NDM-1, since we expected to view the protein band between 25 and 35 kDa.

Figure 5 shows the results of the purification on 12% SDS-PAGE. Because the gene coding for NDM-1 was cloned into the pET100D vector, if the gene had expressed the reading frame codons correctly, we would have expected the molecular weight of the protein to be 32 kDa. The gel showed one district protein band in lane 2. This result indicated that the purification was successful. The band is located between the 17 kDa and 28 kDa, which can be found by comparing the molecular marker in lane 1 to Figure 6 below. Yong et al (2009) determined the molecular weight of NDM-1 without any extra tag on the protein to be around 28 kDa. Our result on the gel, however, showed the molecular weight is below 28 kDa, which differs from the original expectation to be around 28 kDa. The reason is that when we cloned the gene into an expression vector, the signal sequence of the gene was removed, so the molecular weight of the expressed protein is in fact supposed to be below 28 kDa (communication with Sara Schlesinger). Hence, this result is consistent with the expected molecular weight.

Lane 1 Lane 2

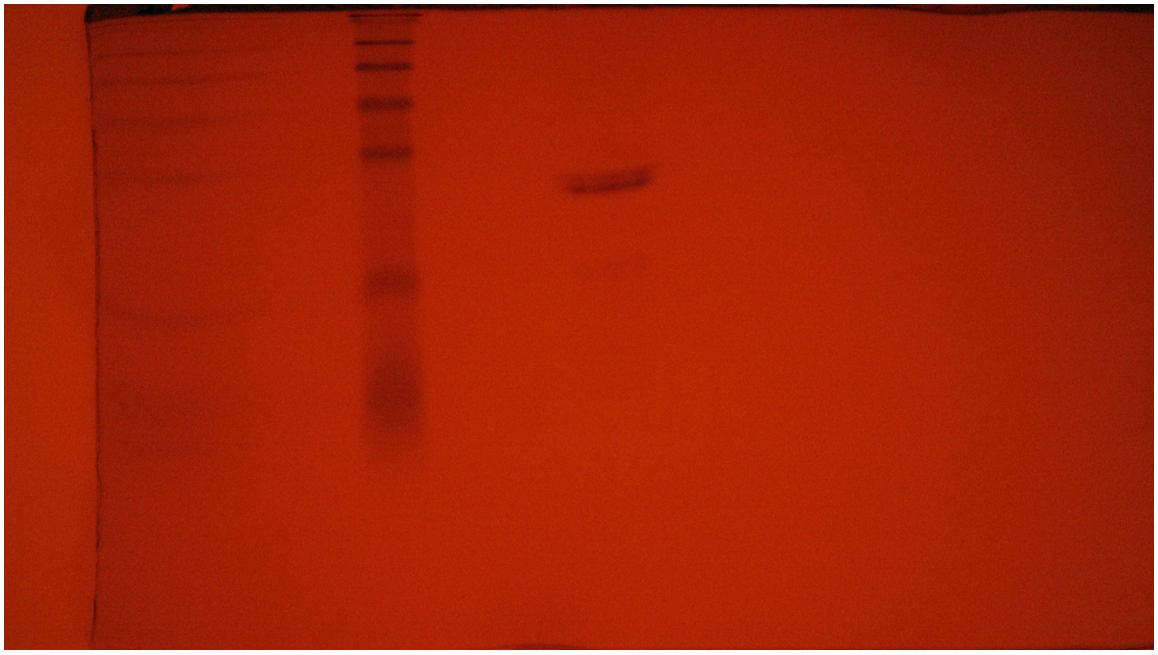

Figure 5. The result of purification. 12% SDS PAGE gel picture. Lane 1. Standard protein marker (see Figure 4b). Lane 2. A sample of NDM-1.

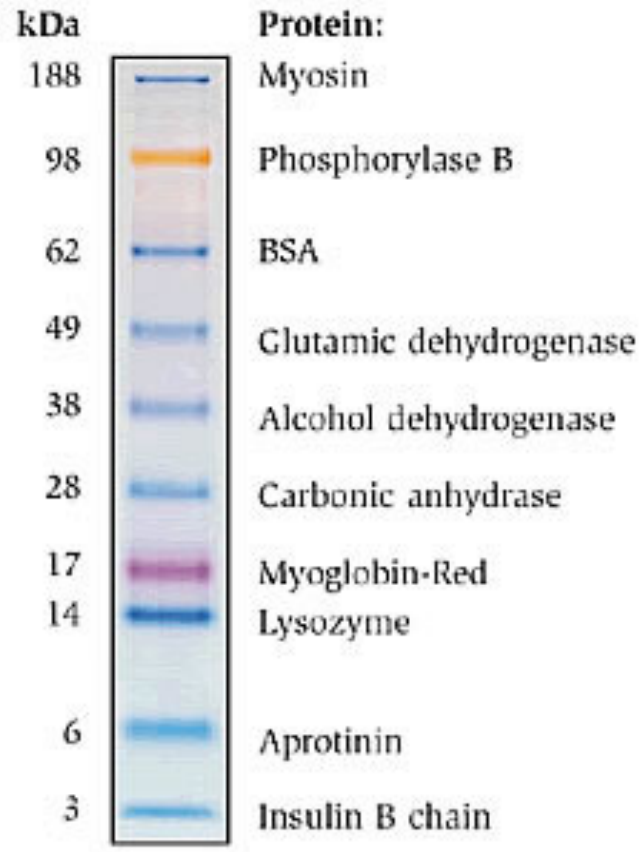

Figure 6. Apparent molecular weights of SEEBlue Plus2 Pre-Stained Standard on a NuPAGE Novel 4-12% Bis-Tris Gel w/ MES,Pre-stained standard Cat. No LC5925.

To ensure that the protein purified indeed was NDM-1, the enzymatic assay was performed. Moreover, we wanted to provide additional detail regarding the kinetics of our protein. It should also be mentioned that it is often rare for enzyme kinetic studies to measure the effects of different buffer concentrations and changes in ionic strength because these effects are usually minor. Thus, these parameters do not have to be strictly monitored. We, however, wanted to probe the effects thoroughly prior to investigating in-

depth kinetic analysis, such as pH-dependent experiments. We reiterate that in the studies requiring meticulous control of kinetic parameters, such as solvent-isotope effects and pK_as from pH rate profiles, ionic and buffer strength control is of great importance.

The kinetic constants were obtained by following the entire course of the reaction. In conditions below substrate saturation, we obtained good pseudo-first order rate constants, k_{obs}. These were shown to be first-order in enzyme concentration. Thus, we derived k_{cat}/K_m, the second-order rate constant, by dividing k_{obs} by concentration of enzyme used.

Ionic-strength effects

The effect of ionic strength on the second-order rate constant k_{cat}/K_m for the New Delhi metallo-beta-lactamase-catalyzed hydrolysis of Penicillin G is shown in Figure 7. At a constant buffer concentration, a change in ionic strength varied from 0.2-1.5 M NaCl. For the MOPS buffer at pH of 7.0, the k_{cat}/K_m value that we determined was 6.802 $M^{-1}s^{-1}$ at 0.2 M, 4.262 $M^{-1}s^{-1}$ at 0.5 M, 4.125 $M^{-1}s^{-1}$ at 1.0 M, and 7.537 $M^{-1}s^{-1}$ at 1.5 M. For the phosphate buffer at pH of 7.0, the k_{cat}/K_m value that we determined was 3.813 $M^{-1}s^{-1}$ at 0.2 M , 8.146 $M^{-1}s^{-1}$ at 0.5 M , 7.097 $M^{-1}s^{-1}$ at 1.0 M, 4.895 $M^{-1}s^{-1}$ at 1.5 M. These values are listed in Table 1.

Prior to choosing a particular buffer, it is important to probe the effect on enzymatic activity by ionic strength from different buffers. We tested the ionic strength effect of the MOPS buffer and phosphate buffer, as shown in Figure 7. We chose these two buffers because it is known that the stability of the enzyme is best around pH 7.0, and these buffers are standard to use at this pH. For MOPS, it has been revealed that the

ionic strength effect increased from 0.2 M to 0.5 M and decreased from 0.5 M to 1.6 M. In the case of the phosphate buffer, the ionic strength effect decreased from 0.2 M to 0.8 M and increased from 0.8 M to 1.6M.

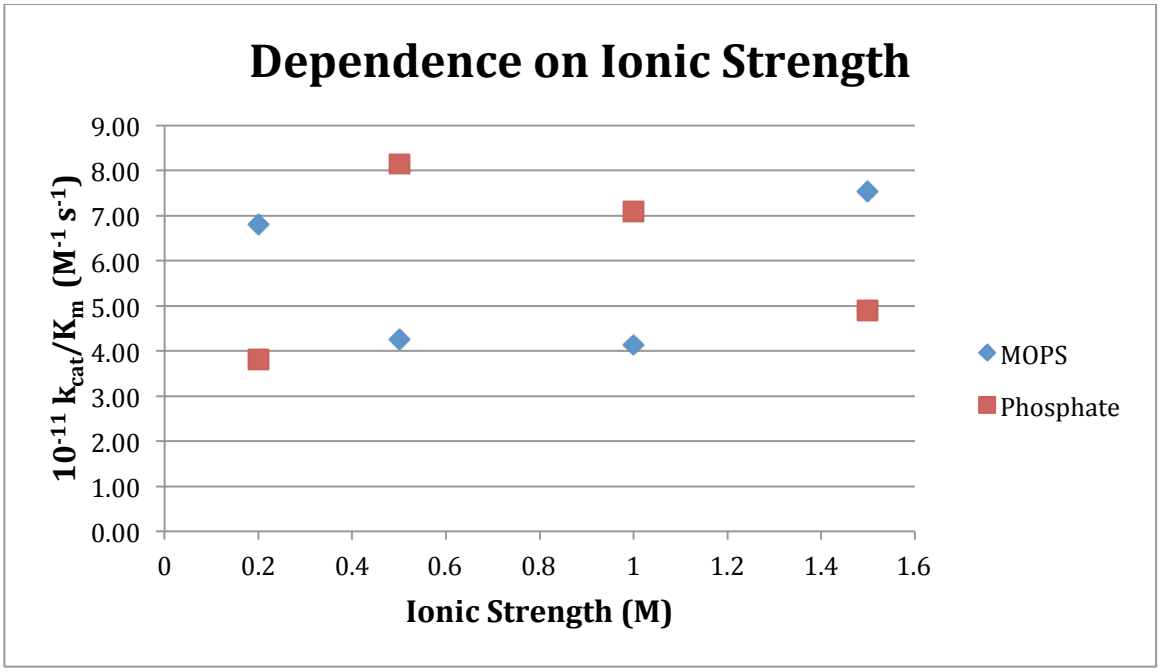

Figure 7. Dependence on ionic strength for the β-lactamase-catalyzed hydrolysis of Penicillin G. The dependence of k_{cat}/K_m for the β-lactamase II catalyzed hydrolysis of Penicillin G on the ionic strength adjusted with NaCl at 25°C, pH 7.0, with MOPS or phosphate buffer at a constant buffer concentration. We used the equation $0.5\ \Sigma\ C_iZ_i^2$ to calculate the total ionic strength for all ionic species, including the buffer.

Table 1. The k_{cat}/K_m values for ionic strength for the β-lactamase-catalyzed hydrolysis of Penicillin G.

Ionic Strength (M)	MOPS 10^{-11} k_{cat}/K_m (M^{-1} s^{-1})	Phosphate buffer 10^{-11} k_{cat}/K_m (M^{-1} s^{-1})
0.2	6.802	3.813
0.5	4.262	8.146
1.0	4.125	7.097
1.5	7.537	4.895

Buffer Effects

The kinetic parameters of NDM-1 were also determined, such as K_m and k_{cat} values. For the MOPS buffer at pH of 7.0, the k_{cat}/K_m value that we determined was 10.880 $M^{-1}s^{-1}$ at 0.025 M, 8.590 $M^{-1}s^{-1}$ at 0.05 M, 3.552 $M^{-1}s^{-1}$ at 0.1 M, 7.322 $M^{-1}s^{-1}$ at 0.15 M, and 13.186 $M^{-1}s^{-1}$ at 0.2 M. For the phosphate buffer at pH of 7.0, the k_{cat}/K_m value that we determined was 11.180 $M^{-1}s^{-1}$ at 0.025 M, 6.642 $M^{-1}s^{-1}$ at 0.05 M, 6.025 $M^{-1}s^{-1}$ at 0.1 M, 5.481 $M^{-1}s^{-1}$ at 0.15 M, and 5.423 $M^{-1}s^{-1}$ at 0.2 M. Table 2 summarizes the values that we found.

Figure 8 shows the effect of varying the buffer concentration and buffer type on the rate of enzyme-catalyzed hydrolysis of Penicillin G in a solution maintained at a constant total ionic strength of 1.0 M by adjusting the concentration of NaCl to compensate for changes in ionic strength brought about by changes in the buffer

concentration. The MOPS buffer decreased the rate of hydrolysis from 0.05 M to 0.1 M, and then increased the rate of hydrolysis from 0.1 M to 0.2 M. The phosphate buffer showed a continual decrease in the rate of hydrolysis. The phosphate buffer with a concentration of 0.15 M caused a 50% reduction in the k_{cat}/K_m compared with the minimum buffer concentration. The effect of MOPS is varied at lower buffer concentrations and higher concentrations; however, at the lower concentration, the reasonably good enzymatic activity was also shown. Thus, the 0.05 M concentration was chosen for the following kinetic studies.

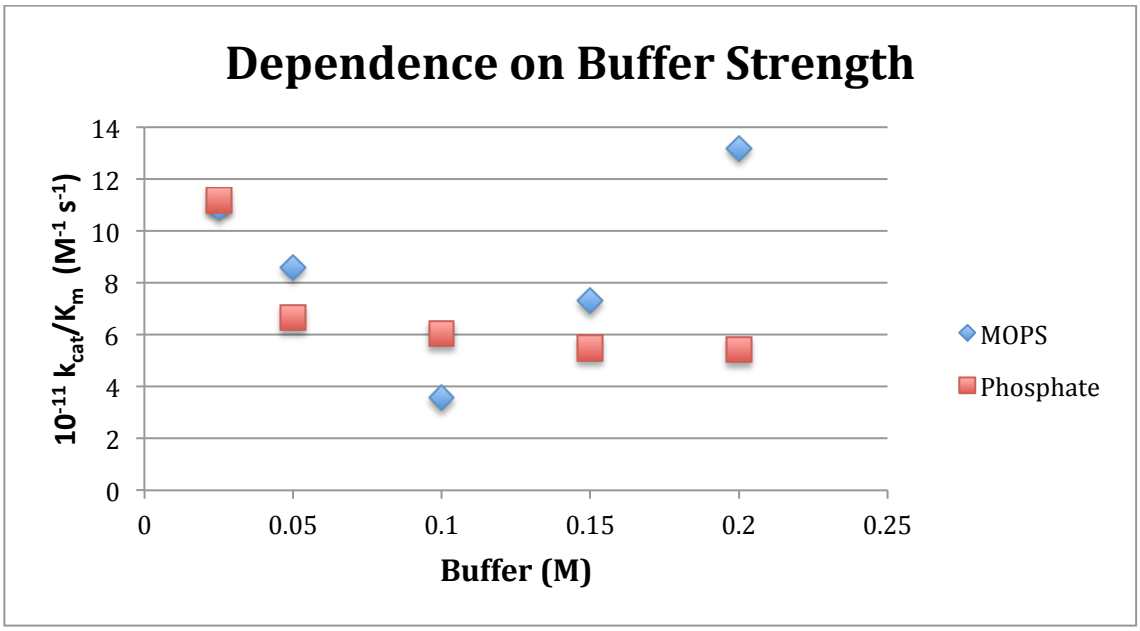

Figure 8. Dependence on buffer concentration of the β-lactamase-catalyzed hydrolysis of Penicillin G. The dependence of k_{cat}/K_m for the β-lactamase II catalyzed hydrolysis of Penicillin G on buffer concentration at 25°C, pH 7.0 for MOPS and phosphate buffer. By changing the concentration of NaCl, the ionic strength was kept constant at 1.0 M.

Table 2. k_{cat}/K_m values for buffer strength for the β-lactamase-catalyzed hydrolysis of Penicillin G

buffer (M)	MOPS 10^{-11} k_{cat}/K_m (M^{-1} s^{-1})	phosphate buffer 10^{-11} k_{cat}/K_m (M^{-1} s^{-1})
0.025	10.880	11.180
0.050	8.590	6.642
0.100	3.552	6.025
0.150	7.322	5.481
0.200	13.186	5.423

Effects of pH on different buffers

Figure 9 shows pH versus the rate constant, k_{cat}/K_m, for the hydrolysis of penicillin G, catalyzed by class B beta-lactamase from NDM-1 by MOPS, MES, TAPS, and Acetate buffer. There was no discernible relationship, which may indicate that the protein is not stable at different pH.

Table 3 summarizes the k_{cat}/K_m values at each of the pH values for different types of buffer used. For MOPS, the k_{cat}/K_m for pH at 6.5, 7.0, and 7.5 were 4.116×10^{-09}, 1.527×10^{-9}, and 2.543×10^{-9} $M^{-1}s^{-1}$. For MES, the k_{cat}/K_m for pH at 5.0, 5.5, 6.0 and 6.5 were 5.039×10^{-9}, 6.624×10^{-9}, 9.747×10^{-9}, and 1.580×10^{-9} M^{-1} s^{-1}. For TAPS, the k_{cat}/K_m for pH 7.5, 8.0, 8.5, and 9 were 2.221×10^{-9}, 2.161 ×10-8, 3.920×10^{-8} $M^{-1}s^{-1}$, and 3.790×10^{-8}. For Acetate buffer, the k_{cat}/K_m for pH at 5.0 and 5.5 were 4.138×10^{-8}, and 2.329×10^{-8} $M^{-1}s^{-1}$.

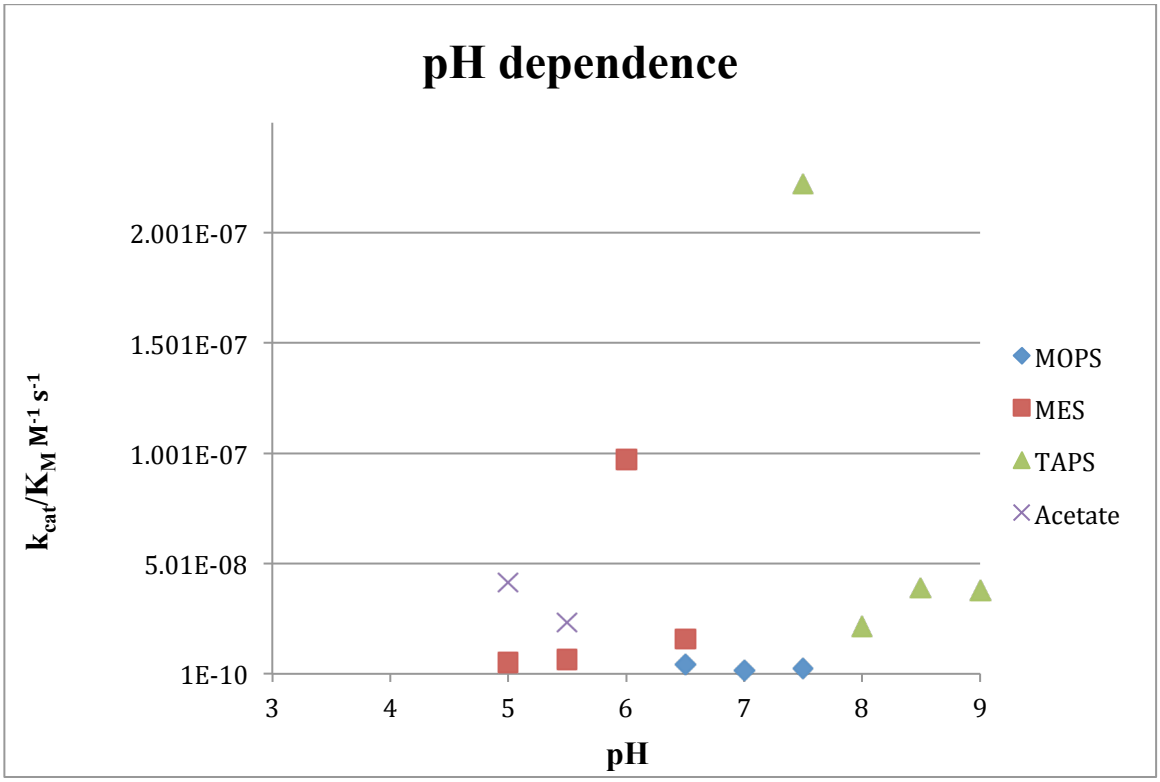

Figure 9. Dependence on pH of the β-lactamase-catalyzed hydrolysis of Penicillin G
The pH dependence of the k_{cat}/K_m for the beta-lactamase-catalyzed hydrolysis of penicillin G at 25°C and at a constant ionic strength of 1.0 M NaCl.

Table 3. k_{cat}/K_m values for differing pH conditions for the β-lactamase-catalyzed hydrolysis of Penicillin G

Type of buffer	pH	k_{cat}/K_m
MOPS	6.5	4.116×10^{-9}
	7.0	1.527×10^{-9}
	7.5	2.543×10^{-9}
MES	5.0	5.039×10^{-9}
	5.5	6.624×10^{-9}
	6.0	9.747×10^{-9}
	6.5	1.580×10^{-9}
TAPS	7.5	2.221×10^{-9}
	8.0	2.161×10^{-8}
	8.5	3.920×10^{-8}
	9.0	3.790×10^{-8}
Acetate	5.0	4.138×10^{-8}
	5.5	2.329×10^{-8}

CHAPTER FOUR

Discussion and Conclusions

The effect of ionic strength on the second-order rate constant k_{cat}/K_m for the New Delhi metallo-beta-lactamase catalyzed hydrolysis of Penicillin G is shown in Figure 7. I have demonstrated that the purification of NDM-1 was successful and that the determination of ionic strengths and buffer strength has been successfully made.

Figure 7 shows that at a constant buffer concentration for MOPS, a change in ionic strength from 0.2-0.5 M NaCl decreased k_{cat}/K_m by nearly half, and a change in ionic strength from 1.0-1.5 M nearly doubled k_{cat}/K_m, with no change observed between 0.5 M and 1.0 M. For the phosphate buffer, at a constant buffer concentration, a change in ionic strength from 0.2-0.5 M NaCl nearly increased k_{cat}/K_m twofold, and a change in ionic strength from 0.5-1.5 M showed a trend of decrease in k_{cat}/K_m. These results are different from the results of the plot of k_{cat}/K_m, for the *Bacillus cereus* 568/H beta-lactamase catalyzed hydrolysis of cephalosporin, where for both the MOPS and phosphate buffer at pH 7.0, at constant buffer concentration, saw a change in ionic strength 0.01-1.0M NaCl changed the k_{cat}/K_m about twofold (Bounaga 1998). The behavior is different, and a possible explanation is that the beta-lactamases are structurally different, although both are metallo-beta-lactamases. Additionally, while Bounaga studied the dependence on ionic strength for cephaloridine, we used penicillin G.

Figure 8 shows the effect of varying the buffer concentration and buffer type on the rate of the enzyme-catalyzed hydrolysis of Penicillin G in a solution maintained at a constant total ionic strength of 1.0 M, by adjusting the concentration of NaCl to compensate for changes in ionic strength from differences in buffer concentration. At a constant ionic strength for MOPS, a change in buffer strength from 0.025 -0.1 M NaCl decreased k_{cat}/K_m by nearly one-third, and a change in ionic strength from 0.1-0.2 M increased k_{cat}/K_m three-fold. For the phosphate buffer, at a constant ionic strength, a change in buffer strength from 0.2 -0.5 M NaCl nearly increased twofold, and a change in ionic strength from 0.025-0.2 M continually decreased k_{cat}/K_m. These results are different from the results of the plot of k_{cat}/K_m for the *Bacillus cereus* 568/H beta-lactamase catalyzed hydrolysis of cephalosporin, which showed both MOPS and phosphate buffers decrease the rate of hydrolysis; 0.15 M phosphate causes a 50% reduction in k_{cat}/K_m compared with zero buffer concentration (Bounaga 1998). The effect of Mops was slightly less, particularly at low buffer concentrations. For our data, MOPS saw a 50% reduction k_{cat}/K_m compared with the buffer concentration at about 0.15 M, and phosphate at 0.05 M. Some of the behavior trends are different, which may due to the fact that the beta-lactamases are different in character, although they are both classified as metallo-beta-lactamases. Additionally, while Bounaga studied the dependence on ionic strength for cephaloridine, we used a different antibiotic – penicillin G.

The pH dependence of the k_{cat}/K_m for the beta-lactamase-catalyzed hydrolysis of penicillin G at 25°C and at constant ionic strength of 1.0 M NaCl were determined. Figure 4 shows pH versus the rate constant k_{cat}/K_m for the hydrolysis of penicillin G,

catalyzed by class B beta-lactamase from NDM-1 by MOPS, MES, TAPS, and Acetate buffer. There is no discernible relationship between k_{cat}/K_m and pH conditions, which may indicate that the protein is not stable in acidic or basic conditions from pH of 7.0.

In conclusion, as the importance of understanding the NDM-1 mechanism urgently increases, the details of ionic strengths and the choice of buffer will likely be of great value. The observation of the possibility of the instability of the enzyme at high and low pH will lead us to further investigation and study of the NDM-1.

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