ABSTRACT

Characterization and Purification of TEM-1 β-Lactamase

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One method of antibiotic resistance in bacteria to the β-lactam class of antibiotics is through the production of β-lactamase enzymes. One of the β-lactamases is an enzyme from *Escherichia coli* (TEM-1) that plays a role in the hydrolysis of β-lactam antibiotics by using a serine residue at the active site. To better understand the mechanism of the enzyme by kinetic analyses, TEM-1 has been cloned, overexpressed, and purified. The effects of different buffers and changes in ionic strength were tested to determine if changes in these conditions had an effect on enzyme activity. As a result, we found that the K_m value was 20.2 μM and the V_{max} was 608.8 μM/min (30 mM Tris, 250 mM NaCl, pH 7.5). The pH dependence of TEM-1 was determined as the pH versus the log of k_{cat}/K_m with two pK_a values of 5.5 and 9.0. These studies will provide an avenue for better understanding the acid base mechanism of TEM-1 β-lactamase.

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CHARACTERIZATION AND PURIFICATION OF TEM-1 β-LACTAMASE

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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December 2013

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ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to Dr. Sung-Kun Kim for offering me a position in his research group and for his continual support and guidance as my thesis advisor. I would also like to thank Sara Schlesinger Mieke Lahousse and Nicole Solida who have provided guidance throughout my undergraduate research career. I would also like to thank all the faculty members of Baylor University who have provided mentorship and have reached out to me throughout my undergraduate career. Lastly I would like to thank my parents for their love and support in all that I have done.

DEDICATION

I dedicate this work to my mother who has been a true pillar of strength to me in times of great difficulty. I hope that I will continue to honor you through all my endeavors. Thank you for the courage and perseverance you instill in me and for sacrificing so much for my wellbeing.

CHAPTER 1

Introduction

The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. Antibiotics have revolutionized medicine in many respects, and countless lives have been saved; their discovery was a turning point in human history. Regrettably, the use of these wonder drugs has been accompanied by the rapid evolution of resistant strains of bacteria. Since the discovery of penicillin there has been an antimicrobial war waged against pathogenic microorganisms. Penicillin was crucial for combating staphylococcal infections and proved to be an effective therapeutic agent.[1] This antibacterial weapon triggered the genesis of a group of antibiotics named β-lactams which are currently the most widely used antibacterial agents.[2] This group of antibiotics which include penicillins, cephalosporins, and carbapenems, represent 60% of the overall antibiotic usage worldwide.[3] The group consists of a diverse array of molecules whose structures are progressively different from the original molecule and share only the common feature of the β-lactam ring which is a four membered ring containing an amide group.

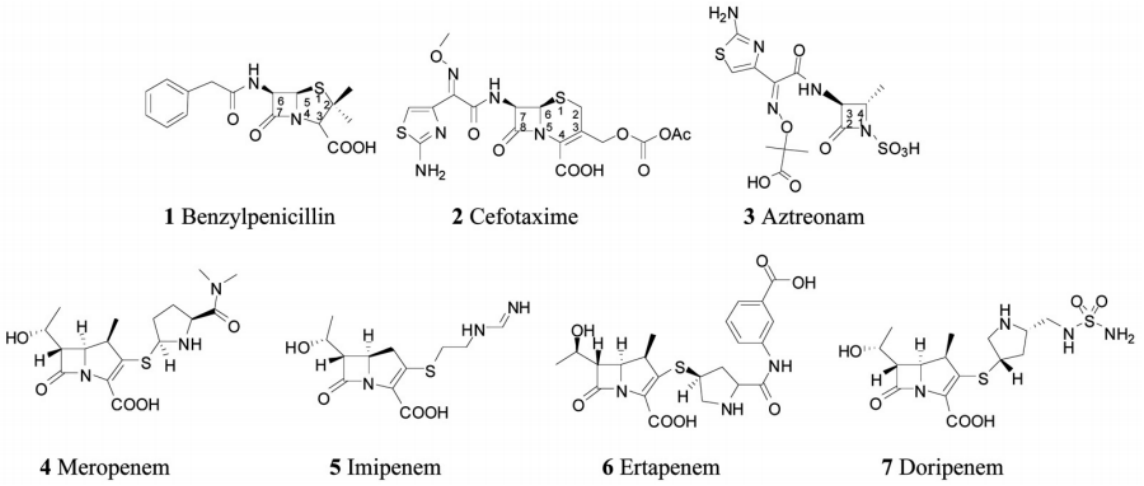


Figure 1. Chemical structures of common β-lactam antibiotics. Compounds 1 to 7, a representative penicillin (compound 1), an extended spectrum cephalosporin (compound 2), a monobactam (compound 3), and carbapenems (compounds 4 to 7). The numbering scheme for penicillins, cephalosporins, and monobactams is shown. (From Drawz, S. M.; Bonomo, R. A. *Clin. Microbiol. Rev.* **2010**, *23*, 160-201.)

β-lactam antibiotics disrupt the biosynthesis of peptidoglycan which is a major structural component of bacterial cell walls. This peptidoglycan layer is primarily composed of alternating β (1,4)-linked monosaccharides, specifically N-acetylglucosamine and N-acetylmuramic acid. The latter is modified by a pentapeptide that always ends with two D-alanine residues. Cross-linking of peptidoglycan units is catalyzed outside the cytoplasmic membrane by cell wall transpeptidase enzymes. In this cross-linking process, a peptide bond is formed between penultimate D-alanine on one chain and the pimelic acid (in Gram-negative) or L-lysine (in Gram-positive) residue on the other.[4] The terminal D-alanine is cleaved off after the linkage is formed with the penultimate residue. β-lactam antibiotics effectively inhibit bacterial transpeptidases, consequently they are often called penicillin binding proteins (PBP).[4] The cell wall

functions in maintaining the rigidity and shape of the cell wall and protecting the bacterium from its own osmotic pressure.

Soon after clinical usage had begun penicillin resistant strains of *Staphylococcus aureus* emerged. An enzyme that could neutralize the bacteriocidal activity of Penicillin was isolated from *S.aureus* and was initially labeled "penicillinase" due to its penicillin hydrolyzing properties.[5] Later it was discovered that these enzymes could inactivate certain cephalasporins as well which led to the more general term that encompassed all classes of β-lactam hydrolyzing enzymes called "β-lactamases."[5] These enzymes have been designated by the Nomenclature Committee of the International Union of Biochemistry as "enzymes hydrolyzing amides, amidines and other C-N bonds."[6]

Classification Schemes for β-lactamases

The β-lactamase enzymes work by hydrolyzing the four membered β-lactam ring rendering the antibiotic ineffective and making the bacterium resistant against the antibiotic. Historically, β-lactamase enzymes have been classified according to their structure and function. The Bush-Jacoby-Medeiros Classification is the primary scheme of classification based on substrate and inhibitor profiles.[7] The problem with phenotypic classifications systems like this is that point mutations greatly alter substrate specificity and inhibitor susceptibility.[6] The Ambler classification scheme proposed in 1980 classified the enzymes according to molecular structure and amino acid sequence and will be used for our purposes.[6] This sequence based classification is unaltered by mutations and is the easiest method of describing these enzymes since it divides the enzymes into molecular cases A through D. Classes A, C and D have an active site serine based

mechanism and class B utilizes a divalent metal cation such as Zn^{2+} for its catalytic mechanism.[1] Class B enzymes are the metallo-β-lactamases and are structurally and enzymatically different from the serine β-lactamases.[8] Table 1 summarizes some of the historical classification schemes for β-lactamases.

Bush-Jacoby-Medeiros group	1989 Bush group (44)	Richmond-Sykes class (253)	Mitsuhashi-Inoue type (194)[a]	Molecular class (2, 121, 132)	Preferred substrates	Inhibited by:		Representative enzymes
						CA[b]	EDTA	
1	1	Ia, Ib, Id	CSase	C	Cephalosporins	−	−	AmpC enzymes from gram-negative bacteria; MIR-1
2a	2a	Not included	PCase V	A	Penicillins	+	−	Penicillinases from gram-positive bacteria
2b	2b	III	PCase I	A	Penicillins, cephalosporins	+	−	TEM-1, TEM-2, SHV-1
2be	2b′	Not included except K1 in class IV	CXase	A	Penicillins, narrow-spectrum and extended-spectrum cephalosporins, monobactams	+	−	TEM-3 to TEM-26, SHV-2 to SHV-6, *Klebsiella oxytoca* K1
2br	Not included	Not included	Not included	A	Penicillins	±	−	TEM-30 to TEM-36, TRC-1
2c	2c	II, V	PCase IV	A	Penicillins, carbenicillin	+	−	PSE-1, PSE-3, PSE-4
2d	2d	V	PCase II, PCase III	D	Penicillins, cloxacillin	±	−	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	2e	Ic	CXase	A	Cephalosporins	+	−	Inducible cephalosporinases from *Proteus vulgaris*
2f	Not included	Not included	Not included	A	Penicillins, cephalosporins, carbapenems	+	−	NMC-A from *Enterobacter cloacae*, Sme-1 from *Serratia marcescens*
3	3	Not included	Not included	B	Most β-lactams, including carbapenems	−	+	L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*
4	4	Not included	Not included	ND[c]	Penicillins	−	?	Penicillinase from *Pseudomonas cepacia*

[a] Csase, cephalosporinase; PCase, penicillinase; CXase, cefuroxime-hydrolyzing β-lactamase.
[b] CA, clavulanic acid.
[c] ND, not determined.

Table 1. Classification schemes for bacterial β-lactamases

Mechanism of Class A β-lactamases

The mechanism of Class A β-lactamases closely resembles that of serine proteases.[9] There are two proposed mechanisms for the class A β-lactamase catalytic mechanism: acylation and deacylation.[10] During the acylation step, a proton is removed from the catalytic Ser^{70} residue.[11] The process by which the proton is removed is

currently under debate as to whether it is transferred to the side chain amine group of Lys^{73} directly or if it is transferred to a water molecule that is coordinated by Ser^{70}, Glu^{166}, and Asn^{170}.[12] Currently, there is evidence for both pathways, and it has been proposed that perhaps both actions are possible and function together to remove the proton from Ser^{70}. The oxygen of Ser^{70} then attacks the carbonyl group to break the amide bond of the β-lactam forming the acyl-intermediate.[12] The water that was coordinated by Ser^{70}, Glu^{166}, and Asn^{170} in acylation is activated to attack the covalent bond formed in the acyl-intermediate structure, causing hydrolysis of the β-lactam antibiotic and regeneration of the enzyme. [12]

Spread of Antibiotic Resistance.

The problem of resistance to β-lactams is ubiquitous and has spurred a cycle of antibacterial therapy and subsequent antibiotic resistance. Genetic dissemination of β-lactamase genes has given rise to resilient strains of bacteria. This genetic transmission can occur both within the same species and between other species via plasmids and transposable elements.[1] Pathogenic bacteria employ unique and intricate mechanisms to enhance their rate of survival and improve their resilience. One method used to accomplish this is the ability of porin proteins in the bacterial cell membrane to alter their configuration in order to decrease the permeability of antibiotics such as β-lactams.[2] Some bacteria including *Pseudomonas aerugionosa* have the ability to pump β-lactamase enzyme into their biofilm and periplasmic space using efflux pumps.[3]

The spread of resistance is further exacerbated by the promiscuous use of antibiotics. Pharmaceutical companies attempted to combat the development of resistance

by introducing a wide range of β-lactam antibiotics such as ceftazidime and cefotaxime, as well as new classes of extended spectrum β-lactams with increased stability towards β-lactamase enzymes, including monobactam agents such as aztreonam and carbapenams which proved to be very effective against serine based β-lactamases.[3] Within a short period of time bacteria had already begun developing β-lactamases that conferred resistance against these antibiotics. Clinicians utilized a combination of extended spectrum β-lactams as well as β-lactamase inhibitors against these newly resistant strains of bacteria. However, the overuse of these antibiotics has placed significant selection pressure on bacteria resulting in the development of resistance.[1] Resistance is more common where antibiotic usage is more frequent.[7] Intensive care units, hematology departments and burn units, as well as developing nations where infection control is at a low level exhibit higher incidence of resistance.[5] Furthermore, researchers have discovered that various substitutions in the active site of the enzyme arising from point mutations confer this resistance by making the active site more accessible for the substrate β-lactams.[3]

TEM-1 β-Lactamase

The first plasmid-mediated β-lactamase was identified in E. coli in 1963, and was named "TEM" after the patient from whom it was isolated and has since then been acquired by numerous pathogens.[6] TEM-1 catalyzes the hydrolysis of β-lactams near the diffusion limit (10^8 M^{-1} sec^{-1}).[13] TEM-1 is encoded by the bla_{TEM-1} gene and present on transposons Tn2 and Tn3 and is the most common plasmid-mediated β-lactamase.[1] Many extended spectrum β-lactamases have originated from this enzyme through the

accumulation of amino acid substitutions near the active site, increasing the catalytic efficiency (k_{cat}/K_m) of the hydrolysis reaction.[1] Currently, there are over 170 TEM extended spectrum β-lactamases (ESBL) or inhibitor-resistant TEM (IRT) variants identified from clinical isolates.[6] Point mutations through amino acid substitutions that have occurred at or near the active site have broadened the substrate specificity, and increased resistance against extended spectrum β-lactams as well as inhibitors.[6] However it has been demonstrated that substitutions that alter the catalytic activity of the enzyme result in a subsequent decrease in enzyme stability.[6] In response to this, global suppressors of protein instability have evolved as secondary antibiotic resistance mutations that stabilize the enzyme and facilitate the evolution of TEM ESBLs.[6]

The proliferation of new variants of TEM-1 β-lactamase has been investigated for years. Structural studies of the enzyme using X-ray crystallography and other methods have helped elucidate the catalytic mechanism of TEM-1 β-lactamase and have helped researchers identify important residues. These residues occasionally mutate to produce broad spectrum β-lactamases with extended substrate specificities or inhibitor resistant types of enzyme. The general mechanism of class A β-lactamases which are penicillinases involve the nucleophilic attack by Ser^{70} on the carbonyl carbon of the amide bond in the β-lactam ring resulting in the formation of an acyl-enzyme intermediate.[1] For TEM-1 the reaction begins with the formation of the precovalent encounter complex, and moves through a high-energy acylation tetrahedral intermediate to form an ester through the catalytic residue Ser70.[14] This acyl-enzyme is then attacked by a hydrolytic water to form a high energy deacylation intermediate which collapses to form the hydrolyzed product.[15] This mechanism requires a catalytic base to activate the

serine nucleophile to attack the amide bond of the substrate and, following formation of the acyl-enzyme, to activate the hydrolytic water for attack on the ester center of the adduct.[15] Lys^{73} and Glu^{166} are important residues that play a role in the catalytic activity by activating the serine residue.[1] There has been controversy regarding the exact role of these residues with Lys^{73} originally being thought to provide an appropriate environment for acylation and that Glu^{166} located on the omega loop was positioned to catalyze the deacylation of the enzyme substrate complex by acting on the serine residue either directly or through a water molecule.[1] Mutagenic studies and spectroscopic studies have shown that Lys^{73} functions in the acylation and deacylation of the enzyme and Glu^{166} functions as a general base in both these steps as well and plays a critical role in the positioning and activation of a water molecule.[1] Acylation and deacylation steps have similar and high rate constants (for example $k_2=2800s^{-1}$, $k_3=1500s^{-1}$ for *E.coli* TEM-1 with Penicillin G as a substrate).[16] The fact that both these steps occur so fast suggests that the chemical events occurring in the catalytic mechanism have minimum activation energy barriers. Electrostatic analysis of the enzyme structure has proven to be useful in elucidating this mechanism which involves general acid base catalysis and electrostatic stabilization.[16]

TEM-1 like most other β-lactamase enzyme exhibits Michaelis-Menten kinetics where the rate is given by the Michaelis-Menten equation $V = V_{max}*S/(K_m + S)$. Occasionally however the enzyme veers from the general trend by exhibiting burst kinetics with acylation much faster than deacylation or because of a branched pathway with either the acyl-enzyme intermediate or the Michaelis complex.[6]

The pK_a values (ionization constants) of the active-site residues in an enzyme are of importance to the functionality of the catalytic mechanism of the enzyme. Often catalysis is initiated by the transfer of a proton from a protein residue (the proton donor) to the substrate, and one of the steps in an enzymatic reaction mechanism is normally a nucleophilic attack on a substrate atom or the stabilization of a positively charged intermediate. For catalysis to take place the proton donor must be protonated, and the protein residue with a free lone-pair or a negative charge must perform the nucleophilic attack and stabilization of the intermediate. Many enzymes function optimally at pH values around 7, and use an aspartic acid or a glutamic acid as the proton donor. For these enzymes, it is necessary to shift the pK_a values of the proton donor upward to a value that is nearer to neutral pH than the normal Glu and Asp pK_a values of 4.0 and 4.4. Knowledge of the pK_a values of the active-site residues can therefore aid in the identification of the reaction mechanism of the enzyme. In the case of TEM-1 the p*K*a's of key residues in the active site include 12.0 (Lys^{73}), 4.6 (Glu^{166}), 11.6 (Lys^{234}).[9]

β-lactamase Inhibitors

There are two main ways that β-lactamase mediated resistance is targeted using compounds that bind to the active site of the enzyme. First by creating substrates that reversibly and/or irreversibly bind the enzyme with high affinity but form unfavorable steric interactions as the acyl-enzyme, or secondly developing mechanism-based or irreversible "suicide inhibitors.[17]" Examples of the former are extended-spectrum cephalosporins, monobactams, or carbapenems which form acyl-enzymes and adopt catalytically incompetent conformations that are poorly hydrolyzed.[17] Irreversible

"suicide inhibitors" can permanently inactivate the β-lactamase through secondary chemical reactions in the enzyme active site. Irreversible inhibitors can be characterized by first-order rate constants for inhibition (k_{inact}, the rate of inactivation achieved with an "infinite" concentration of inactivator) and K_I values (the concentration of inactivator which yields an inactivation rate that is half the value of k_{inact}).[18] The K_I closely approximates the meaning of K_m for enzyme substrates. The 50% inhibitory concentration (IC_{50}) measures the amount of inhibitor required to decrease enzyme activity to 50% of its uninhibited velocity.[18]

Two structurally distinct classes of β-lactamase inhibitors, clavams represented by clavulanic acid and penicillanic sulfones represented by sulbactam and tazobactam, have been widely used clinically.[18] All three β-lactamase inhibitor compounds share structural similarity with penicillin and are effective against many susceptible organisms expressing class A β-lactamases including ESBL derivatives of TEM-1

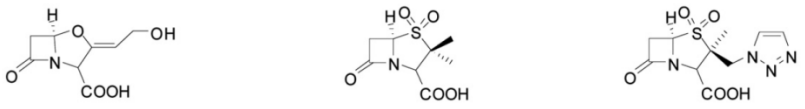


Figure 2. Chemical Structures of β-lactamase inhibitors.[12]

In combination with a β-lactam antibiotic, these inhibitors have successfully overcome bacterial β-lactam resistance caused by β-lactamase-mediated β-lactam hydrolysis. Amoxicillin/clavulanic acid is one such commonly used combination.[19] Several new antibiotic inhibitor combinations include ceftolozane–tazobactam,

ceftazidime–avibactam, ceftaroline–avibactam, and imipenem–cilastatin.[20] Inevitably, the use of such drug combinations has selected mutant derivatives of the TEM and SHV families of class A β-lactamases that have become relatively resistant to inactivation by mechanism-based inactivators and thereby confer resistance to β-lactam–β-lactamase inactivator combinations. Many inhibitor-resistant clinical isolates and laboratory mutants that display such resistance have emerged as the result of single or multiple mutations in the structural genes for their β-lactamases. The TEM-1 β-lactamase is a very plastic enzyme and is capable of tolerating multiple mutations that enhance its activity or broaden its spectrum. As of 2010 over 170 TEM extended-spectrum β-lactamases or inhibitor resistant TEM (IRT) variants have been identified from clinical isolates. Each of these has a primary sequence that differs slightly from the wild type TEM-1.[8]

CHAPTER 2

Materials and Method

Expression and Purification of TEM-1

The plasmid pET100-TEM-1 was transformed in *E. coli* BL21 (DE3) (Appendix I). For large scale expression, a 40 mL overnight culture of *E. coli* grown in 10 uL ampicillin (50 μg/mL) was used to inoculate 4 × 1 L of Luria-Bertani medium containing 1ml of ampicillin (50 μg/mL) per liter of medium. The culture was grown at 37°C with shaking until it reached an optical density (OD_{600nm}) of 0.6 – 1.0. Protein production was induced by the addition of IPTG. The cultures were further incubated at 31°C with shaking for another 10 hours. The bacteria were then harvested by centrifuging the cultures at 4750 revolutions per minute (RPM) for 20 minutes. The supernatant was discarded and the pellet was stored in a -80 ºC freezer.

The frozen cells were resuspended with 5 mL of buffer A (30 mM Tris, 250 mM NaCl, pH 7.5) per 1 L of medium. The cells were lysed by sonication and centrifuged at 20,000×g for 20 min. The supernatant was collected and filtered twice, first with 0.8 μm filters followed by 0.45 μm filters. The filtered supernatant was loaded on to a Ni^{2+} affinity column previously equilibrated with 50 ml of buffer A by injecting it manually through a syringe. The Ni^{2+} column was then washed with 15 ml of buffer A. The elution was carried out manually with 50 ml of a solution of 3% imidazole in buffer A. The imidazole acted as a competitive agent helping to remove bound proteins.[21] The eluate containing the protein was collected and concentrated in centrifugal filters at 4750 RPM for 20 min. The protein was reloaded for buffer exchange and concentrated at the same

setting for a total of three times. The summary of purification of TEM-1 produced in *E. coli* BL21* is shown in Table 3.

Determination of Protein Concentration.

Extinction coefficient used for TEM-1: ε232nm = 27960 M-1cm-1. Protein concentrations were determined from the UV absorbance at 280 nm with a SHIMADZU UV-2450 UV/Vis spectrometer.

Electrophoretic Analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by with a fixed 30% (wt/vol) polyacrylamide gel in the presence of 12% SDS, The molecular weight of TEM-1 was determined by comparison of its relative mobility with that of a standard protein mixture.

TEM-1 Activity Assay.

The activity of TEM-1 was monitored using a UV/Vis spectrometer to measure the change in absorbance upon hydrolysis of the substrate penicillin G by the enzyme. The assay involves monitoring the rate of hydrolysis of the substrate by TEM-1. In a typical experiment calculated volumes of the enzyme and buffer solution (30mM Tris pH 7.0) were added to the cuvette before the substrate was introduced and the decline in A_{232} as a function of time was monitored immediately at 25 °C. The initial rates of hydrolysis were obtained from the slopes of the initial reaction profile. SigmaPlot 11.0 was used to

produce the Michaelis-Menten plot of the average activity of the enzyme in units of μM/min as a function of substrate concentration.

Buffer and Ionic-Strength Effects

It is relatively rare for enzyme kinetic studies to involve the effects of different buffers, buffer concentrations and changes in ionic strength. In most cases these effects are usually minor therefore the lack of control of these parameters is of little consequence. However, for those studies requiring the careful deduction of kinetic parameters, for example, solvent-isotope effects, and pK_a's from pH-rate profiles, buffer and ionic-strength control is important.

The kinetic constants were obtained from initial rates. The K_m value was determined using SigmaPlot 11.0 using one site saturation binding. The effect of ionic strength on the second-order rate constant k_{cat}/K_m for the *E. coli* TEM-1 β-lactamase catalyzed hydrolysis of penicillin G was investigated (Figure 5). At a constant buffer concentration, a change in ionic strength from 0.01-1.5 M (NaCl) brought about a negligible change in the k_{cat}/K_m.

pH Dependence of Enzyme Activity and Solvent Kinetic Isotope Effects

The pH dependence of TEM-1 was studied using acetate (pH 4.0-5.03), MES (pH 5.5-6.5), MOPS (pH 6.5-7.5), and Taps [N-Tris (hydroxymethyl) methyl-3-aminopropane] (pH 7.5-8.5) buffers, at 30 °C and 0.05 M, with the ionic strength maintained at 1.0 M with NaCl. The concentration of enzyme used was 1.07 mg/ml and

penicillin G concentration of 50 mM. Hydrolysis of the substrate was followed by measuring the decrease in absorbance at 232 nm as a function of time.

CHAPTER 3

Results

The purification of TEM-1 is summarized below for the various fractions collected during the purification process including the cell extract following sonication, the supernatant after ultracentrifugation, the nickel column eluate, and the buffer exchanged protein. The amount of protein was calculated as the product of the fraction volume and the concentration of protein. The total activity which is a measure of how many units of protein are present in the sample was calculated as the product of activity in the fraction and the volume of protein in the fraction. The specific activity was calculated by dividing the activity by the concentration of protein in the fraction.[22] The fold purification was calculated relative to the specific activity of the cell extract. The percent yield was calculated for each fraction using the total activity of starting step.[22] The concentrations for each fraction are listed in Table 2.

Fraction	$Absorbance_{280}$	Concentration (mg/ml)
Cell extract	0.599	348.31
Ultracentrifugation	0.109	63.27
Nickel exchange	0.052	30.12
Buffer exchange	0.033	18.93

Table 2. Spectrophotometric determination of protein concentration

Stage of purification	Volume (mL)	Total protein (mg)	Total enzyme activity (μmol min^{-1} ml^{-1})	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Fold purification	Percent yield
Cell extract	56.0	19505.1	95.1	5325.1	0.3	1.0	100.0
Ultracentrifugation	44.0	2784.1	63.5	2792.0	1.0	3.7	52.4
Nickel column	51.5	1551.2	31.4	1616.8	1.0	3.8	30.4
Buffer exchange	1.0	18.9	21.5	21.5	1.1	4.2	0.4

Table 3. Summary of purification of TEM-1 produced in E. coli BL21*

The total amount of protein and total protein activity decreases with each subsequent stage of purification. The specific activity increases as the protein becomes more pure and non-specific proteins are removed. We observed an increase in the fold purification as each stage of the purification process was complete, however the yield of protein was observed to decrease.

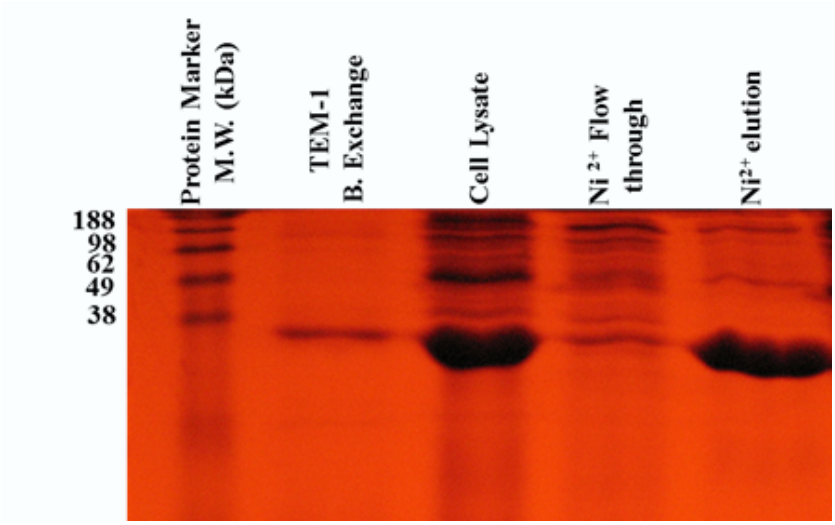


Figure 3. SDS/polyacrylamide-gel electrophoresis of TEM-1 β-lactamase fractions eluted from the affinity column.

The observed results for the SDS-PAGE indicated that the pure protein product was isolated in the Ni^{2+} elution step as indicated by the appearance of a single dense band around molecular weight of 31 kDa. The molecular weight was calculated using the ExPASy ProtParam tool and was found to be 31515 Da. This value is higher than the published value for TEM-1 enzymes which is around 29700 Da.[23] The added weight observed in our enzyme was due to the polyhistidine-tag which adds about 1 kDa of weight to the protein.[24]

The kinetic parameters of TEM-1 were determined by measuring the rate of hydrolysis of the substrate β-lactam antibiotic penicillin G using a UV/Vis spectrophotometer at 232 nm. The kinetic constants were obtained from initial rates. The K_m and V_{max} values were determined using SigmaPlot 11.0 using one site saturation binding. The K_m was found to be 20.2 μM and the V_{max} value was found to be 608.8 μM/min.

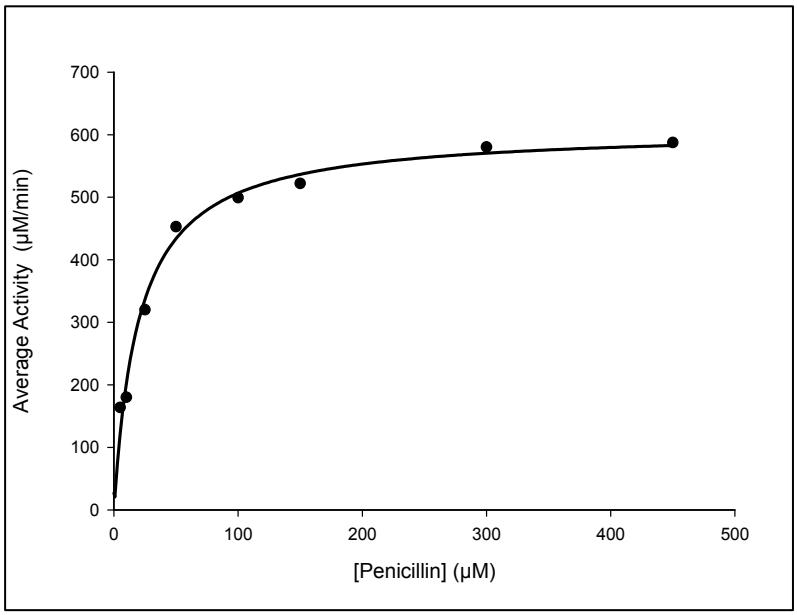


Figure 4. Kinetic assay for the β-lactamase-catalyzed hydrolysis of penicillin G.

It is relatively rare for enzyme kinetic studies to involve the effects of different buffers, buffer concentrations and changes in ionic strength. For studies requiring the careful deduction of kinetic parameters, for example, solvent-isotope effects, p*K*a's from pH-rate profiles etc., buffer and ionic-strength control is important.

Buffer and Ionic Strength Effects

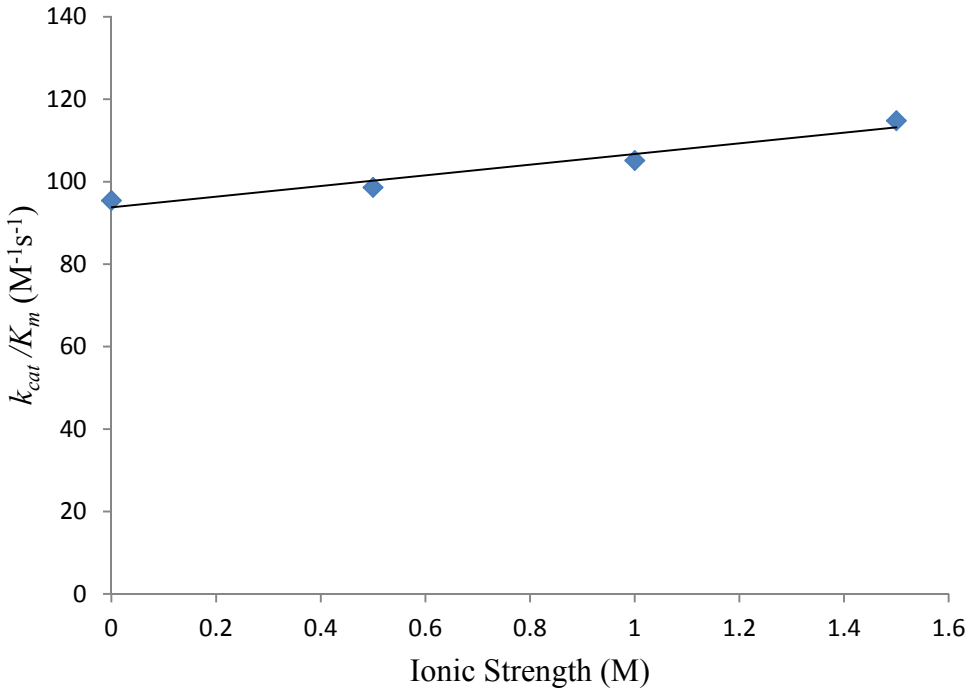


Figure 5. Ionic strength dependence for the β-lactamase-catalyzed hydrolysis of penicillin G.

The dependence of the second order rate constant k_{cat}/K_m for the β-lactamase catalyzed hydrolysis of penicillin G upon the ionic strength of the medium (adjusted with NaCl) at 25 °C, pH 7.0, with MOPS at a constant buffer concentration. The ionic strength was calculated using the equation given below for all ionic species present:

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \qquad (1)$$

where *ci* is the molar concentration of ion i (mol/L) z_i is the charge number of that ion, and the sum is taken over all ions in the solution.

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 constant ionic strength was tested. The two types of buffers tested were MOPS and phosphate buffers.

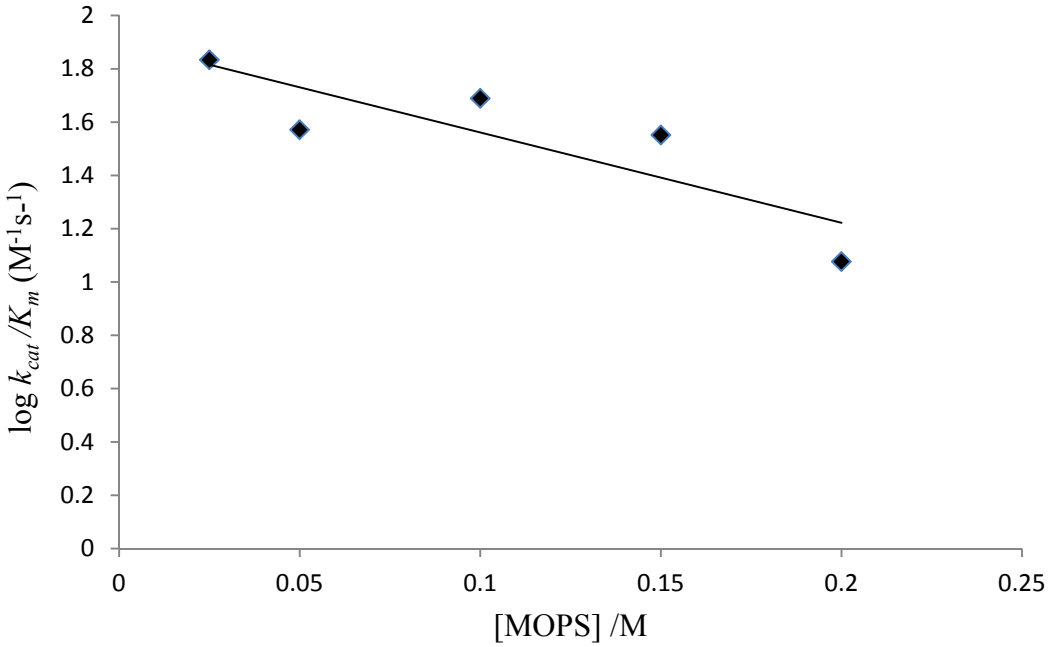


Figure 6. Dependence on MOPS buffer concentration for the β-lactamase catalyzed hydrolysis of penicillin G.

Figure 6 shows the effect of varying MOPS buffer concentration 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 caused by changes in the buffer concentration. Increasing MOPS buffer concentration decreased the rate of hydrolysis.

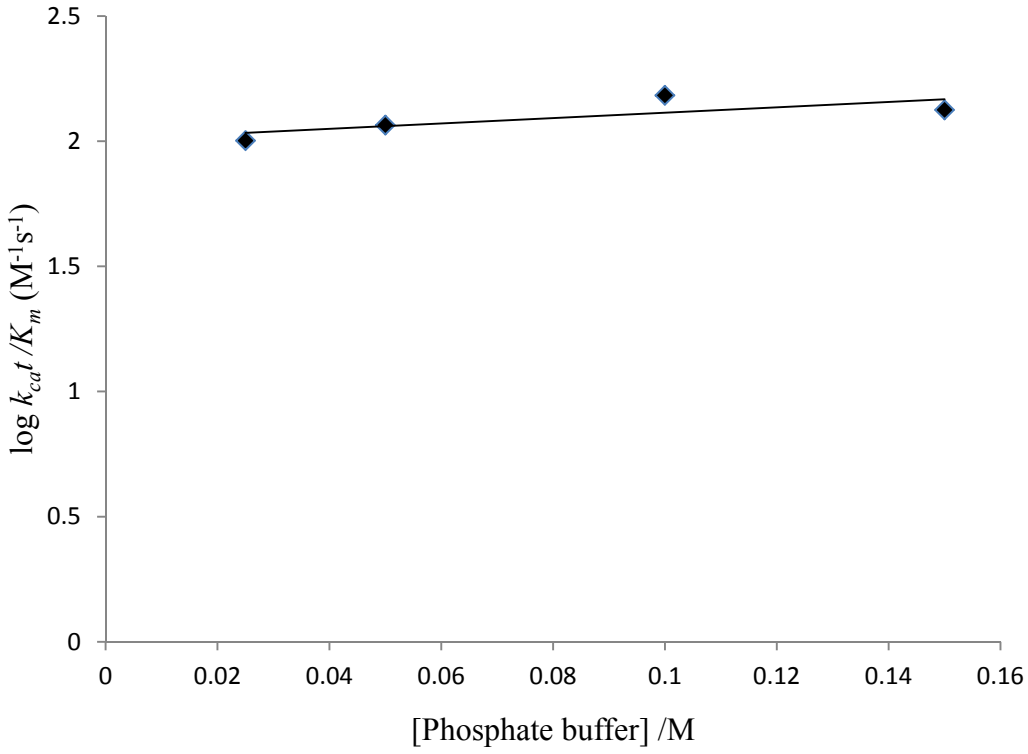


Figure 7. Phosphate buffer concentration dependence of the β-lactamase catalyzed hydrolysis of penicillin G

Figure 7 shows the effect of varying phosphate buffer concentration 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 caused by changes in the buffer concentration. Increasing phosphate buffer concentration increased the rate of hydrolysis.

pH Dependence Studies

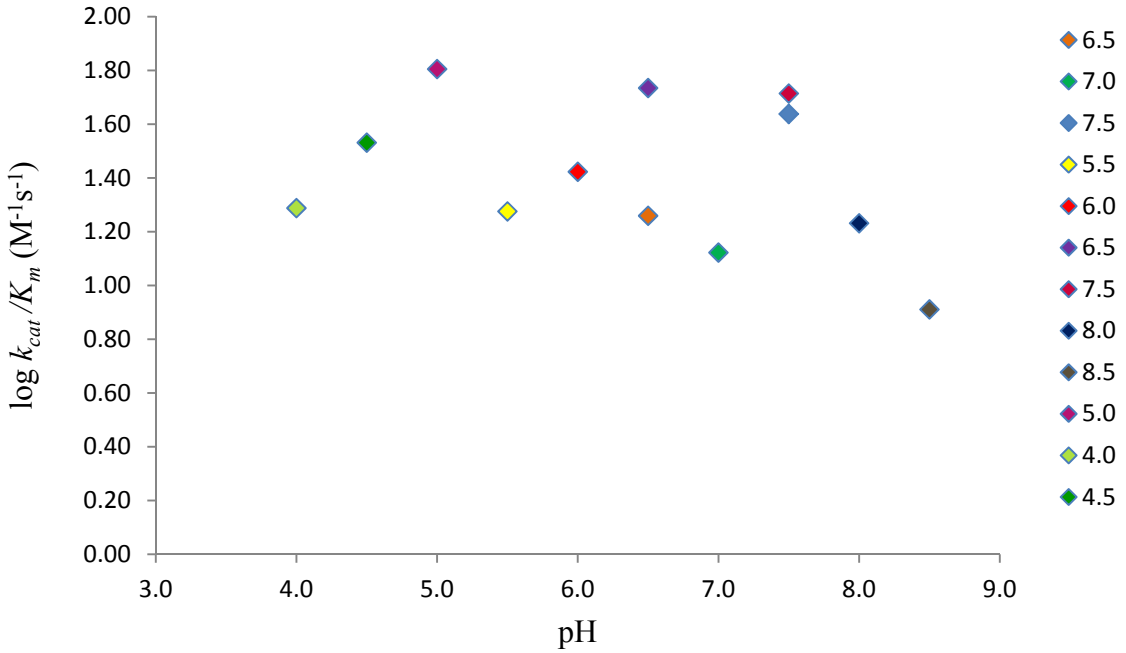


Figure 8. pH dependence for the β-lactamase-catalyzed hydrolysis of penicillin G.

Buffer	pH	Km (µM)	Vmax(µmol/min)	Kcat	Kcat/Km	logKcat/Km
MOPS	6.5	18.99	2930.90	1588.31	83.63	1.92
MOPS	7	25.15	3092.79	1676.04	66.66	1.82
MOPS	7.5	28.58	2290.71	1241.38	43.44	1.64
MES	5.5	17.69	1780.97	965.14	54.56	1.74
MES	6	31.72	2392.44	1296.51	40.88	1.61
MES	6.5	25.17	2521.59	1366.50	54.29	1.73
TAPS	7.5	18.22	1741.41	943.70	51.80	1.71
TAPS	8	76.71	2412.77	1307.53	17.05	1.23
TAPS	8.5	70.98	1066.93	578.19	8.15	0.91
Acetate	5	17.24	2031.18	1100.74	63.85	1.81
Acetate	4	34.22	1224.82	663.76	19.40	1.29
Acetate	4.5	28.17	1767.17	957.67	33.99	1.53

Table 4. pH dependence for the β-lactamase-catalyzed hydrolysis of penicillin G

Kinetic studies were carried out using TEM-1 β-lactamase. The buffers used were acetate (pH 4.0-5.0), MES (pH 5.5-6.5) MOPS (pH 6.5-7.5) and TAPS (pH 7.5-8.5) at 30 °C and 0.05 M, with the ionic strength maintained at 1.0 M with NaCl. The substrate used was penicillin G at a concentration of 0.05M and the hydrolysis was followed by measuring the decrease in absorbance at 232nm respectively as a function of time. The graph fit the characteristic bell shaped curve previously reported.[13]

CHAPTER 4

Discussion

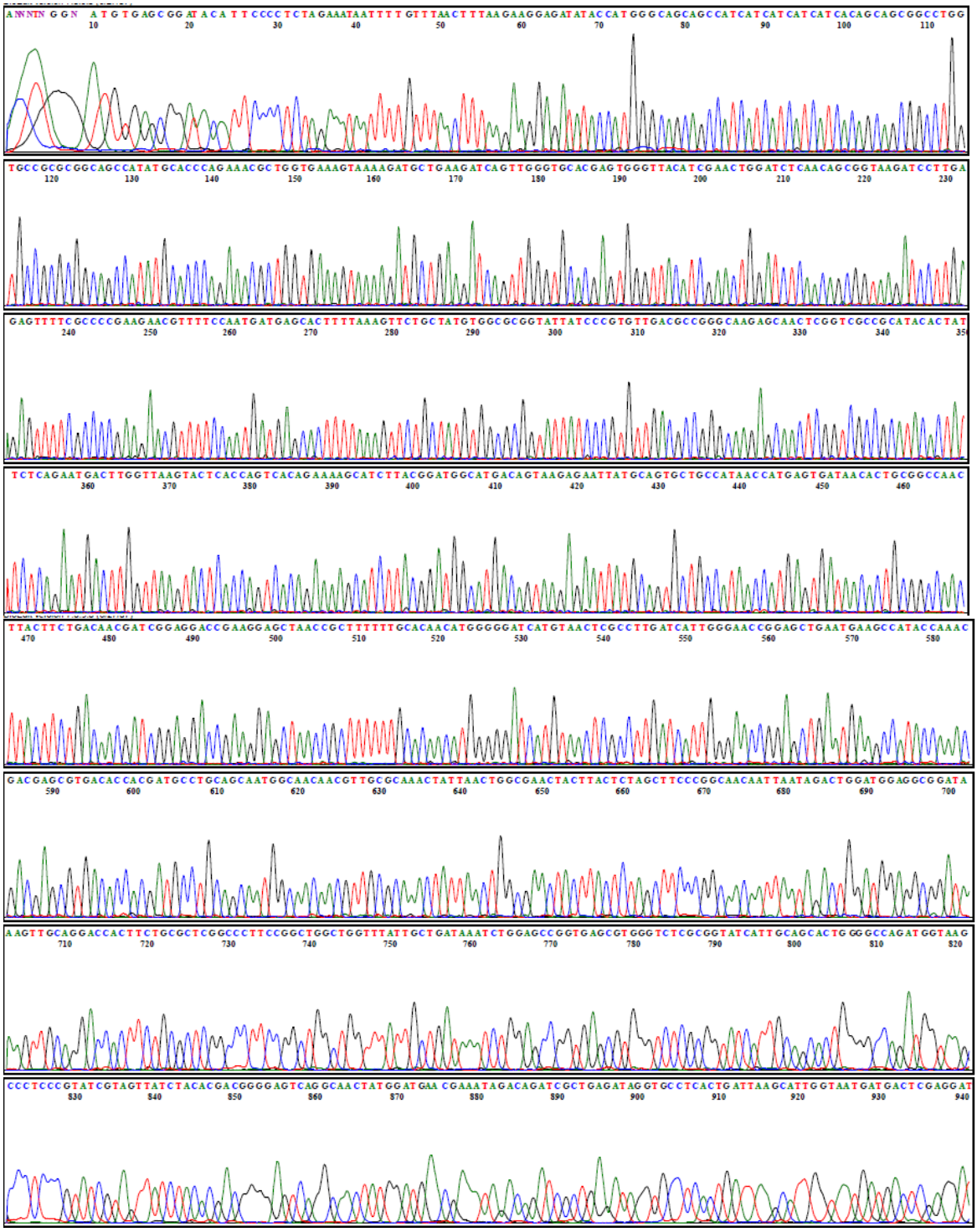
The focus of this study was to determine the kinetic parameters of TEM-1 β-lactamase to better understand its activity with the substrate penicillin G in order to develop new inhibitors to combat β-lactamase mediated drug-resistance in pathogenic bacteria. The hydrolysis of penicillin G was monitored and its K_m was found to be 20.2 μM and the V_{max} value was found to be 608.8 μM/min in 30 mM Tris, 250 mM NaCl buffer. These K_m and V_{max} values are very similar to the previously reported literature values reflecting that our enzyme functions without any abnormality.[13] We tested the dependence of the second order rate constant k_{cat}/K_m for the β-lactamase catalyzed hydrolysis of penicillin G upon the ionic strength of the medium. The k_{cat}/K_m was used since it allows direct comparison of the effectiveness of an enzyme toward different substrates. Although there was an increase in k_{cat}/K_m from 0 - 1.5 M ionic strength adjusted with NaCl we decided that the increase was not significant for the purposes of our study. We then tested the effect of change in buffer concentration on the second order rate constant k_{cat}/K_m for MOPS and phosphate buffer. Increasing Mops buffer concentration decreased the rate of hydrolysis while increasing phosphate buffer concentration increased the rate of hydrolysis. The decrease in the rate of hydrolysis for MOPS buffer was greater than the increase for phosphate buffer. Given the increase in the rate of hydrolysis for phosphate buffer we decided that phosphate buffer was a stable buffer for use in further kinetic studies. We studied the pH dependence for TEM-1 in the presence of different buffers ranging from pH values of 4.0-8.0 and obtained a bell

shaped distribution showing that there is an acid-base mechanism with two pK_a values of 5.5 and 9.0. For comparison Imtiaz, *et al* performed a similar experiment based on pH dependence in the pH range of 5.5 - 9.[13] However, the profile at pH values below 5.0 was not shown indicating that they may have not been able to maintain protein stability at the lower pH's. In our research we did not see significant instability in the lower pH's providing the chance to find the pK_a values from lower to higher pH range. In previous studies several amino acid residues were discussed for the acid-base catalysis. Glu^{166}, Lys^{73}, and Lys^{234} have been implicated as key residues for catalysis.[9] The p*K*a values for these residues in the TEM-1 β-lactamase in the presence of penicillin G were found to be 12.0, 4.6, and 11.5 respectively for Lys^{73}, $Glu1^{66}$, and Lys^{234}.[9]

For further investigation, we can study the possible amino acid residues by site-directed mutagenesis, which may provide a conclusive idea as to how the acid-base catalysis of this enzyme works. To elucidate the mechanism of action of TEM-1 further studies with inhibitors are needed. These studies will provide an avenue for better understanding the acid-base mechanism of TEM-1 which will aid in the discovery of novel drugs to combat antibiotic resistance in bacteria.

APPENDIX

The gene sequence coding for TEM-1 from *E. coli* was identified (GenBank Accession X57972.1). The first 23 amino acids whose sequence is MSIQHFRVALIPFFAAFCLPVFA were removed due to the presence of a signal peptide, and the remaining 810 bp were synthesized and cloned into the pET15b vector by GENEWIZ. Inc. using NdeI and XhoI as restriction sites. After receiving the constructed plasmid, primers were designed in order to amplify TEM-1 and clone the sequence into pET100 (Invitrogen). The forward primer sequence is 5' CAC CCA TCC AGA AAG GCT GGT GAA AG 3' where the underlined portion represents the 5' overhang necessary to clone into the pET100 vector system, and the reverse primer sequence is 5' TTA CCA ATG CTT AAT CAG TGA 3'. PCR was performed using GoTaq, 0.5 μM forward primer, 0.5 μM reverse primer, 5 μL of purified pET15b containing TEM-1, and water at a final PCR volume of 50 μL. 2.5 μL of the PCR reaction was used in TOPO cloning into pET100 and transformation into BL21 Star™ (DE3) chemically competent *E. coli* according to manufacturer instructions. A stock cell solution was made from positive clones for protein expression.



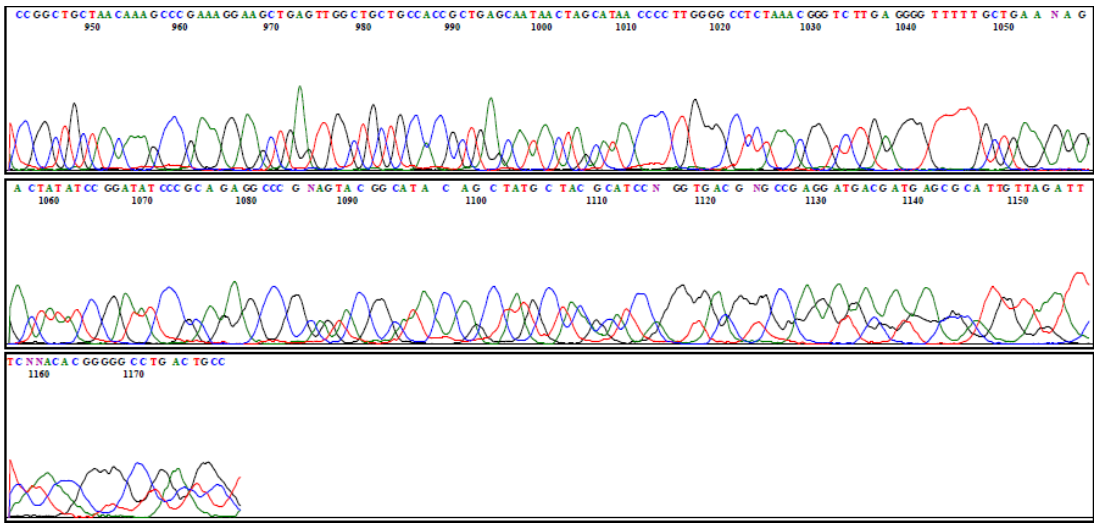


Figure 9. Automated DNA sequencer trace

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