ABSTRACT

Characterization of the Metallo-β-Lactamase from *Pseudomonas aeruginosa*, IMP-1

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The rate at which pathogenic bacteria are gaining antibiotic resistance has become increasingly alarming. Major contributors of this antibiotic resistance in microbes are a class of enzymes known as β-lactamases. These enzymes are effective in breaking down the most commonly prescribed antibiotics at present. This work investigates two separate metallo-β-lactamase enzymes, first IMP-1, which provides antibiotic resistance to *Pseudomonas aeruginosa*, and second Bla2, which grants antibiotic resistance to *Bacillus anthracis*. The main focus of this work was to investigate and characterize IMP-1 according to its activity and stability in temperature and pH. This was done in an effort to increase the general knowledge for potential inhibitors to be designed. The secondary focus of this work was to examine the ability of novel hydroxamate compounds to inhibit the growth of bacterial cells expressing Bla2. In addition to this work, aptamers were investigated as a potential means of future inhibitor design.

Characterization of the Metallo-β-Lactamase from *Pseudomonas aeruginosa*, IMP-1

by

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A Thesis

Approved by the Department of Chemistry and Biochemistry

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

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LIST OF ABBREVIATIONS

Bla2 – metallo-β-lactamase from *Bacillus anthracis*

βME – β-mercaptoethanol

CFU – colony forming units

DMSO – dimethyl sulfoxide

EDAC – 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride

EDTA – ethylenediaminetetraacetic acid

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC_{50} value – concentration of inhibitor necessary to inhibit 50% of enzyme activity

IMP-1 – metallo-β-lactamase from *Pseudomonas aeruginosa*

IPTG – isopropylthio-β-D-galactopyranoside

LB – Luria-Bertani media

MBL – metallo-β-lactamase

MES – 2-(N-Morpholino)ethanesulfonic acid

MH – Mueller-Hinton broth

MIC – minimum inhibitory concentration

MOPS – 3-(N-Morpholino)propanesulfonic acid

NHS – N-hydroxysuccinimide

OD – Optical density

ss – single stranded

TAPS – N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid

TE – Tris-EDTA

ACKNOWLEDGMENTS

This work was in part funded by the Undergraduate Research and Scholarly Achievement grant at Baylor University. Acknowledgment goes to Christopher Taylor and Drs. Jugeshwar Kshetrimayum, Nelson van der Velde, Sung Joon Kim, and Sung-Kun Kim for their collaboration and contribution to this work.

I would like to further acknowledge my mentor, Dr. Sung-Kun Kim, for his guidance and support, and without whom this work would not have been possible. I have a great appreciation for the commitment he made to my graduate education and experience. I also would like to recognize the other members of my research group, past and present, for their continued support throughout this journey. Thank you Dr. Mieke Lahousse, Myung Cho, M.S., and Sara Schlesinger (soon to be Ph.D.) for welcoming me into your lab space with open arms. I am forever grateful to you for your kindness and willingness to teach a 'newbie' the lab skills necessary for survival in a biochemistry research lab.

Finally I would like to express my greatest appreciation for my Mom and Dad, my brother, Allen, and my boyfriend, Brandon. Without the encouragement of these people, I may never have even entered graduate school, and I am grateful for their commitment to me throughout this process. They have been the greatest support structure I could have asked for, and I hope that my work in this thesis will make them proud.

CHAPTER ONE

Introduction

Due to the overuse and misuse of antibiotics, drug resistance among infectious pathogens, including *Pseudomonas aeruginosa*, has been increasing[1–3]. This resistance is a serious problem, especially in nosocomial and immunosuppressed patients, who have an increased risk of infection, and in which infection is more harmful[4]. One of the most commonly prescribed classes of antibiotics are β-lactam antbiotics, which are among the most effective antibiotics for Gram-negative bacterial infections[5]. The problem, however, lies in the ability of these bacteria to gain resistance to antibiotics through a variety of methods, one of which is to produce enzymes that break down a drug and inactivate it [6]. β-lactamases are among those enzymes that have developed for this purpose, specifically neutralizing β-lactam antibiotics, such as penicillins, cephalosporins, and carbapenems, by hydrolyzing the amide bond of the lactam ring, leaving behind an inactive β-amino acid. In response to this increasing bacterial resistance, there has been development of β-lactamase inhibitors. Common β-lactamase inhibitors include penicillanic acid sulfone and clavulanic acid [7]. These inhibitors work for serine-β-lactamases by binding to the serine residue in the active site, but the compounds fail to inhibit another type of β-lactamase known as metallo-β-lactamases (MBLs) [7]. In fact, MBLs belong to group B out of the four types of β-lactamases in their classification system (A through D), where group A, C, and D belong to serine-β-lactamases. The group B MBL has three subclasses, B1-B3 [8]. The subclass B1 enzymes

have two zinc ions, where a tightly bound zinc with a tetrahedral geometry coordinated by three His residues and one solvent molecule is called Zn1, and a relatively loose bound zinc with a distorted trigonal bipyramidal geometry coordinated by three different amino acid residues (His, Cys, Asp), a water molecule and a solvent such as glycerol is called Zn2. The subclass B2 enzymes have only one zinc ion, and the subclass B3 enzymes have two tightly bound zinc ions in the active site.

IMP-1 is a subclass B1 MBL[9], and was initially isolated only from *Pseudomonas aeruginosa* [10,11]. In the amino acid sequence, IMP-1 possesses the key conserved sequences that hold two zinc ions (Fig 1) and shows its ability to hydrolyze carbapenems, cephalosporins, and penicillins [1,12]. The 3D structure of IMP-1 has been solved, as shown in Figure 2, and shows that the overall structure of IMP-1 is similar to other B1 MBLs in its characteristic α-β-β-α folding [13]. IMP-1 has shown its ability to be spread through horizontal gene transfer through plasmids and transposons [10,11]; thus, the gene $bla_{\text{IMP-1}}$ has been isolated in several pathological bacteria, including *P. aeruginosa* and *Klebsiella pneumoniae* [1].

IMP-1 is clinically important because it has an extended spectrum of substrate specificity, lacks clinically useful inhibitors, and, as mentioned previously, can be spread between species and genera of Gram-negative bacteria [3]. With increasing resistance in bacteria, especially opportunistic pathogens that can infect those with weakened immune systems, it is important to combat this resistance by finding inhibitors that will allow for current drugs to be useful again. Inhibitor design has been extensively studied and includes thioester derivatives, trifluoromethyl alcohols and ketones, thiols, sulfonyl hydrazones, tricyclic natural products, succinic acid derivatives, biphenyl tetrazoles,

cysteinyl peptides, mercaptocarboxylates, 1-β-methylcarbapenem, cefotetan, thioxocephalosporins, and penicillin derivatives [3,14–16]. None of these compounds, however, are thus far approaching phase 1 clinical trials. Thus, if we take into consideration the time-consuming process to develop a drug, it is critical that we discover effective inhibitors of MBLs as soon as possible.

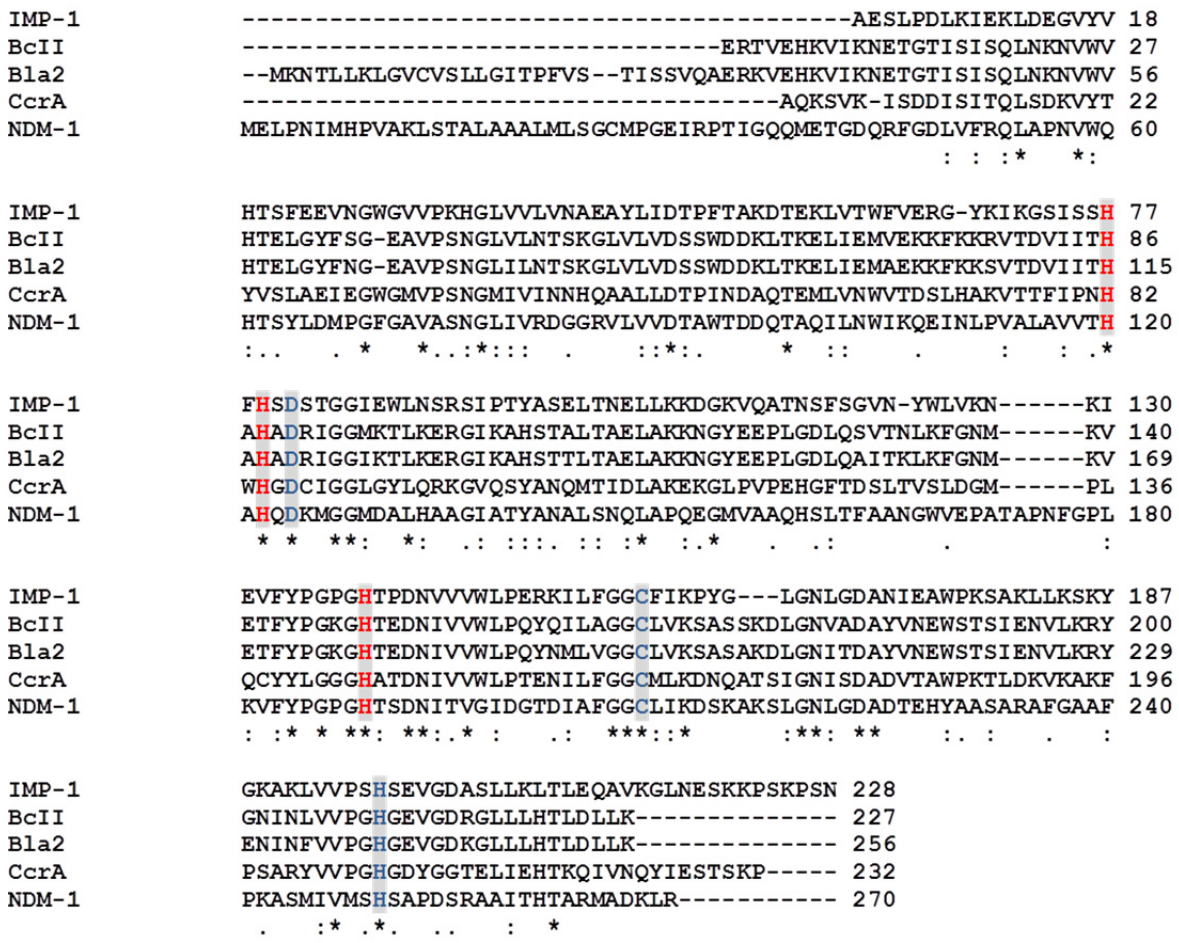

Fig 1. Amino acid sequence alignment of IMP-1 with other class B1 MBLs. Highlighted in blue are the amino acid residues that bind to Zn1, and in red, the amino acid residue responsible for coordinating to Zn2. IMP-1 is from *Pseudomonas aeruginosa*; BcII is from *Bacillus cereus*; Bla2 is from *Bacillus anthracis*; CcrA is from *Bacteriodes fragilis*; NDM-1 is from *Klebsiella pneumonia.*

Due to the crucial role that the active site zinc ions play in reaction catalysis, one method of enzyme inhibition is to chelate these metal ions and render them inactive. In the past, effort has been placed on carboxylic acid-containing inhibitors and thiol-containing compounds, which inactivate the nucleophilic hydroxide group responsible for hydrolysis of the substrate [17]. These types of inhibitors are typically very specific for particular enzymes, however, and do not have promising potential as broad-spectrum inhibitors. One possible approach to designing broad-spectrum inhibitors is to chelate the Zn^{2+} ions that MBLs employ and therefore render the enzyme inactive. The hydroxamic acid functional group is known as a zinc binding group and, when linked to a peptide backbone, inactivates the catalytically essential zinc ion of matrix metalloproteinases [18]. These types of compounds were synthesized and tested for effectiveness of inhibition against class B1 MBLs.

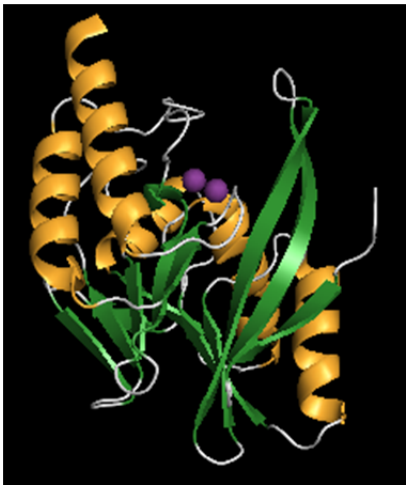

Fig 2. The 3D structure of IMP-1. Highlighted in gold are the α-helices, in green are the β-sheets, and in purple are the Zn1 and Zn2 ions that are coordinated at the active site. This structure was determined by Concha et al.[13]

This body of work is focused on describing an improved purification method for IMP-1 from *P. aeruginosa* and characterizing this enzyme with respect to optimal temperature and pH and reporting enzyme kinetic parameters that provide insight into the acid-base chemical mechanism of IMP-1. Furthermore, the susceptibility of IMP-1 to ethylenediamineteraacetic acid (EDTA) and β-mercaptoethanol (βME) was investigated and results are reported in the form of an IC_{50} determination. In addition, the MBL from *Bacillus anthracis*, known as Bla2, was expressed in *Bacillus subtilis* cells and compounds containing hydroxamic acid moieties were utilized for examining their effect on the growth of these bacterial cells. Lastly, a G-quadruplex aptamer was developed for potential research projects involving the neuraminidase enzyme (H5N1) and a DNA affinity column was generated for purification purposes.

CHAPTER TWO

Materials and Methods

Expression and Purification of IMP-1

Plasmid pET15b-IMP-1, a pET 15b vector containing a cloned copy of the bla_{IMP} gene of *P. aeruginosa* (GenBank: DQ842025.1), was purchased from Genewiz, Inc. (South Plainfield, NJ). The plasmid pET15b-IMP-1 was transformed in *E. coli* BL21(DE3). For large scale expression, a 40 mL overnight culture of *E. coli* BL21(DE3) with the pET15b-IMP-1 plasmid was used to inoculate 4 × 1 L of Luria-Bertani (LB) medium containing ampicillin (50 μg/mL), where LB is composed of trypsin, yeast extract and sodium chloride: 10g:5g:10g in one liter. The culture was grown at 37 °C with shaking at 250 rpm using a New Brunswick Scientific Excella E25 incubator/shaker until it reached an optical density at 600 nm (OD_{600nm}) of 0.6 - 1.0. The optical density was measured using an Eppendorf BioPhotometer plus photometer. Protein expression was induced by the addition of isopropylthio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. The cultures were further incubated at 30 °C with shaking at 250 rpm for another 6 hrs. The bacteria were then harvested by centrifuging the cultures in Beckman bottles at 4750 rpm for 20 min using a Beckman Coulter Allegra® X-15R centrifuge. The supernatant was discarded and the cell pellet stored in a -80 ºC freezer.

IMP-1 was purified according to Schlesinger et al.[19] with several modifications. The frozen cells were thawed and transferred into a beaker with 5 mL of buffer A (30 mM Tris-HCl at pH 8.0 with 100 mM NaCl) per liter of medium used, for a total 20 mL.

The resuspended cells were then lysed through a French press (Thermo Electron Corporation French Press Cell Disruptor) at 1200 psi for 6 min. This process was repeated for a total of four times. The cell lysate was then centrifuged (Beckman Coulter Avanti® J-26 XP) at 20,000 rpm for 20 min. The supernatant was then filtered three times with 0.8 μm Millipore™ Millex® Syringe-driven Filter units and twice more with 0.45 μm Millipore™ Millex® Syringe-driven Filter units before column chromatography. The filtered supernatant was loaded on to a Ni^{2+}-affinity column (GE Healthcare) attached to a liquid chromatography system (BioLogic LP, Bio-Rad) that had been previously equilibrated with buffer A. The column was washed with a linear gradient of 1-3% buffer B (30 mM Tris-HCl at pH 8.0 with 100 mM NaCl and 500 mM imidazole) with buffer A over 60 min, which was then followed by elution of the protein using a linear gradient of 3.0 - 50% buffer B with buffer A. The protein was eluted with 26.5 mM NaCl and 132.5 mM imidazole. The fractions containing IMP-1 were collected and concentrated using Ultra-15 centrifugal filters (EMD Millipore) by centrifuging at 4,000 rpm for 20 min. Precipitation occurred and the samples were centrifuged at 5000 rpm for 5 min in micro-centrifuge tubes. The protein from the micro-centrifuge tubes were consolidated and concentrated again at the same settings as before. The protein was extracted from the concentrator and the protein was reloaded for buffer exchange. Buffer A was added to the concentrator and then centrifuged at the same settings as before for a total of two times. Once buffer exchange was complete, the protein was concentrated once more at the same settings. Glycerol was added to 10% (v/v) and the resulting protein solution was stored at -20^{o}C. The protein concentration was measured at 280 nm and calculated using an extinction coefficient of 45,000 $M^{-1}cm^{-1}$ and mass of

27,692.52 g/mol for IMP-1. Throughout the purification process, 50 µl aliquots were taken from the lysed cells, the supernatant, the column fractions and the concentrated protein to be analyzed for enzymatic activity.

Enzyme Assay with Penicillin G

IMP-1, in 30 mM Tris-HCl buffer at pH 8.0 containing100 mM NaCl and 10% (v/v) glycerol, was assayed in in a mixture that contained 500 µl of 30 mM Tris-HCl (pH 7.0), 7 µl 0.4025 mg IMP-1/ml (a 20x dilution from the original concentration), varying volumes of 50 mM penicillin G, and water to give a final volume of 1 mL (a final buffer concentration of 15 mM Tris-HCl). The assay was performed at room temperature and the reaction was initiated with the addition of enzyme. UV spectroscopy (Shimadzu UV-2450) was used to monitor the reaction by measuring the decrease in absorbance due to hydrolysis of penicillin G at 235 nm. The extinction coefficient (ε) used to perform calculations was 775 M^{-1} cm^{-1} [20]. Averages are reported based on three independent measurements. One activity unit is defined as the amount of enzyme required to catalyze the hydrolysis of 1µM penicillin G in 1 min at 25°C.

Temperature Stability

To evaluate the temperature stability of IMP-1, the enzyme was incubated in water baths (Fisher Scientific Isotemp 202) of varying temperatures (20, 30, 40, 50, 60, and 70°C) and tested for activity every 30 min for up to 4 hrs. The enzymes assays were performed using 500 µL of 30 mM Tris-HCl (pH 7.0), 40 µL of 50 mM penicillin G, 7 µL 0.4025 mg IMP-1/ml, and 453 µL of dH_2O for a total volume of 1 mL (a final concentration of 2 mM penicillin G as substrate). Again, the change in absorbance at 235

nm was monitored, however the temperature of the UV/Vis chamber was adjusted to match the assay being performed using a Fisher Scientific Isotemp 3016S system.

pH Dependence

To analyze the pH dependence of the enzyme activity, IMP-1 was mixed into buffers of varying pH and enzyme assays were performed. The assays to investigate the pH dependence of the rate were performed similarly to the enzyme assay to find the kinetic parameters, with the only difference being the buffers. k_{cat} and K_m were found using penicillin G as a substrate from pH 5.5 to 9.0, with a 0.5 step. To test the activity between the pH range from 5.5 to 6.5, 100 mM 2-(N-Morpholino)ethanesulfonic acid (MES) buffer was used, 100 mM 3-(N-Morpholino)propanesulfonic acid (MOPS) buffer was employed for the pH range between 6.5 and 7.5, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was utilized for the pH range from 7.0 to 8.0, and 100 mM N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer was used for the pH range between 8.0 and 9.0.

Data for k_{cat} and k_{cat}/K_m pH profiles were fitted using Equation 1.

$$\log y = \log[C/1 + (K/H)] \qquad \text{(Eq. 1)}$$

In Equation 1, K represents the dissociation constant of the enzyme, y is the value of the parameter observed as a function of pH, H is the hydrogen ion concentration, and C is the pH-independent value of y.

Inhibition Tests with EDTA and βME

EDTA and βME were used to test for inhibition of IMP-1 activity. An enzyme assay was performed in the form of an IC_{50} determination, which defines the

concentration of inhibitor necessary to inhibit 50% of the enzyme's activity. Varying concentrations of EDTA from 1.0 nM – 1.0 mM or of βME from 1.0 nM – 10 mM were used before initiating the reaction with penicillin G. Reported values are based on the averages of three trials.

MIC Testing of B. subtilis

The MIC of β-mercaptoethanol (βME) and compounds 4 and 8 was determined for *Bacillus subtilis* cells with or without the Bla2 plasmid. The vector pHT01 containing the Bla2 gene was constructed and transformed into *Bacillus subtilis* cells by Soo-Keun Choi in the Systems and Synthetic Biology Research Center of the Korea Research Institute of Bioscience and Biotechnology located in Daejeon, Republic of Korea. Bacterial cells were incubated 24 hours in Luria-Bertani (LB) media with or without chloramphenicol (5 μg/mL) as a selection agent. A volume of 100 μL of this culture was then spread on agar plates with or without chloramphenicol (30 μg/mL) and incubated (Fisher Scientific Isotemp Incubator) at 37°C for 12 hours to determine the CFU of the 24 hour culture. The 24 hour culture was stored at room temperature while the CFU was being determined.

After determining the CFU, the 24 hour culture was able to be diluted with sterile LB media to give approximately 10^6 bacteria per mL. Ampicillin was prepared fresh to a final concentration of 1.5 μg/mL in Mueller-Hinton broth. β-mercaptoethanol solution was also freshly prepared to a stock concentration of 17.2 mg/mL and then serially diluted twofold in MH broth.

Two novel hydroxamate compounds, named compound 4 and compound 7, were synthesized under the guidance of Dr. Shin at McMurry University. Solutions of

compounds 4 and 7 were prepared by dissolving the compound in 30% DMSO in water, followed by dilution to the final stock concentration in MH broth without additional DMSO, and then immediately added to a 96-well microplate. A volume of 50 μL of Ampicillin was added to the wells of a 96-well microplate with either 50 μL βME for the MIC determination of βME, 50 μL MH broth containing compound 4 for the MIC determination of compound 4, or 50 μL MH broth containing compound 7 for the MIC determination of compound 7. After the addition of 50 μL of the bacterial cell samples, the wells were mixed and incubated at 37°C for 24 hours. The plates were measured every 4 hours (except hour 20) at 620 nm with a Thermo Scientific Multiscan MCC/340 microplate reader after 20 seconds of shaking at 1200 rpm.

Preparation of DNA Affinity Column

The DNA affinity column was prepared according to Chockalingam et al.[21] with several modifications. The oligonucleotide 5'-NH_2-C_6-GCAAGCTTCCCCCCA-3', an amine-modified "linker" that contains a sequence complementary to the template DNA, was purchased from IDT (Integrated DNA Technologies), Inc. (Coralville, IA). This oligonucleotide was prepared to a concentration of 100 μM in sterilized water prior to preparation of the column, and stored at -20°C.

The base silica used was from VWR (300 Å pore, cat. no. AA44099-09). 1 gram of dry silica was measured and transferred to a glass pear-shaped flask. The system was placed under vacuum. To the flask, 2 mL of 2-propanol containing 96 mg (0.5 mmol) of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDAC) was added and mixed thoroughly. This was followed by bath sonication for 1 minute. To this mixture, 2 mL of 2-propanol containing 58 mg (0.5 mmol) of N-hydroxysuccinimide (NHS) was

added and mixed. Bath sonication was continued for an additional minute. The mixture was then removed from the sonicator and placed above a stir plate. While still under vacuum, the mixture was stirred at room temperature (19-22°C) for 1 hour.

The silica was then removed from the vacuum system and transferred to a 15 mL polypropylene Falcon tube. The silica was washed with 4 mL portions of 2-propanol (3 times), methanol (2 times), DI water, and 0.5 M sodium phosphate buffer, pH 7.5, by centrifugation at top speed for 1 minute. After removing the last wash, 2 mL of 0.5 M sodium phosphate buffer, pH 7.5, and containing 11 nmol of 5'-NH_2-C_6-GCAAGCTTCCCCCCA-3' was added to the silica. This mixture was shaken for 75 minutes at room temperature. 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE) buffer was added to the silica mixture and stored refrigerated at 4°C until needed.

Determination of Amine-modified Linker Coupling

The 58 base ssDNA oligonucleotide containing 12 bases of randomized sequence (N_6), 5'-GCGGATCCGGGGGGT(N_6)TGGGGGGATGGGGGGT(N_6)TGGGGGG-AAGCTTGC-3', was obtained from IDT (Integrated DNA Technologies), Inc. (Coralville, IA). This DNA was prepared to a concentration of 500 ng/μL (27.4 μM) in TE buffer and stored at -20°C until needed.

100 mg of the amine-modified silica (11 nmol/g) was transferred to a 2 mL microcentrifuge tube and washed three times with 1 mL portions of Buffer A (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, pH 8.3) via centrifugation in order to remove the storage buffer. The silica was then mixed with 5 nmol of the DNA oligonucleotide.

Prior to mixing, the DNA was pretreated to form single-stranded DNA fragments. Buffer A was added to the 5 nmol of DNA to obtain a final volume of 1 mL. The mixture was heated to 90°C for 10 minutes, and then cooled quickly on ice for 30 minutes. The absorbance at 260 nm was measured.

After the full volume of DNA (1 mL) was added to the silica mixture, the entire system was heated to 65°C for 10 minutes and then cooled slowly to room temperature over the next 30 minutes. The silica was then washed (5 times) with 0.8 mL portions of Buffer A by centrifugation for 1 minute at max speed. The washes were collected and the absorbance was measured at 260 nm after diluting to a final volume of 1 mL. This will allow us to observe the change in absorbance value due to the annealing of the DNA to the column.

The column material was then washed with 0.8 mL TE buffer at 42°C via centrifugation. The wash was collected and diluted to a final volume of 1 mL for measuring the absorbance at 260 nm. This process was repeated with TE buffer at 90°C and water at 90°C. Absorption values at 260 nm from the high temperature washes will indicate success of the column. The template should elute from the amine-modified silica in 90°C TE or water. TE buffer was added to the silica for storage in refrigeration.

To confirm that the template DNA had eluted from the column, PCR was performed and PAGE was run to visualize the amplification of DNA. PCR was performed using the TE buffer wash at 42°C, the TE buffer wash at 90°C, the first water wash at 90°C, and the second water wash at 90°C as the template DNA sample. The forward and reverse primers used were generated by IDT (Integrated DNA Technologies), Inc. (Coralville, IA). The forward primer sequence was

5’-GCGGATCCGGGGGGT-3’ and the reverse primer sequence was 5’-GCAAGC TTCCCCCCA-3’. A PCR control test was run at the same time as the wash samples to either confirm or negate PCR success. For the control test, 1 µL of 50 ng/mL template DNA, 1 µL of 25 µM forward primer, 1 µL of 25 µM reverse primer, 25 µL of GoTaq Green Master Mix (Promega), and 22 µL of dH_2O were pipetted into a PCR tube. These conditions were kept constant for all experimental samples on which PCR was run. The PCR machine was setup to perform the following program: (1) initial denaturation at 95°C for 10 minutes, (2) denaturation at 95°C for 40 sec, (3) annealing at 55°C for 40 sec, (4) elongation at 72°C for 20 sec, (5) steps 2-4 were repeated an additional 39 times to have a total of 40 cycles, (6) final elongation step at 72°C for 5 minutes, (7) lower temperature and hold at 4°C.

The PCR products obtained were run on polyacrylamide gel to confirm amplification of the template DNA. A NuPage 4-12% 1.0 mm Bis-Tris gel (Novex by Life Technologies) was set up, and 12 µL each of control PCR test, product of TE buffer wash at 42°C, product of TE buffer wash at 90°C, product of first water wash at 90°C, and product of second water wash at 90°C were loaded into separate wells. The gel was run at 100 volts for 1 hour 15 minutes. Afterwards, the gel was stained with ethidium bromide in 1x TBE buffer for 30 minutes and an image was taken under UV light.

CHAPTER THREE

Results

Purification of IMP-1 and Kinetic Parameters

A simplified purification method was developed for IMP-1 with only one affinity chromatography step. Although a significant loss of activity was observed during the purification process, this simplified, optimized method allowed us to obtain highly purified enzyme with a large quantity of pure protein, i. e., about 29 mg of protein per liter of culture (Table 1).

Table 1. Summary of purification of IMP-1. The activity was measured using penicillin G as a substrate. These values were the result of 6 trials.

Protein Sample	Volume (mL)	Total protein (mg)	Total Activity (μmol min^{-1})	Specific Activity (μmol min^{-1} mg^{-1})	Purification (Fold)
Cell extract	37	8616	223,300	25.9	1.00
Centrifuge Supernatant	26	2647	112,700	42.6	1.64
Ni^{2+} column elution	30	63	11,300	179.1	6.91
Buffer exchange	3.6	29	5,300	183.6	7.12

The specific activity of the purified enzyme toward penicillin G was 183.63 μmol min^{-1} mg^{-1}. SDS-PAGE analysis of IMP-1 displayed one Coomassie-staining band with an apparent molecular mass of 28 kDa (Fig. 3).

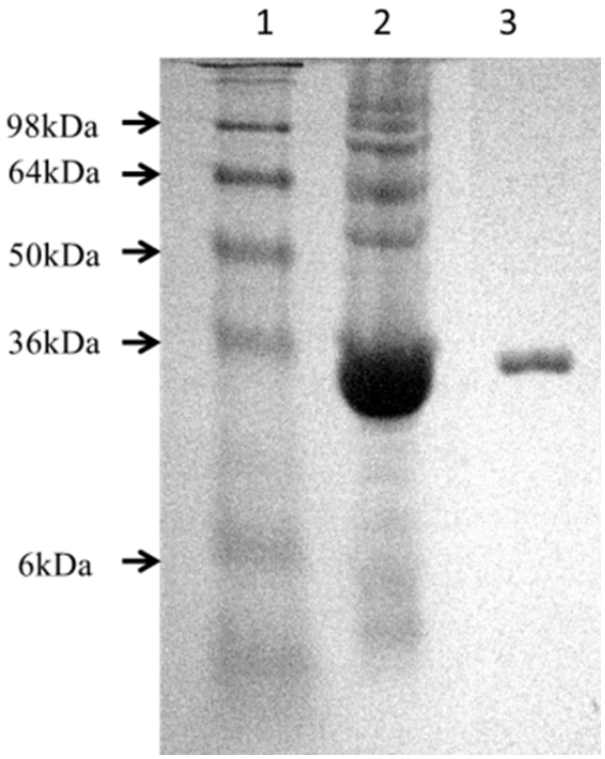

Fig 3. SDS-PAGE analysis of purified IMP-1. Lane 1 = standard marker; Lane 2 = sample of crude extract; Lane 3 = sample of the elution from Ni^{2+}-column. The molecular weight of IMP-1 is approximately 28 kDa.

Mass spectrometry revealed the molecular weight of the purified protein to be 27,629.2 Da ± 10, which is in excellent agreement with the calculated molecular weight of 27,692.5 Da. After confirmation of purification, we determined the Michaelis-Menton kinetic parameters of IMP-1 using penicillin G as a substrate. The results showed that the K_m and V_{max} parameters for IMP-1 with penicillin G were 390 μM and 156 μmol min^{-1} mg^{-1} (Fig 4), and the k_{cat} and the catalytic efficiency (k_{cat}/K_m) were 72.1 s^{-1} and 0.186 s^{-1} μM^{-1}, respectively.

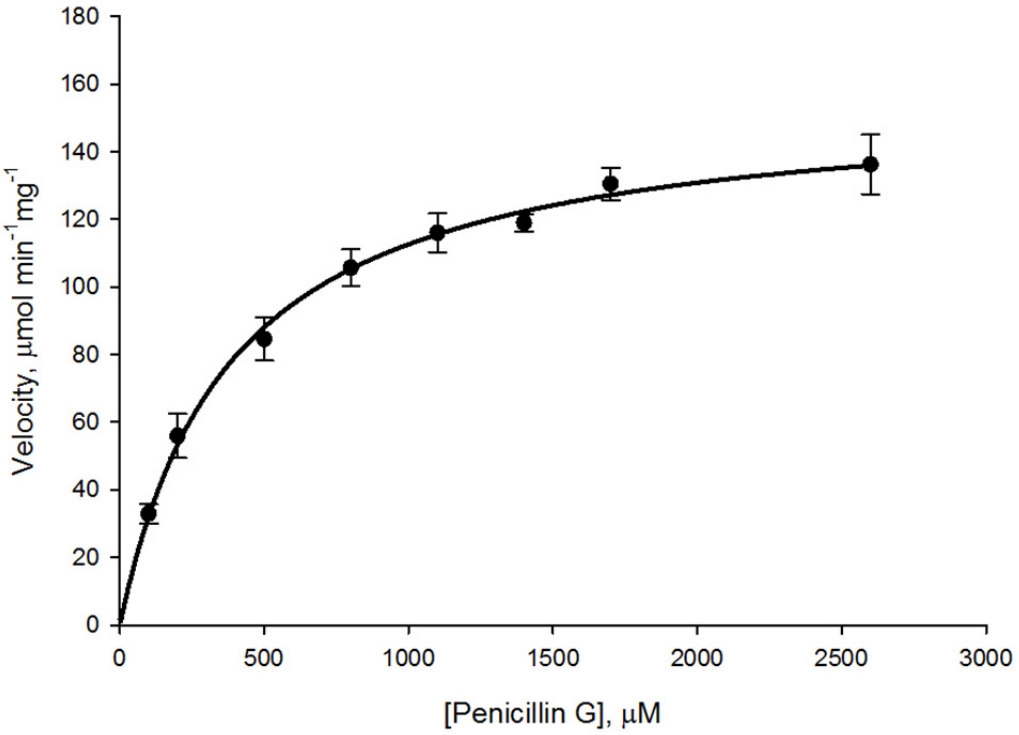

Fig 4. Kinetic activity of IMP-1 against penicillin G at room temperature and pH 8.0. The K_m and V_{max} are reported as 390 µM and 156 µmol/min/mg, respectively. These values were the average results over three separate trials

Effects of Temperature and pH

Other well studied MBLs have been characterized according to their thermostability, and despite their sequence similarities, some are very stable at temperatures up to 60 °C, while others are unstable at these high temperatures[22–24]. Here we measured the enzyme activity every 30 min for up to 4 h at 20, 30, 40, 50, 60, and 70 ^{o}C (Table 2).

These results showed that IMP-1 was able to withstand prolonged exposure to 60 °C and maintain high levels of activity (Fig. 5).

Table 2. Summary of temperature dependence assays of IMP-1. The activity was measured using penicillin G as a substrate.

	Specific Activity, μmol min^{-1} mg^{-1}					
Time (h)	20°C	30°C	40°C	50°C	60°C	70°C
0	111.5	148.2	305.2	424.4	510.0	595.9
0.5	123.9	189.4	282.7	377.4	516.5	564.4
1	145.0	194.1	296.5	426.9	579.2	488.4
1.5	153.6	183.6	319.5	482.0	542.1	454.8
2	163.4	216.8	318.7	425.0	596.3	506.0
2.5	169.3	223.2	317.6	476.6	548.9	489.5
3	163.8	223.6	295.2	494.2	581.4	397.2
3.5	166.7	220.4	310.8	497.6	501.4	356.8
4	148.1	220.5	291.6	465.7	553.1	322.6

However, at 70 °C, the activity immediately was reduced after 30 min and continued to decline. At 60 °C, IMP-1 reaches a maximum activity after 2 h and maintained a high level of activity during the 4 h period with the final activity measured being 92.8% of the maximum activity. Based on these results, we are able to conclude that IMP-1 is a thermostable MBL.

The activity of IMP-1 was tested with the pH range between 4.5 and 9.0. The enzyme was most active between pH 6.0 and 7.0 as shown in Fig 6A. Over the pH range of 4.5 – 9.0, we observed that the k_{cat} values for IMP-1 decrease to a slope of 1 at high pH but the k_{cat} values also decrease to a slope of 2 at low pH. These data are fitted well into Equation 1, giving p*K*s of 8.2 and 5.3. Like the k_{cat} pH-rate profile, the k_{cat}/K_m pH-rate profile showed that k_{cat}/K_m values decrease to a slope of 1 at high pH with a p*K* of 8.4, and the k_{cat}/K_m values decrease to a slope of 2 at low pH with a p*K* of 5.2 (Fig 6B).

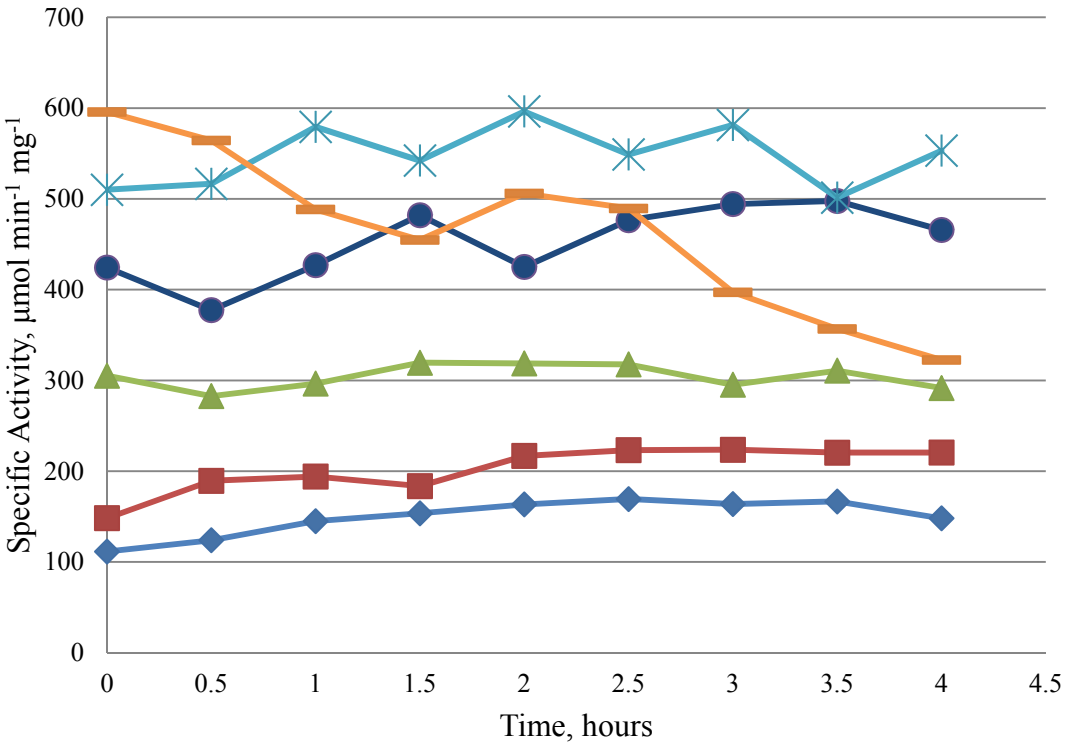

Fig 5. Graphic analysis of IMP-1 temperature stability data. The temperatures represented are 20 °C (♦), 30 °C (■), 40 °C (▲), 50 °C (●), 60 °C (☼), 70 °C (▬).

This k_{cat} pH-rate profile indicates the requirement of one group unprotonated and one group protonated for catalysis. Similarly, the k_{cat}/K_m profile indicates that unprotonated and protonated groups are involved for substrate binding and catalysis. The similarity of the pK values observed for both k_{cat} and k_{cat}/K_m pH-rate profiles suggests that in the course of the complex formation of the substrate and enzyme, there are no substantial changes in the environment of the active site pocket.

On the acidic side of the k_{cat}/K_m pH-rate profile, we were able to determine the pK value to be 5.2. This result is consistent with the fact that Laraki et al. [20] showed a decrease in the activity of IMP-1 at pH below 5.5.

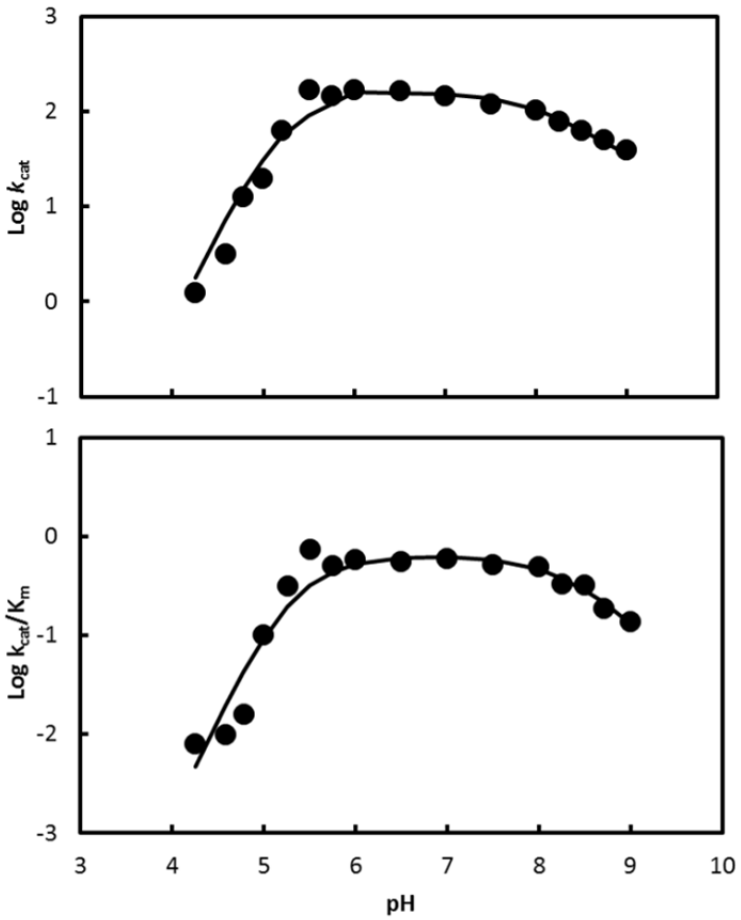

Fig 6. pH dependence of kinetic parameters for IMP-1. Data were obtained at room temperature for k_{cat} (A) and for k_{cat}/K_m (B). Units for k_{cat} and k_{cat}/K_m are s^{-1} and $\mu M^{-1} s^{-1}$, receptively. Points are experimentally determined values, and the curve for panel A is theoretical and based on fits to Equation 1.

IC_{50} of EDTA and βME

To understand the inhibition in the active site of the enzyme, we examined the effects of inhibition of IMP-1 using EDTA and βME. EDTA is a well-known inhibitor of MBLs due to the chelation of zinc ions in the active site [7] and βME was selected for inhibition due to the possibility of a zinc ion binding to sulfur group of βME. The average enzyme activity was calculated from triplicate experiments using EDTA. Based on these data, IC_{50} of EDTA was determined to be 3.0 μM (Fig. 7). For the inhibition

assay in the presence of βME, the average enzyme activity values were calculated from triplicate experiments, and the IC_{50} of βME was determined to be 315 μM. These observations suggest that even though thiol is a good functional group to bind zinc ions, the chelating molecule exhibits somewhat more effective binding to zinc ions in the active site of IMP-1.

MIC Testing of B. subtilis

The minimum inhibitory concentration (MIC), which is the lowest concentration at which no cell growth is detectable, was determined for β-mercaptoethanol (βME) in combination with 0.5 μg/mL ampicillin and either compound 4 or 8 in combination with 0.5 μg/mL ampicillin. It was determined that 12 hours was the best incubation time and gave the most reproducible results for *B. subtilis*. The MIC for βME + ampicillin against wild type *B. subtilis* was 1.43 mg/mL and 0.72 mg/mL against *B. subtilis* expressing Bla2, as expressed in Table 3.

Compounds 4 and 7 were also tested in combination with ampicillin. For compound 4 + ampicillin, however, there was no detectable inhibition of either the wild type or Bla2 carrier (Table 4). This failure was caused by the compound having become colored (dark brown) over time, and at its highest concentrations, the color of compound 4 was affecting the OD_{600} measurements of the assay. Meanwhile, the administration of compound 7 + ampicilllin exhibited an MIC of 2.0 mg/mL after 12 hours against wild type *B. subtilis* and an MIC of 1.0 mg/mL after 12 hours against the Bla2 carrier (Table 5).

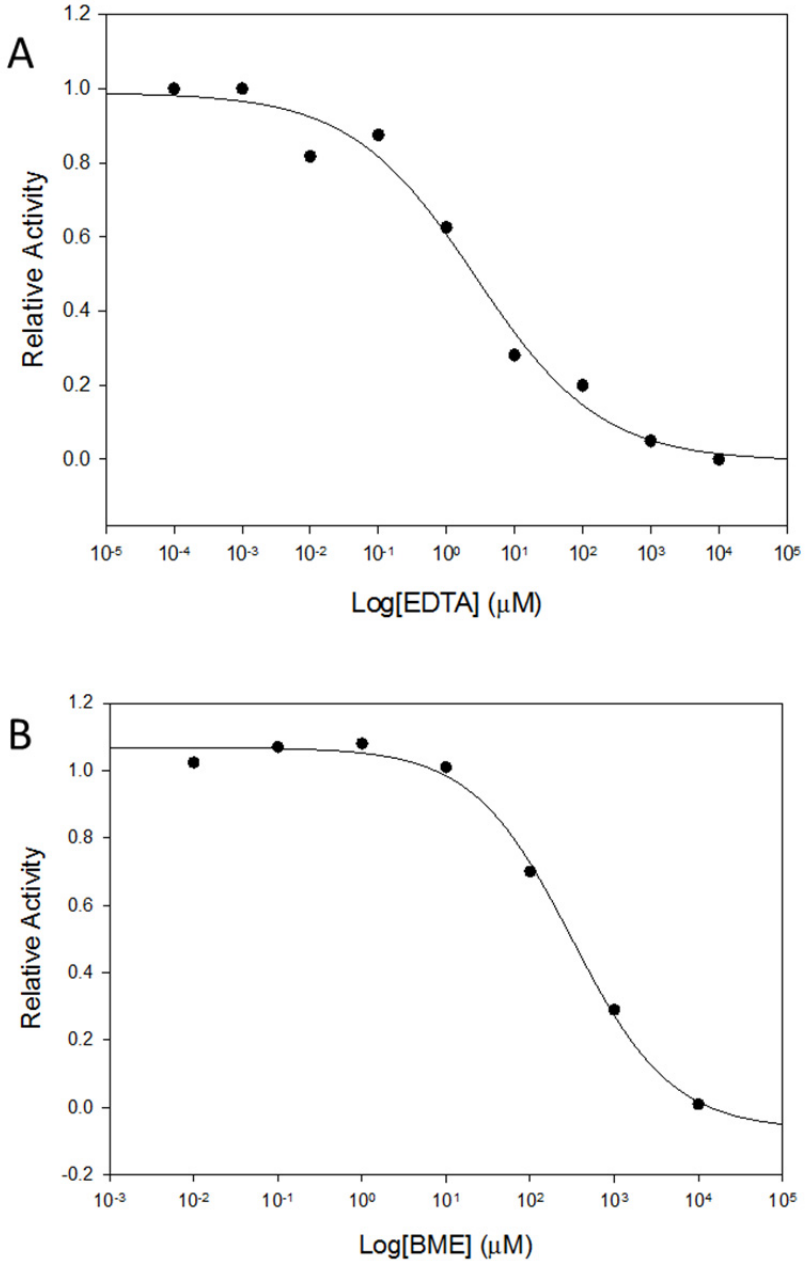

Fig 7. Determination of IC_{50} of EDTA and βME for IMP-1. The enzyme (10 mL of 0.4025 mg/mL IMP-1) was incubated with various EDTA (A) and βME (B) in 50 mM MOPS buffer (pH 7.0) at room temp before being assayed with penicillin G as a substrate.

Table 3. OD_{620} of bacterial cell solutions under the influence of 0.5 μg/mL ampicillin with various concentrations of βME over 20 h.
Bold values are indicative of the MIC value at the 16 h measurement.

		Concentration of βME, mg/mL							
Bacteria	Time, h	5.73	2.87	1.43	0.717	0.358	0.179	0.0896	0
Wild type *B. subtilis*	4	0.050	0.046	0.045	0.043	0.047	0.043	0.045	0.084
	8	0.049	0.046	0.044	0.043	0.045	0.043	0.045	0.211
	12	0.050	0.046	**0.051**	0.094	0.162	0.176	0.187	0.201
	16	0.051	0.052	0.125	0.175	0.165	0.168	0.181	0.252
	20	0.069	0.09	0.234	0.245	0.22	0.249	0.236	0.392
B. subtilis + Bla2	4	0.047	0.043	0.042	0.041	0.040	0.040	0.041	0.062
	8	0.047	0.044	0.044	0.043	0.043	0.041	0.043	0.199
	12	0.048	0.043	0.044	**0.044**	0.042	0.043	0.044	0.212
	16	0.049	0.045	0.059	0.138	0.161	0.164	0.168	0.245
	20	0.05	0.054	0.193	0.218	0.296	0.264	0.24	0.354

Table 4. OD_{620} of bacterial cell solutions under the influence of 0.5 μg/mL ampicillin with various concentrations of Compound 4 over 24 h.
There were no MIC values detected for this compound.

		Concentration of Compound 4, mg/mL							
Bacteria	Time, h	2.00	1.00	0.500	0.250	0.125	0.0625	0.0313	0
Wild type *B. subtilis*	4	0.303	0.222	0.108	0.087	0.056	0.045	0.041	0.061
	8	0.277	0.207	0.105	0.080	0.054	0.047	0.043	0.169
	12	0.258	0.197	0.101	0.076	0.051	0.047	0.071	0.217
	16	0.238	0.187	0.099	0.072	0.051	0.11	0.179	0.276
	24	0.19	0.165	0.096	0.071	0.103	0.29	0.316	0.356
B. subtilis + Bla2	4	0.328	0.221	0.114	0.086	0.061	0.042	0.040	0.080
	8	0.311	0.207	0.108	0.080	0.059	0.043	0.042	0.184
	12	0.296	0.196	0.101	0.075	0.056	0.047	0.084	0.192
	16	0.283	0.187	0.096	0.071	0.063	0.139	0.13	0.272
	24	0.255	0.171	0.093	0.067	0.181	0.253	0.293	0.374

Table 5. OD_{620} of bacterial cell solutions under the influence of 0.5 μg/mL ampicillin with various concentrations of Compound 7 over 24 h.
Bold values are indicative of the MIC value at the 12 h measurement.

		Concentration of Compound 7, mg/mL							
Bacteria	Time, h	2.00	1.00	0.500	0.250	0.125	0.0625	0.0313	0
Wild type *B. subtilis*	4	0.045	0.047	0.043	0.047	0.046	0.046	0.049	0.070
	8	0.044	0.047	0.046	0.046	0.047	0.047	0.048	0.196
	12	0.049	**0.047**	0.086	0.09	0.096	0.123	0.125	0.195
	16	0.046	0.112	0.153	0.146	0.15	0.155	0.157	0.259
	24	0.053	0.236	0.255	0.246	0.248	0.228	0.238	0.374
B. subtilis + Bla2	4	0.041	0.041	0.040	0.042	0.041	0.041	0.041	0.057
	8	0.041	0.041	0.041	0.042	0.042	0.043	0.043	0.186
	12	0.043	0.042	**0.052**	0.086	0.116	0.115	0.112	0.218
	16	0.042	0.085	0.153	0.171	0.186	0.185	0.191	0.280
	24	0.046	0.199	0.29	0.311	0.334	0.314	0.311	0.394

Confirmation of Linker Coupling to DNA Affinity Column

The DNA affinity column generated was assessed for its ability to effectively bind the G-quadruplex DNA oligonucleotide, and then subsequently release the purified oligonucleotide via washes with TE buffer at 42 and 90 °C and water at 90 °C (Table 6). The DNA binding and removal events were monitored by measuring the absorbance at 260 nm. The results indicate that after washing the column material with the template DNA in buffer A, more than half of the DNA material remained on the column (was not present in the wash solution as seen by lowered absorbance values). The column was then washed with TE buffer and water at elevated temperatures in an effort to remove the bound DNA. Some of the DNA was able to be removed from the column, however, only approximately half of the original template DNA was recovered.

Table 6. Summary of results from assay measuring the amount of template DNA being attached and subsequently removed from the prepared DNA affinity column.

Trial	Absorbance at 260 nm				
	Template DNA in Buffer A	Column Washes: Buffer A, 20°C	Column Wash: TE, 42°C	Column Wash: TE, 90°C	Column Wash: Water, 90°C
1	1.715	0.540	0.205	0.152	0.185
2	1.798	0.540	0.292	0.241	0.388
Average	1.757	0.540	0.249	0.197	0.287

The washes used to remove the template DNA from the column were then used to perform PCR, in an effort to confirm the presence of the template DNA. A control test was run alongside the experimental tests as confirmation of the PCR program used. The gel image obtained confirmed the amplification of the control test, as well as all of the experimental samples run (Fig 8).

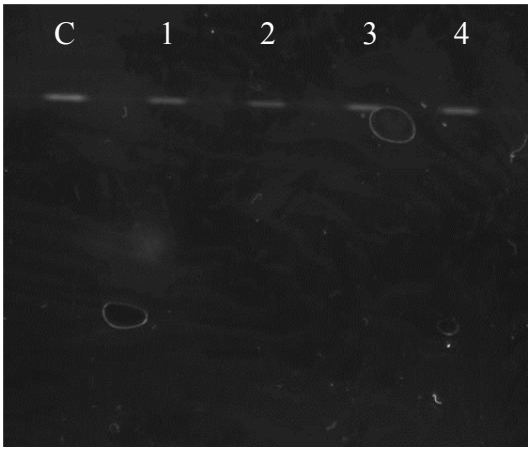

Fig 8. PCR results of washes removing template DNA from affinity column. C is a control test, run to confirm PCR program; 1 is the product of using the TE wash at 42°C as a template; 2 is the product of using the TE wash at 90°C as a template; 3 is the product of using the first water wash at 90°C as a template; 4 is the product of using the second water wash at 90°C as a template.

CHAPTER FOUR

Discussion

Purification of IMP-1 and Kinetic Parameters

Previously reported purification methods for constructs expressing IMP-1 by Watanabe et al. and Juan et al. made use of sulfate precipitation followed by two or more column chromatographs[11,25]. In a more developed method than those, reported by Laraki et al., the sulfate precipitation step was eliminated, however, two columns were still necessary for full purification[20]. Through this work, we have developed a simplified purification method for IMP-1 with only one affinity chromatography step, based on the protocol exampled for the MBL Bla2 laid out by Schlesinger et al[19].

In our hands, although a significant loss of activity was observed during the purification process, this simplified, optimized method allowed us to obtain a large quantity of highly purified enzyme, i.e., about 29 mg of pure protein per one liter of culture, comparable to the quantities reported by Laraki et al[20]. The specific activity of the purified enzyme toward penicillin G was 183.63 μmol min^{-1} mg^{-1}.

We determined the Michaelis-Menton kinetic parameters of IMP-1 using penicillin G as a substrate; hydrolysis was measured at 235 nm and the extinction coefficient used to calculate activity was 775 $M^{-1}s^{-1}$, according to Laraki et al[20]. The results showed that the K_m and V_{max} parameters for IMP-1 with penicillin G were 390 μM and 156 μmol min^{-1} mg^{-1} (Fig 4), and the k_{cat} and the catalytic efficiency (k_{cat}/K_m) were 72.1 s^{-1} and 0.186 s^{-1} μM^{-1}, respectively. We can compare these results to the K_m, k_{cat},

and the catalytic efficiency (k_{cat}/K_m) reported by Rasmussen and Bush, which were 440 μM, 370 s^{-1} and 0.840 s^{-1} μM^{-1}, respectively[26]. Similarly, the K_m, k_{cat}, and the catalytic efficiency (k_{cat}/K_m) reported by Laraki et al. for IMP-1 with penicillin G were 600 μM, 280 s^{-1} and 0.470 s^{-1} μM^{-1}, respectively.

In addition, the specific activity observed for IMP-1 after purification by the Ni^{2+}-affinity column was 183.63 units/mg and had a more than 7-fold increase in specific activity over the course of the purification (Table 1). The purified IMP-1 appeared as one primary band on SDS-PAGE at approximately 28 kDa (Fig 3), which is in excellent agreement with the calculated molecular weight of 27,692.5 Da. This shows that the application of the Ni^{2+} affinity column as a simple purification method for IMP-1 is an effective means as well.

Effects of Temperature and pH

Some studied β-lactamases have been characterized as thermostable proteins [22]. Previously published literatures showed that while the MBL from *Bacillus cereus,* BcII, is stable at 60 °C [22], the MBL from *B. anthracis,* Bla2, is unstable at 60 °C [19]. Even though BcII shares 92% amino acid sequence similarity with Bla2 [27,28], the 8% sequence difference showed a significant change in thermostability [19]. It seems likely that the environment of active sites significantly contribute to zinc ion binding stability, particularly for the second zinc ion binding site [29]. Thus, it is of interest to determine whether IMP-1 is stable at high temperatures.

The results obtained here show that IMP-1 was able to withstand prolonged exposure to 60 °C with high levels of activity (Fig. 5). Once at 60 °C, IMP-1 reaches a maximum activity after 2 h and maintained a high level of activity during the 4 h period,

with the final activity measured being 92.8% of the maximum activity. From this we are able to conclude that IMP-1 is a thermostable MBL, and that the zinc ion binding in the active site of IMP-1 may be stronger than other MBLs that are not able to withstand these high temperatures.

When the kinetic assay for IMP-1 was altered to determine the effect of pH, we observed the enzyme to be most active between pH 6.0 and 7.0 as shown in Fig 6A. Over the tested pH range of 4.5 – 9.0, the k_{cat} values for IMP-1 decreased to a slope of 1 at high pH, but the k_{cat} values also decreased to a slope of 2 at low pH. This data was fitted into Equation 1, and showed p*K*s of 8.2 and 5.3. Similarly, the k_{cat}/K_m pH-rate profile showed that k_{cat}/K_m values decreased to a slope of 1 at high pH with a p*K* of 8.4, and the k_{cat}/K_m values decreased to a slope of 2 at low pH with a p*K* of 5.2 (Fig 6B).

Unlike the typical bell-shaped curve that is characteristic to pH-rate profiles, where the slope is only 1 at low pH [30], the k_{cat} pH-rate profile obtained for IMP-1indicates the requirement for one group unprotonated and one group protonated for catalysis. Similarly, the k_{cat}/K_m profile provides idea that unprotonated and protonated groups are involved for substrate binding and catalysis. The similarity of the p*K* values observed for both k_{cat} and k_{cat}/K_m pH-rate profiles suggests that in the course of the complex formation of the substrate and enzyme, there are no substantial changes in the environment of the active site pocket.

In addition, previous studies by Bounaga et al. [30] reported that the k_{cat}/K_m pH-rate profile using BcII for penicillin G showed a pK value of 5.6 on the acidic side with a slope of 2, which suggests that two protonation events would occur with similar pK value. Further studies in this report assigned the p*K* value to both Zn1-hydroxide and to

Asp90 of BcII, which is corresponding to Asp81 of IMP-1 as shown in Figure 1. Hence, it appears that the water molecule bound to Zn1 and Asp81 would account for the slope of 2. There are no published sources to date that have reported a k_{cat}/K_m pH-rate profile using IMP-1 for penicillin G hydrolysis and showed a pK value in the acidic range of pHs.

IC_{50} of EDTA and βME

We examined the effects of inhibition of IMP-1 using EDTA and βME to gain an understanding of the active site of the enzyme, as well as to weigh the effects of these inhibitors on IMP-1 versus other MBLs. EDTA is a well-known inhibitor of MBLs due to the chelation of zinc ions in the active site [7] and βME was selected for inhibition testing due to the possibility of a zinc ion binding to the sulfur group of βME.

The IC_{50} of EDTA against IMP-1 was determined to be 3.0 μM and the IC_{50} of βME against IMP-1 was determined to be 315 μM (Fig. 7). Compared to other subclass B1 MBLs, IMP-1 appears more susceptible to EDTA. For example, the IC_{50} of EDTA for DIM-1 from *P. stutzeri* and VIM-1, -2 and -13 from *P. aeruginosa* are 176, 9.3, 50 and 253 μM, respectively [25,31]. However, IMP-1 is less susceptible to EDTA than Bla2 (IC_{50} of EDTA for Bla2 is 0.63 μM) [19] There were no other studies that tested βME as an inhibitor of IMP-1. The results of our βME inhibition test suggest that even though thiols are good functional groups for binding zinc ions, the chelating molecule (EDTA) exhibits somewhat more effective binding to zinc ions in the active site of IMP-1.

MIC Testing of B. subtilis

An MIC is the lowest concentration of an inhibitor at which no bacterial growth is detectable (by a measure of optical density, or OD). In this study the MIC was determined for βME in combination with 0.5 μg/mL ampicillin and either compound 4 or 7 in combination with 0.5 μg/mL ampicillin. These tests were performed on *Bacillus subtilis* cells that were either wild type or expressing Bla2 as part of a collaborative study to test these hydroxamate compounds for their effectiveness as potential broad spectrum MBL inhibitors in both Gram positive and Gram negative bacterial strains.

The MIC for βME + ampicillin against wild type *B. subtilis* was 1.43 mg/mL and 0.72 mg/mL against *B. subtilis* expressing Bla2, as expressed in Table 3. These MIC values were detected at 12 hours, which over multiple trials was found to be the best incubation time for giving reproducible results. In this set of experiments, βME is used as a control test. The IC_{50} value of βME against Bla2 had been determined as 218 μM as part of a collaborative test performed by Sara R. Schlesinger. Due to the micromolar concentration range of the IC_{50} value, we would expect for βME to also inhibit microbial growth in cells expressing this enzyme. The MIC value of 720 μg/mL is more than three times the IC_{50} value, and while βME is able to inhibit cellular growth at a micromolar concentration, this large gap between MIC and IC_{50} values may indicate that the interactions of βME with the cell are nonspecific for Bla2.

The MIC test for compound 4 + ampicillin against both the wild type and Bla2 expressing *B. subtilis* showed no detectable inhibition (Table 4). This failure was caused by the compound having become colored (dark brown) over time, and at its highest

concentrations, the color of compound 4 was affecting the OD_{620} measurements of the assay.

Alternatively, the administration of compound 7 + ampicilllin exhibited an MIC value of 1.0 mg/mL after 12 hours against the Bla2 carrier, as seen in Table 5. We can compare this MIC value to a previously determined IC_{50} value for of compound 7 against Bla2, performed by Megan Hermann. The IC_{50} value determined was 0.48 ± 0.2 nM. In comparison, the MIC value obtained was substantially greater than the IC_{50}value. Again, this discrepancy could be attributed to nonspecific binding of compound 7 to *B. subtilis*, decreasing the concentration of compound available to inhibit the enzyme. However, if we are able to employ concentrations of compound 7 that are within the enzyme inhibition range, the ampicillin used in combination with compound 7 will be free to inhibit cell growth without risk of hydrolysis.

DNA Affinity Column

We constructed a DNA affinity column as the first step towards a future project that is focused on the study of G-quadruplex aptamers as potential enzyme inhibitors. The DNA affinity column serves as a mode of purification for the template DNA, which had twelve randomized nucleotide bases and whose sequence follows: 5'-GCGGAT-CCGGGGGGT(N_6)TGGGGGGATGGGGGGT(N_6)TGGGGGGAAGCTTGC-3'. This affinity column was generated using silica that had a short oligonucleotide sequence, complementary to the template DNA, synthetically linked. This oligonucleotide allowed for the binding and subsequent removal of the template DNA to the column. The results of our assay indicated that we were successful in generating an affinity column that was specific for our DNA template, as seen in Table 6. More than half of the template DNA

that was loaded onto the column had attached, evidenced by low absorbance values in the wash buffer after having been blanked with the original template DNA solution. In addition, subsequent washes of the column with buffer at elevated temperatures allowed for the removal of approximately half the attached DNA. While these results do not have maximal recovery of purified template DNA, there is promise. Further modifications are necessary to optimize the column conditions in order to gain a useful tool in the purification of aptamer sequences.

CHAPTER FIVE

Conclusions

The metallo-β-lactamase from *Pseudomonas aeruginosa*, IMP-1, has drawn increasing attention due to its increasing prevalence and its ability to spread across bacterial species. Thus, in order to effectively study and characterize this enzyme, it is necessary to obtain a pure sample of enzyme. In this study, we have successfully introduced a facile method for obtaining large quantities of pure IMP-1. To characterize the IMP-1 enzyme, temperature stability and pH dependent studies were completed. This enzyme was thermostable, showing activity for prolonged periods at high temperatures, indicating that the zinc binding in the active site of IMP-1 is as stable, or more stable, than other MBLs. The pH dependent experiments revealed that the enzyme is most stable within a pH range of 6.0-7.0, based on activity levels. These experiments also provided insight into the acid-base chemistry of the active site, suggesting that IMP-1 requires one protonated and one unprotonated group for catalysis to occur. In addition, we were able to identify a pK in the acidic range of 5.2. No other published works have reported an acidic pK value for the hydrolysis of penicillin G by IMP-1. The inhibition studies with EDTA and βME show that IMP-1 is susceptible to these compounds, with the zinc chelating molecule being the more effective inhibitor, which provides us with information for designing effective inhibitors of IMP-1 in the future.

In a collaborative study of novel hydroxamate compounds as inhibitors of the metallo-β-lactamase from *Bacillus anthracis*, Bla2, MIC testing was done to investigate

the potential of these compounds (4 and 7) as inhibitors of Gram positive cell growth. This testing was performed on *Bacillus subtilis* cells either with or without Bla2. While compound 4 displayed no inhibition of cell growth, compound 7 was successful in deterring cell growth in the presence of 0.5 μg/mL ampicillin. Thus, compound 7 could be further investigated as a lead for the development of effective MBL inhibitors.

In a final study, we focused on the development of G-quadruplex aptamers as potential enzyme inhibitors. We began foundation work by designing a template DNA with twelve random nucleotide bases, and generated a DNA affinity column for use as a tool in purifying this specific ssDNA sequence. Analysis of the affinity column displayed its ability to effectively purify the template by binding and subsequently releasing the target DNA. This tool can be further optimized for its use in purification of aptamer molecules designed to target enzymes of interest.

APPENDIX

APPENDIX

Supplementary Figures

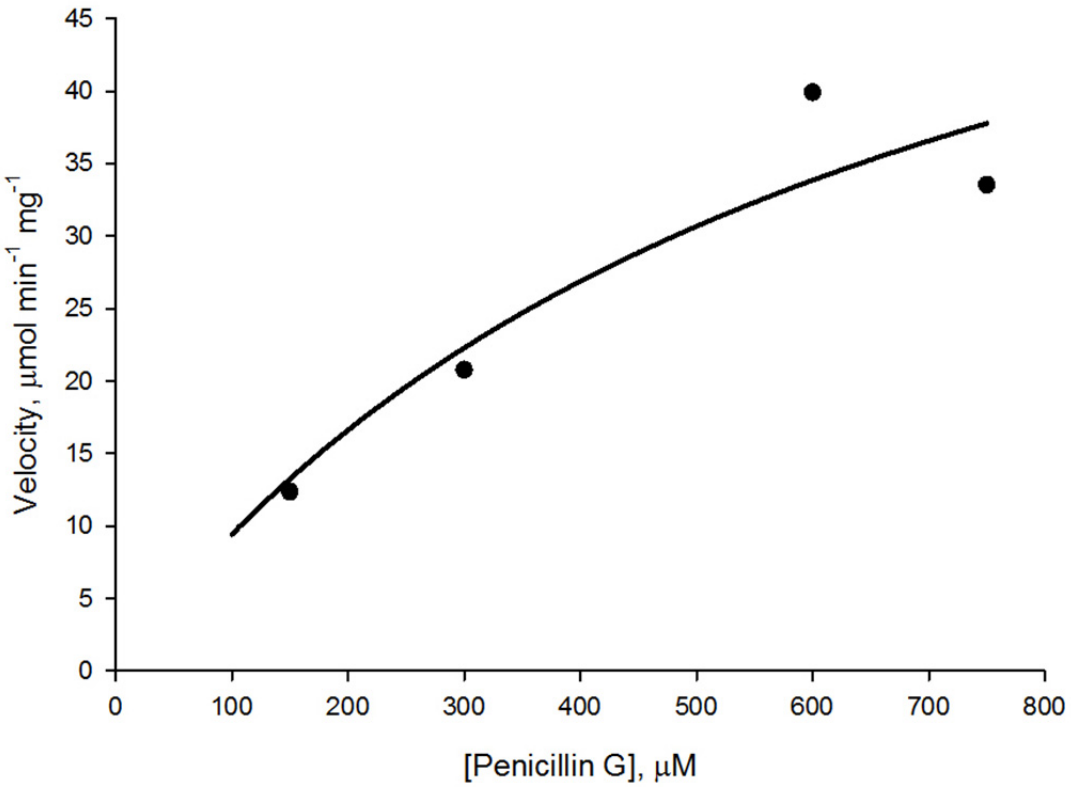

Fig. A.1 Michaelis-Menton curve of IMP-1 at pH 4.5. The K_m and V_{max} at pH 4.5 is 648.2 µM and 70.45 µmol/min/mg, respectively.

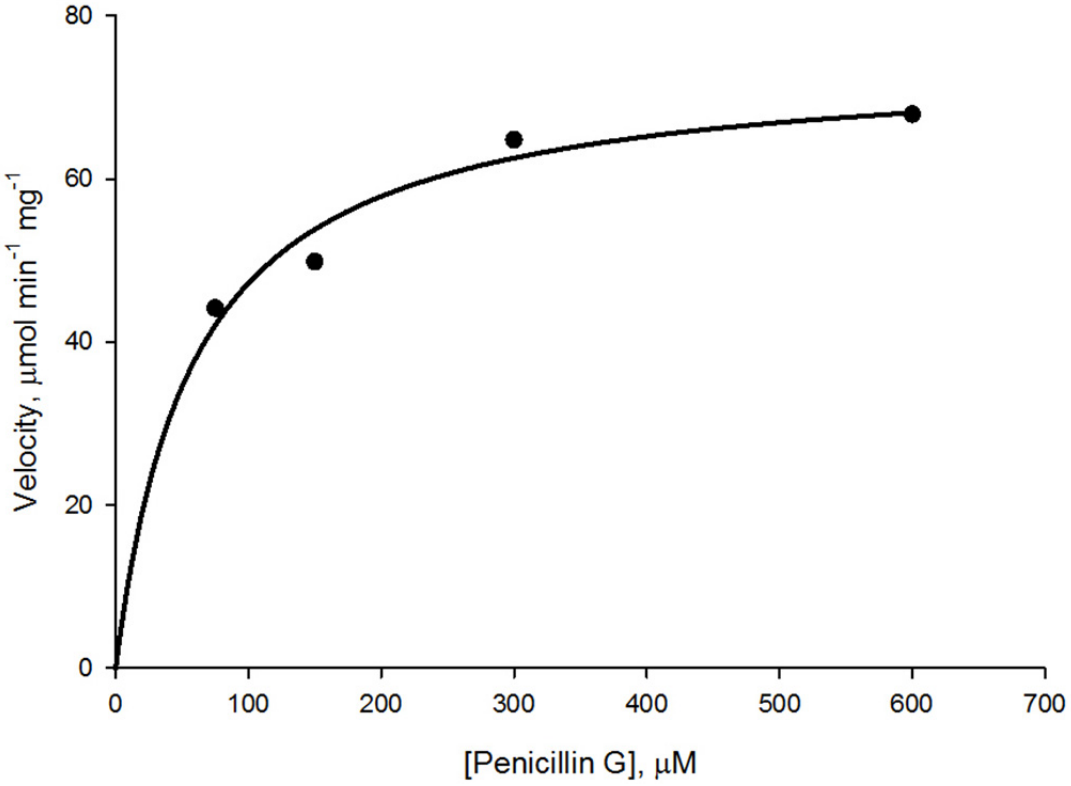

Fig A.2 Michaelis-Menton curve of IMP-1 at pH 4.75. The K_m and V_{max} at pH 4.75 is 58.2 µM and 74.71 µmol/min/mg, respectively.

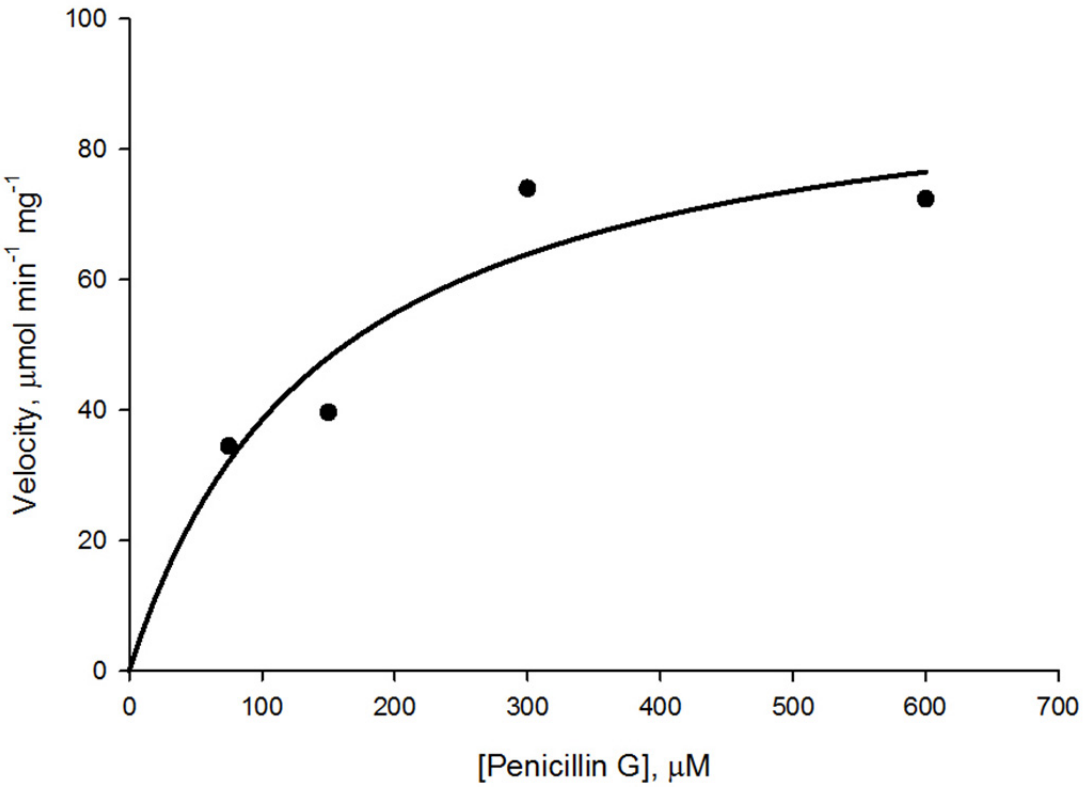

Fig A.3 Michaelis-Menton curve of IMP-1 at pH 5.0. The K_m and V_{max} at pH 5.0 is 147.5 µM and 95.35 µmol/min/mg, respectively.

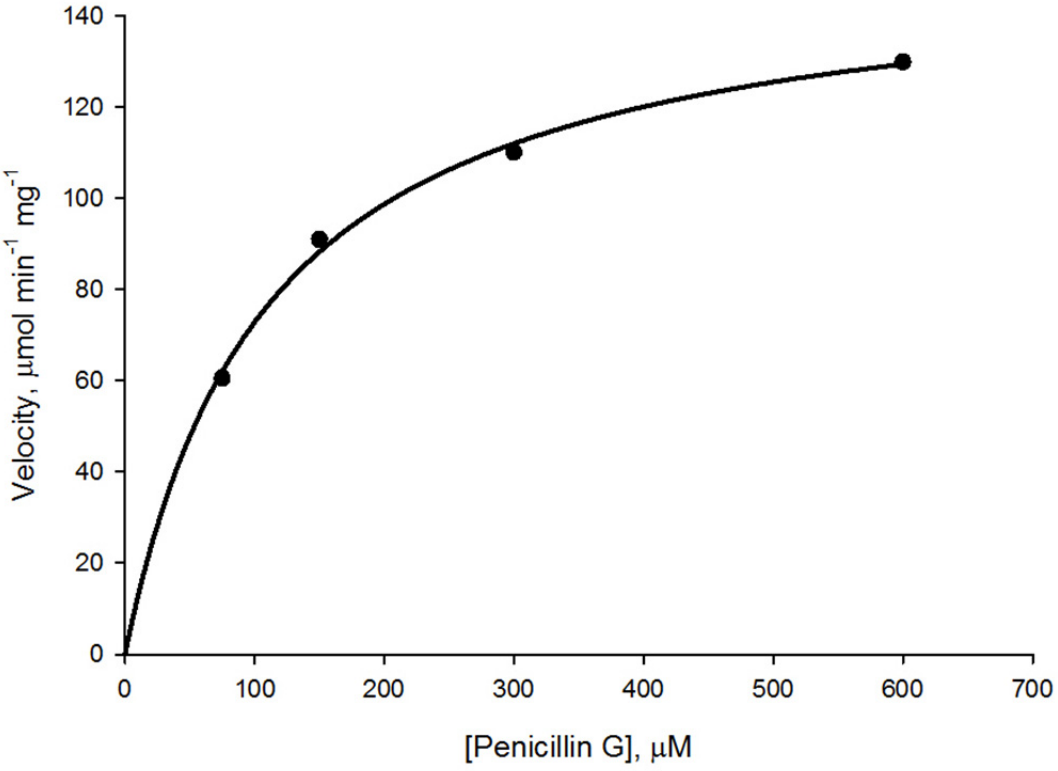

Fig A.4 Michaelis-Menton curve of IMP-1 at pH 5.25. The K_m and V_{max} at pH 5.25 is 110.4 µM and 153.29 µmol/min/mg, respectively.

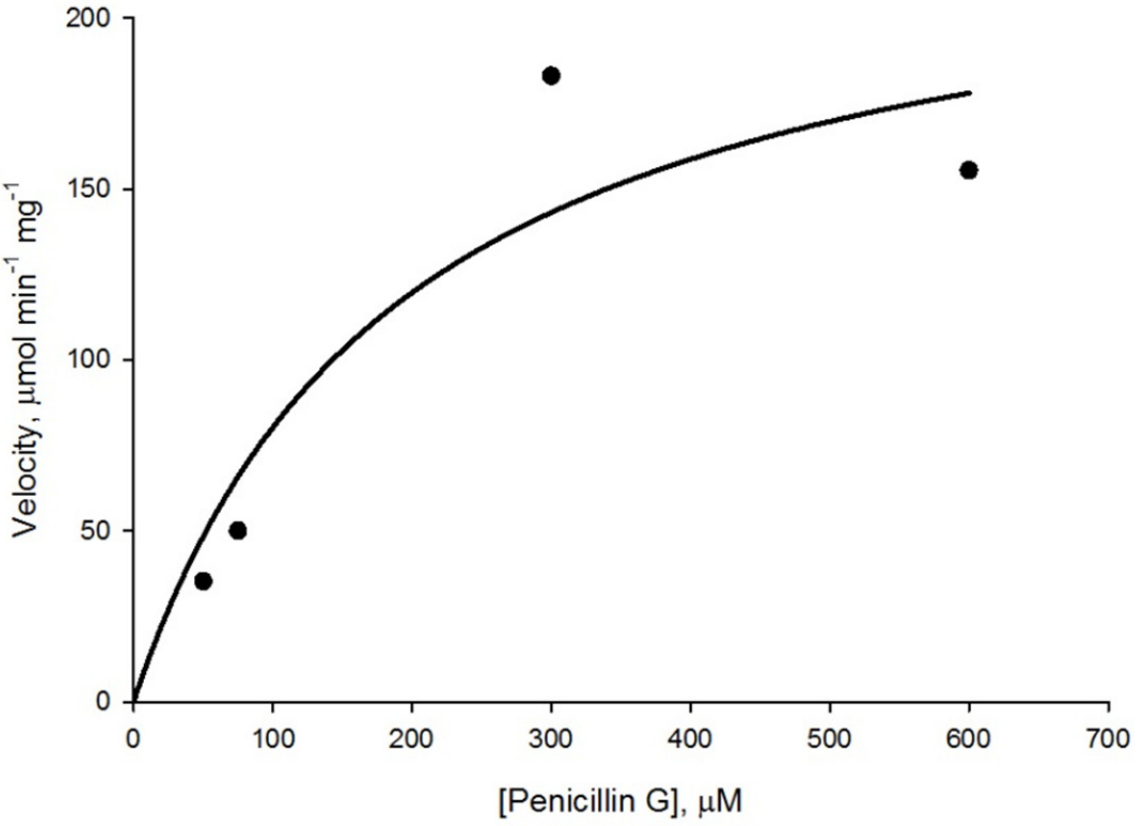

Fig A.5 Michaelis-Menton curve of IMP-1 at pH 5.5. The K_m and V_{max} at pH 5.5 is 193.4 µM and 235.46 µmol/min/mg, respectively.

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