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**MARMARA UNIVERSITY**

**INSTITUTE FOR GRADUATE STUDIES IN**

**PURE AND APPLIED SCIENCES**

# PRODUCTION AND PURIFICATION OF RTEM-1 β-LACTAMASE AND ITS INHIBITION BY PEPTIDES

**Naze Gül AVCI**

## THESIS

**FOR THE DEGREE OF MASTER OF SCIENCE**

**IN**

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**SUPERVISOR**

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**Co-ADVISOR**

**Prof. Dr. Dilek KAZAN**

**İSTANBUL 2011**

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**June, 2011** **Naze Gül AVCI**

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## RTEM-1 B-LAKTAMAZ'IN ÜRETİMİ, SAFLAŞTIRILMASI VE PEPTİTLER İLE İNHİBİSYONU

β-laktam antibiyotikleri yüksek etki, düşük fiyat ve minimum yan etkilerinden dolayı yaygın kullanılan antibiyotiklerdendir. Bu antibiyotiklerin sık ve aşırı kullanımı ise bakterilerin bu ilaçlara direnç geliştirmesine neden olmaktadır. β-laktam antibiyotiklerine karşı en yaygın rastlanan direnç; Gram-pozitif ve Gram-negatif bakteriler tarafından sentezlenen ve β-laktam antibiyotiklerinin β-laktam halkalarının kırılmasına neden olan β-laktamaz enziminin sentezi ile meydana gelmektedir. 263 amino asit uzunluğunda periplazmik bir protein olan RTEM-1 β-laktamaz, A sınıfı bir β-laktamaz olup penisilin, sefalosporin gibi antibiyotikleri hidrolize edebilmektedir. *Streptomyces clavuligerus* tarafından üretilen 165 amino asitlik β-laktamaz inhibitör proteini (BLIP) TEM-1, SHV-1 gibi A sınıfı β-laktamazlara farklı ilgilerle bağlanarak onları inhibe edebilmektedir.

Bu çalışmanın amacı RTEM-1 β-laktamazı saflaştırmak ve BLIP ile RTEM-1 β-laktamazın hücre içi koşullarda bağlanma ve inhibisyon kinetiğini aydınlatmaktır. İki molekülün birbiriyle etkileşimi daha önce yapılmış *in-vitro* çalışmalarla doğrulanmıştır. Bu amaçla pUC18 vektörü içeren *E. Coli* TB1 hücrelerinde üretilen RTEM-1 β-laktamaz, periplazmik bir protein olması nedeniyle, ozmotik şok ile hücre dışına alındıktan sonra ultrafiltrasyonu takiben yapılan iyon değişim kromatografisi ile 5,1 kat saflaştırılmıştır. Enzimin saflığı sodyum dodesil sulfat poliakrilamit jel elektroforezi (SDS-PAGE) ile kontrol edilmiştir. BLIP ile yapılan çalışmalar sonucunda, BLIP'in saflaştırılan enzimi yarışmasız inhibe ederken, saf ticari enzimi yarışmalı inhibe ettiği görülmüştür.

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# ABSTRACT

## PRODUCTION AND PURIFICATION OF RTEM-1 β-LACTAMASE AND ITS INHIBITION BY PEPTIDES

β-lactam antibiotics are the most commonly used antibiotics due to their high effectiveness, low cost, ease of delivery and minimal side effects. As a result of wide use of these antibiotics, bacteria render resistance to β-lactam antibiotics. One mechanism of β-lactam resistance is the synthesis of β-lactamase by both Gram-positive and Gram-negative bacteria. This enzyme hydrolyzes the amide bond of the β-lactam antibiotics causing them to be ineffective. The most prevalent β-lactamase is the class A RTEM-1 β-lactamase which is a periplasmic protein with 263 amino acids. *Streptomyces clavuligerus* produces a protein inhibitor of β-lactamase called β-lactamase inhibitor protein (BLIP) which is a 165 amino acid protein. BLIP inhibits class A β-lactamases such as TEM-1 or SHV-1 with varying degrees.

The aim of this study was to purify β-lactamase and elucidate the intracellular binding and inhibition kinetics of BLIP and β-lactamase. The interaction of these molecules has previously been verified by *in-vitro* studies. The wild-type form of RTEM-1 β-lactamase has been produced by *E. Coli* TB1 pUC18 cells and purified 5.1 times from the periplasmic protein extract by ultrafiltration and ion exchange chromatography methods. Purified enzymes are used for subsequent β-lactamase assays. Competitive inhibition of commercial pure RTEM-1 β-lactamase by BLIP was demonstrated however BLIP was found to be an uncompetitive inhibitor of the purified periplasmic protein extract.

**June, 2011** **Naze Gül AVCI**

# LIST OF SYMBOLS

| | |
|---|---|
| ***Ki*** | : Inhibition constant |
| $\mathbf{K_m}$ | : Michaelis-Menten constant |
| $\mathbf{V_{max}}$ | : Maximum reaction rate |
| **H** | : His, Histidine |
| **Y** | : Tyr, Tyrosine |

# ABBREVIATIONS

**atm** : Atmosphere
**APS** : Ammonium persulfate
**bp** : Base pair
**BLIP** : β-lactamase inhibitor protein
**EDTA** : Ethylenediaminetetraacetic acid
**E-S complex** : Enzyme-Substrate complex
**Da** : Dalton
**IPTG** : Isopropyl β-D-1-thiogalactopyranoside
**kDa** : Kilodalton
**L** : Liter
**LB** : Luria-Bertani broth
**M** : Molar
**mg** : Milligram
**ml** : Milliliter
**mM** : Milimolar
**MW** : Molecular Weight
**MWCO** : Molecular weight cut-off
**ng** : Nanogram
**nM** : Nanomolar
**OD** : Optical density
**PES** : Polyethersulfone
**rpm** : Rotation per minute
**SDS-PAGE** : Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
**TEMED** : N,N,N,N-tetramethylethylendiamine
**U** : Unit
**µg** : Microgram
**µL** : Microliter
**µM** : Micromolar

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# CHAPTER I

## INTRODUCTION and AIM

Bacteria have a remarkable ability to adapt to adverse environmental conditions such as exposure to drugs. It appears that the emergence of antimicrobial resistant bacteria is inevitable to most every new drug and this is recognized as a major problem in the treatment of microbial infections in hospitals and in the community (Yoneyama *et al*., 2006).

The β-lactam family of antibiotics includes many of the most commonly used antibacterial in clinical medicine and they play an important role because of their high effectiveness as antibacterial agents coupled with their low toxicity on eukaryotic organisms and low cost. The β-lactam structure is being exploited by many drugs with improved efficiency against resistant bacterial strains (Lawung *et al*., 2001).

β-lactam antibiotics account for approximately 65 per cent of global antibiotic consumption (Chandel *et al*., 2008). Since β-lactams represent one of the most important groups of antibiotics prescribed, resistance to β-lactam antibiotics in bacteria is a significant and growing problem in the health care system (Hujer *et al*., 2009).

Due to the extensive use of β-lactam antibiotics bacteria have developed resistance via different mechanism. β-lactamase enzyme production is the most coomon mechanism of resistance to β-lactams (Poole *et al*., 2002). β-lactamases are widespread in the bacterial world. β-lactamases hydrolyze the β-lactam amide bond of antibiotics such as penicillins and cephalosporins that are the most widely used antibacterial agents (Damblon *et al*., 1996).

Combinations of β-lactams and β-lactamase inhibitors have become one of the most successful antibacterial strategies against bacterial infections (Miller *et al*., 2001). Understanding the catalytic mechanism of these enzymes is an important step towards designing improved β-lactam antibiotics or pharmaceutically important inhibitors.

β-lactamases are grouped into four classes (A, B, C and D) based on their primary sequences. Classes A, C and D are active-site serine enzymes that catalyze the hydrolysis of the β-lactam by a serine-bound acyl intermediate (Ghuysen, 1991). β-lactamases have been found widely in both Gram-positive and Gram-negative bacteria such as *Staphylococcus* spp., *Bacillus* spp., Enterococci, Enterobacteria, *Pseudomonas* spp. The most prevalent plasmid encoded β-lactamase in Gram-negative bacteria is the Class A RTEM-1 β-lactamase that catalyzes the hydrolysis of penicillins and cephalosporins. Therefore it is an important source of bacterial resistance to the β-lactam antibiotics. Class B enzymes need metal cations as zinc to catalyze β-lactam hydrolysis.

To combat β-lactamase mediated antibiotic resistance extended-spectrum antibiotics including aztreonam, cefotaxime and ceftazidime have been developed. Unfortunately, after the introduction of extended-spectrum antibiotics, variant forms of β-lactamases capable of hydrolyzing these antibiotics have emerged (Palzkill *et al*., 1998). The use of inhibitors such as clavulanic acid and sulbactam is an additional strategy to overcome drug resistance. Even though these agents do not have antimicrobial activity themselves, they are used in conjunction with various β-lactam antibiotics to bind β-lactamase and prevent the hydrolysis of the antibiotics.

A protein inhibitor, β-lactamase-inhibitor-protein (BLIP) is produced by *Streptomyces clavuligerus*, a microorganism that also produces β-lactam antibiotics such as cephamycins as well as the β-lactam inhibitor, clavulanic acid. BLIP is a 165 amino acid protein composed of two tandemly repeated domains which has been shown to be a potent inhibitor of class A β-lactamases, including the *Escherichia coli* RTEM-1 β-lactamase. BLIP is more active against some of β-lactamases where clavulanic acid is less effective and vice versa (Page, 2000). Protein-protein interactions such as BLIP and β-lactamase interactions are important starting points for drug design.

In this work *E. Coli* TB1 strain harboring the pUC18 plasmid (*E. Coli* TB1 (pUC18)) with the RTEM-1 β-lactamase gene was used for periplasmic β-lactamase production. RTEM-1 β-lactamase that was liberated by osmotic shock method was purified by ultrafiltration followed by ion exchange chromatography method. The purity of β-lactamase enzyme is checked by SDS-PAGE. *E. Coli* BL21(DE3) cells harboring pET-26SJ construct was used for the production of BLIP. *In-vitro* binding of BLIP and BLIP derived peptide to RTEM-1 β-lactamase were investigated. The

peptides derived from BLIP, residues 46 to 51 were synthesized and the inhibition effect was investigated on the pUC18 derived RTEM-1 β-lactamase in the periplasmic protein extract and commercial pure RTEM-1. The results obtained were used to calculate the inhibition parameters $K_m$ and $V_{max}$, to evaluate the potential of peptides for *in-vivo* β-lactamase inhibition.

# CHAPTER II

## GENERAL BACKGROUND

### II.1. ANTIBIOTICS AND β-LACTAM ANTIBIOTICS

The term 'antibiosis' means against life and was first described in bacteria by Louis Pasteur and Robert Koch who observed that an airborne *Bacillus* could inhibit the growth of *Bacillus anthracis*. These drugs were later renamed antibiotics by Selman Waksman, in 1942.

Antibiotics are therapeutic agents against bacteria. Antibiotics are classified based on their mechanism of action, chemical structure or spectrum of activity. Most antibiotics target bacterial functions as protein synthesis and nucleic acid synthesis. The world's first antibiotic, Penicillin, was discovered by the British scientist Alexander Fleming in 1928 (Fleming, 1929). In the 1940s it was found that penicillin had an effect on cell wall structure and morphology. By the 1950s, penicillin was shown to be a specific inhibitor of cell wall synthesis (Sykes, 2010). Over the years, this family of antibiotics has grown, and the antimicrobial activity has expanded. Since their introduction into clinical medicine more than 60 years ago, antibiotics have become the main means of controlling bacterial infection (Navarro, 2006). Today there are at least 20 kinds of penicillin antibiotics such as ampicillin, amoxicillin, etc. However, despite the discovery of many new antibiotics, penicillin has remained the primary choice for treatment of a wide variety of bacterial infections.

The mechanism of antibiotic action in general can be expressed as illustrated in Figure II.1. Ideally, a given antibiotic reaches its target sites unimpeded and intact, binds to and inactivates the essential targets and, as a result, the cell rapidly loses viability and dies.

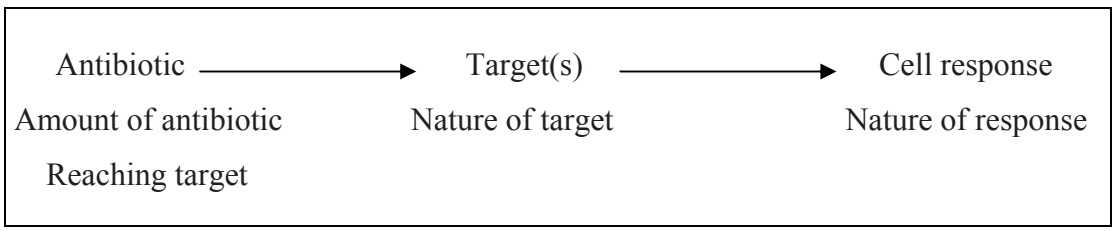


**Figure II.1.** Mode of action of antibiotics and factors that influence their activity

Antibiotics that target the bacterial cell wall as penicillin, cephalosporins, monobactam and carbapenems are β-lactam antibiotics. Since the discovery of penicillin over 50 years ago, bacteria have been consistently exposed to a wide variety of β-lactams and more than 40 β-lactams are in clinical use (Miller *et al*., 2001). β-lactam antibiotics are among the most commonly utilized antimicrobial agents (Rudgers *et al*., 2001). The extensive and irresponsible use of these antibiotics in clinical and agricultural settings has contributed to the emergence of antibiotic-resistant bacteria.

According to a report by Global Industry Analysts, global antibiotics market was estimated to reach US $40.3 billion by 2015 and, β-lactam antibiotics have annual sales of approximately $15 billion and 65 per cent of this market belonged to β-lactam antibiotics (Chandel *et al*., 2008). They are the most varied and widely used of all the different groups of antimicrobial agents in treatment of bacterial infections owing to their comparatively high effectiveness, low cost, ease of delivery and minimal side effects (Wilke *et al*., 2005; Doran *et al*., 1990).

## II.2. MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS

β-lactam antibiotics are among the most commonly prescribed drugs that have been grouped based upon a shared structure, a four-membered β-lactam ring (Figure II.2). The β-lactam ring plays an essential role in the inactivation of a set transpeptidases which is the catalyzing agent of the final cross-linking reactions of peptidoglycan synthesis in bacterial cells (Therrien and Levesque, 2000).

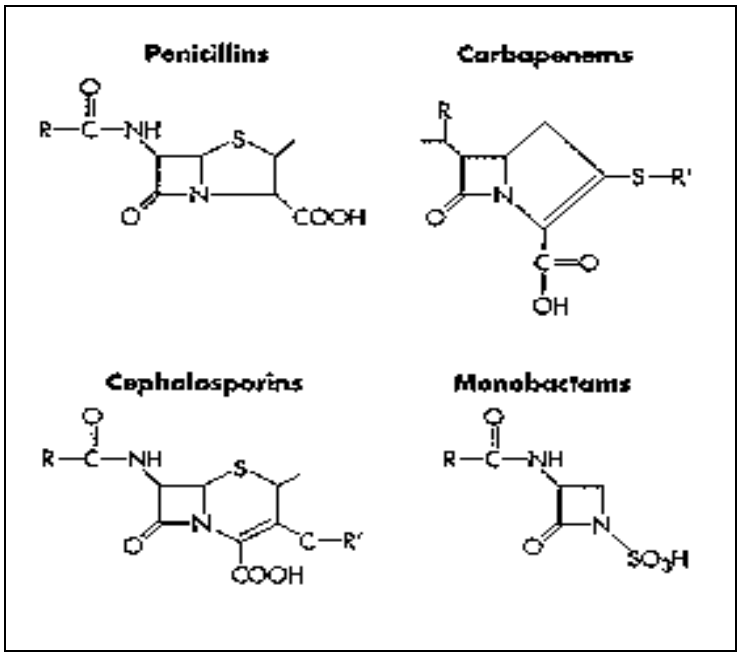


**Figure II.2.** Chemical structures of β-lactam antibiotics.

Bacteria have a cross-linked peptidoglycan layer, structural element to preserve cell shape and rigidity (Wilke *et al*., 2005). Peptidoglycans are made up of a polysaccharide backbone comprised of two derivatives of glucose: *N-acetyl* muramic acid (MurNAc or NAM) and *N-acetyl* glucose amine (GlcNAc or NAG). The NAM and NAG strands are connected by interpeptide bridges (Koch, 1998). In both Gram-positive and Gram-negative bacteria, the synthesis of peptidoglycan layer proceeds by the same general mechanism by amino acid chain that contains D-alanine and D-glutamic acid as in the case of a Gram-negative bacteria (*Escherichia coli*) or L-alanine, D-glutamine, L-lysine and D-alanine as in the case of a Gram-positive bacteria (*Staphylococcus aureus*). Except the L-amino acids, these amino acids are not found in proteins and help protection against attacks by peptidases. The specific amino acid sequences vary with the bacterial species. Transpeptidase enzymes also called penicillin binding proteins (PBPs) help the cross-linking between amino acids. The PBPs are located on the outer side of the cytoplasmic membrane. In Gram-negative bacteria this is in the periplasm. Lethal targets in bacteria are active-site serine enzymes and transpeptidase enzymes perform their catalytic cycle by the way of an acylation/deacylation pathway. In the presence of the antibiotic, the transpeptidases form a covalent penicilloyl-enzyme complex to block the normal transpeptidation reaction (Wise *et al*., 1999). This results in weakly cross-linked peptidoglycan and eventually cell lysis and death. The peptidoglycan, the major

structural component of most bacterial cell wall, makes β-lactam antibiotics non-toxic drugs for humans. No new β-lactam subclass has been discovered in thirty years, research on the β-lactams has declined, and new β-lactam derivatives are a minority of new anti-infectives in clinical development (Devasahayam *et al*., 2010).

**II.2.1.** Inactivation of the β-lactam antibiotics

The primary means of bacterial resistance to β-lactam antibiotics is the ability of bacteria to produce β-lactamases, which hydrolyze the β-lactam moiety of these antibacterial agents, hence rendering them ineffective (Bulychev, 1999).

β-lactam antibiotics are rendered inactive by bacteria in three ways. The most common mechanism of direct inactivation of an antibiotic by bacteria involves the neutralization of penicillin and penicillin-like antibiotics via the action of β-lactamases. The antibiotics act as pseudosubstrates and acylate the PBP transpeptidation active sites, which then deacylate very slowly and are subsequently incapable of performing regular cross-linking functions (Figure II.3.). The PBP-catalyzed acylation reaction is only slowly reversible, while β-lactamases allow a subsequent hydrolysis of the covalent acyl bond by a water molecule; in the latter case, the antibiotic is inactivated and the active site is regenerated (Spratt, 1994). The active sites of class A β-lactamases are very similar to those of PBPs. This similarity poses a challenge for designing new β-lactam antibiotics, which might be less sensitive to β-lactamase inactivation, but must still be specifically bound by the PBP active site. Significant changes to the antibiotic's structure may avoid inactivation by β-lactamases, but at the same time it would not be able to bind to its PBP target site (Kernodle *et al*., 2000).

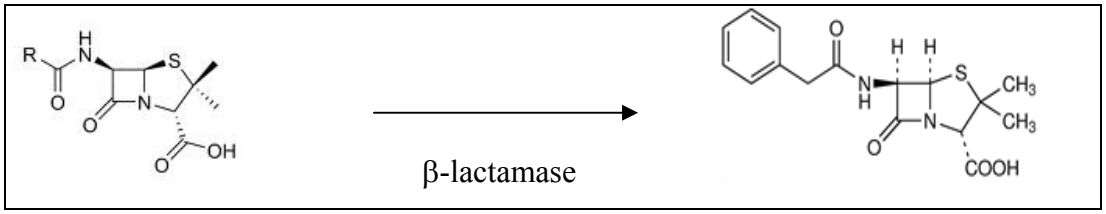


**Figure II.3.** Effect of β-lactamase attack on penicillins and cephalosporins
A stable penicilloic acid is formed.

The second mechanism is alteration of the antibiotic target site. In this case the β-lactam-resistant cell-wall transpeptidases play their role, the major cause of resistance in several pathogens.

The last mechanism is the prevention of access of the antibiotic to the target by way of altered permeability or forced efflux. Mutations of cellular genes, acquisition of exogenous resistance genes, or a combination of these two events are responsible for these mechanisms (Navarro, 2006).

### II.3. β-LACTAMASES

β-lactamases are ancient enzymes, indeed the first β-lactamase was identified in the early 1940s prior to the large scale use of penicillins (Fisher *et al*. 2005). While a handful of β-lactamases were known in the early 1970s, this number at present exceeds 470. β-lactamases are hydrolytic enzymes that disrupt the amide bond of the characteristic β-lactam ring, before the antibiotic can get to the site of cell wall synthesis, rendering the antimicrobial ineffective.

Classification of β-lactamases has been based on either the functional characteristics of the enzymes (Richmond *et al*., 1973, Bush *et al*., 1995) or their primary structure (Ambler, 1980). The simplest classification is The Ambler scheme whereby β-lactamases are grouped into four classes: Classes A, B, C and D. The basis of this classification scheme rests upon protein homology (amino acid similarity) (Paterson *et al*., 2005). Classes A, C and D, three serine-dependent enzymes catalyze the hydrolysis of the β-lactam via a serine-bound acyl intermediate. The active-site serine β-lactamases belong to a larger family of penicillin-recognizing enzymes that includes the penicillin-binding proteins (PBPs) which cross-link bacterial cell wall. Class A TEM-1 β-lactamase that hydrolyzes both penicillins and cephalosporins was identified in the 1970s and early 1980s and is the most common plasmid-mediated β-lactamase found in gram-negative bacteria (Majiduddin *et al*, 2002). Class C and D β-lactamases are chromosomally encoded. Class B β-lactamases are metal-dependent and require zinc for activity. Catalysis does not proceed by a covalent intermediate and these enzymes usually exhibit a broad spectrum of activity.

**II.3.1.** TEM-1 β-lactamase

In terms of bacterial resistance, the most current plasmid-mediated β-lactamase is the Class A TEM-1 β-lactamase, encoded by the $bla_{\text{TEM-1}}$ gene. *Bla* genes have profited from the many mechanisms for horizontal gene transfer between bacteria to spread to new hosts and to become part of multiresistance plasmids common in clinical isolates (Bush *et al*., 2010).

TEM-1 β-lactamases was first reported in 1965 from an *Escherichia coli* isolate from a patient in Athens, Greece named Temoneira (hence the designation TEM) (Datta *et al*., 1965).

TEM-1 β-lactamase has a molecular weight of 28.8 kDa. The crystallographic structure contains 263 amino acid residues.

| | | |
|---|---|---|
| TEM-1 | HPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRID | 60 |
| TEM-1 | AGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGP | 120 |
| TEM-1 | KELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQ | 180 |
| TEM-1 | QLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTG | 240 |
| TEM-1 | SQATMDERNRQIAEIGASLIKHW | 263 |

**Figure II.4.** The sequence of TEM-1 β-lactamase (Reynolds *et al*, 2006)

TEM-1 β-lactamase is able to hydrolyze both penicillin and cephalosporins (Frere *et al*. 1999). Therefore it is an important cause for bacterial resistance to β-lactam antibiotics (Rudgers *et al*., 2001). In response to the appearance of microbial resistance mediated by TEM, extended-spectrum antibiotics were developed (Schroeder *et al*., 2001). TEM-1 β-lactamases cannot hydrolyze the extended-spectrum antibiotics including cefotaxime, ceftazidime and aztreonam. Most extended-spectrum β-lactamases are mutants of TEM-1 with 1- or 4-amino acid substutions (Fisher *et al*., 2005). These changes, amounting to less than 2 % of the protein sequence remodel the enzyme active site to allow attack on cephalosporins.

Ampicillin had been the major antibiotic used to treat *Haemophilus influenzae* meningitis from 1960 to the 1970s. In 1974, a plasmid-mediated β-1actamase was first noted in *H. influenzae*. Since then, resistance to ampicillin has continued to increase (Neu, 1992).

Genetically engineered pUC18 (Figure II.5.) plasmid carries a *bla*TEM-1 gene. The largest component of pUC18 is the region containing the 861 bp TEM-1 β-lactamase gene, which confers resistance to the antibiotic ampicillin (Figure II.6.). The pUC18 derived RTEM-1 β-lactamase has two amino acids substitutions different than TEM-1; I84A and V184A, both of which alter neither activity nor structure of the enzyme significantly.

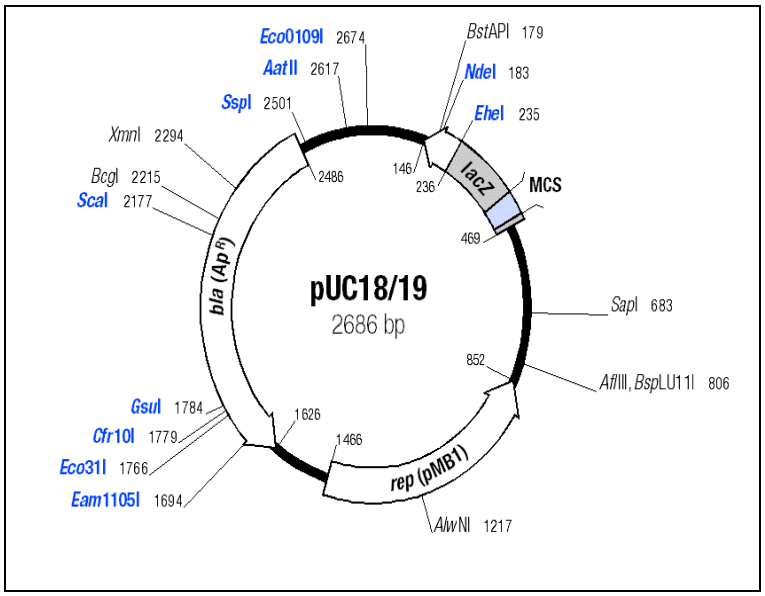


**Figure II.5** Map of pUC18 vector

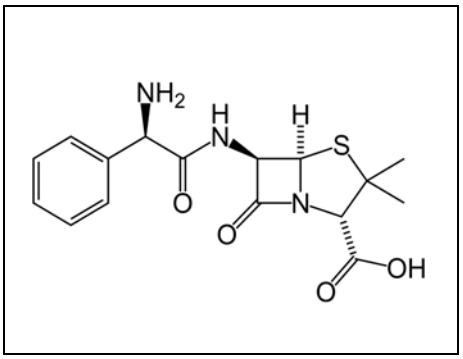


**Figure II.6.** Chemical structure of β-lactam antibiotic used in this study, Ampicillin

## II.4. β-LACTAMASE INHIBITORS

There are two primary strategies for circumventing the antibiotic resistance mechanism. The first is to produce antibiotics that are resistant to β-lactamases, such as carbapenems, extended-spectrum cephalosporins, and oxacillin. The other alternative is to use a β-lactamase inhibitor in combination with a β-lactam antibiotic.

β-lactamase inhibitors alone have little or no antimicrobial activity. However, β-lactam/β-lactamase inhibitor combinations are effective against a wide range of bacterial pathogens. When coadministered with a β-lactam antibiotic, an inhibitor can restore the activity of the β-lactam by binding to and inactivating β-lactamase. Within the last years, potent β-lactamase inhibitors, clavulanic acid, sulbactam and tazobactam (Figure II.7) have become available for halting the action of common β-lactamases. These inhibitors also contain a β-lactam ring structure. Even though they are poor antibiotics, they are good inhibitors of some β-lactam antibiotics (Zang et Palzkill, 2007).

In clinic, penicillins and cephalosporins are used in combination with clavulanic acid obtained from *Streptomyces clavuligerus.* This microorganism has been the subject of extensive research in the last 30 years because of its ability to produce β-lactam metabolites with antibiotic, antifungal and β-lactamase-inhibitory activities, sulbactam and tazobactam, to deal with the problem of the resistance caused by β-lactamases (Jensen *et al*., 2001). Each demonstrates high affinity (nM-μM) for β-lactamases, is hydrolyzed by the enzyme, undergoes different reaction chemistry than β-lactams and occupies the active site significantly longer (Babic *et al*, 2006).

However, these inhibitors are efficient in inactivating only class A β-lactamases and the efficiency of the inhibitor/antibacterial combination can be compromised by several mechanisms, such as the production of naturally resistant class B or class D enzymes, the hyper production of AmpC or even the production of evolved inhibitor-resistant class A enzymes. Thus, there is an urgent need for the development of novel inhibitors (Bebrone, 2010).

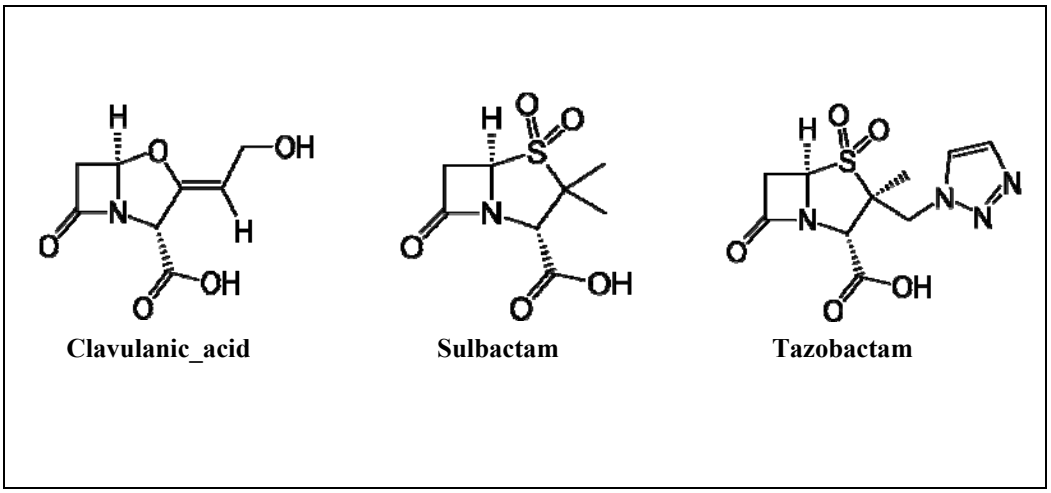


**Figure II.7.** Chemical structures of β-lactamase inhibitors used in clinic

**II.4.1.** β-lactamase inhibitory protein (BLIP)

β-lactamase inhibitory protein (BLIP) was first detected by its ability to inhibit penicillinase derived from *Bacillus cereus*, Bactopenase, the plasmid pUC-, chromosomally mediated β-lactamases of *Escherichia coli* and a wide range of other β-lactamases.

BLIP is a 165 amino acid protein produced by gram-positive bacterium *Streptomyces clavuligerus* (Rudgers *et al*., 2001). This bacterium also produces β-lactam antibiotics such as cephamycins as well as the β-lactamase inhibitor, clavulanic acid which is a small molecule used to overcome the antibiotic resistance mechanism mediated by β-lactamases. This 17 kDa extracellular β-lactamase-inhibiting protein (Doran *et al*., 1990, Strynadka *et al*., 1994, Perez-Llarena *et al*., 1997) binds a variety of β-lactamase enzymes with wide-ranging specificity. Its binding mechanism and interface interactions are a model system for the characterization of protein–protein interactions (Gretes *et al*., 2009). BLIP has been shown to bind and inhibit the catalytic activity of TEM-1 β-lactamase with a Ki of 0.1 to 0.6 nM and also other Class A β-lactamases by binding their active site (Rudgers *et al*., 2001). But it does not efficiently bind to Class B, C or D β-lactamases (Strynadka *et al*., 1994). Thus, the BLIP-β-lactamase interface represents not simply a protein-protein interaction but rather a family of protein-protein interactions.

```
BLIP    AGVMTGAKFTQIQFGMTRQQVLDIAGAENCETGGSFGDSIHCRGHAAGDY        50

BLIP    YAYATFGFTSAAADAKVDSKSQEKLLAPSAPTLTLAKFNQVTVGMTRAQV        100

BLIP    LATVGQGSCTTWSEYYPAYPSTAGVTLSLSCFDVDGYSSTGFYRGSAHLW        150

BLIP    FTDGVLQGKRQWDLV                                           165
```

**Figure II.8.** DNA sequence of *S. clavuligerus* BLIP gene (Doran *et al*, 1990)

While the proteinaceous nature of BLIP limits its value as a therapeutic agent, the fact that BLIP is structurally unrelated to existing small-molecule β-lactamase inhibitors, such as clavulanic acid and tazobactam, suggests that new classes of β-lactamase inhibitors based on small molecule derivatives of BLIP might hold clinical potential (Schroeder *et al*. 2002).

*In-vitro* binding experiments of TEM-1 β-lactamase and BLIP were performed to determine their kinetic parameters (Albeck and Schreiber, 1999). The binding affinity was found to be 0.4 nM. A better understanding of the affinity of TEM-1 and BLIP may help in the design of novel inhibitors.

**II.4.2.** Peptide Inhibitors of β-lactamase

Peptide inhibitors display a significant advantage over small organic molecules since β-lactamase has acquired resistance against most of the small organic molecules.

Peptides derived from BLIP have been shown to inhibit TEM-1 β-lactamase (Rudgers and Palzkill, 2001). By using phage display Huang *et al*. (2003) found that a linear 6-mer peptide with the sequence RRGHYY binds to the class A β-lactamase, TEM-1 and inhibits it with a $K_i$ of 136 μM. Rudgers *et al*. (2001) reported the inhibition potential of peptides based on the 46 to 51 region of BLIP. This peptide in a cyclic form with cysteine residues at both ends inhibits TEM-1 with a $K_i$ of 603 μM, while in reduced form; the peptide binds to TEM-1 with a $K_i$ of 488.

## II.5. ENZYME KINETICS

The mechanism of enzyme catalyzed reactions is commonly studied by kinetic measurements on enzyme-substrate reaction systems. These studies include measuring rates of the enzyme-catalyzed reactions at different substrate and enzyme concentrations. The kinetics of most enzyme reactions is reasonably well represented by the Michealis-Menten equation:

$$v_0 = \frac{V_{max}[S]}{K_M + [S]} \qquad \textbf{(II.1)}$$

$V_o$ = Current reaction rate

$V_{max}$ = Maximum reaction rate

$K_m$ = Michealis constant for the substrate (mM)

[S] = Concentration of the substrate (mM)

Rearrangement of equation (II.1) gives

$$\frac{1}{v} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \qquad \textbf{(II.2)}$$

By the plot of 1/V versus 1/[S], known as Lineweaver-Burk, the effects on the behavior of an enzyme of varying kinetic constants can be explored. A Lineweaver-Burk plot (Figure II.9) determines the parameters $K_m$ and $V_{max}$ from experimental data (Doran, 1995).

It is well known that $V_{max}$ is not a fundamental characteristic of enzyme because it depends on the enzyme purity and concentration (Segel, 1976). $K_m$ is a more fundamental parameter, which is a measure of the affinity of an enzyme for a particular substrate. The smaller the value of $K_m$, the more efficient is the catalyst (Godfrey and West, 1996). The value of $K_m$ for an enzyme depends on the particular substrate. It also depends on the pH of the solution and the temperature at which the reaction is carried out. For most industrially used enzymes, the $K_m$ values of enzymes range lie in the range $10^{-1}$ to $10^{-5}$ M when acting on biotechnologically important substrates, under normal reaction conditions.

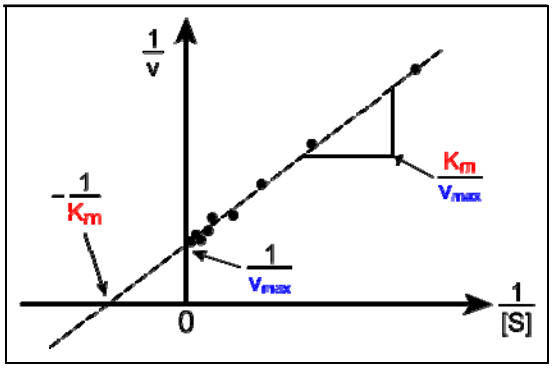


**Figure II.9.** Lineweaver-Burk Plot

**II.5.1.** Enzyme Inhibition

Enzyme reaction rates can be decreased by various types of enzyme inhibitors (Figure II.10);

Competitive inhibitor has a chemical similarity to the substrate and competes with the substrate for binding to the active site of the enzyme. Competitive inhibition is usually reversible if sufficient substrate molecules are available to displace the inhibitor. Therefore, the amount of enzyme inhibition depends upon the inhibitor concentration, substrate concentration, and the relative affinities of the inhibitor and substrate for the active site. $V_{max}$ of the reaction is unchanged, while $K_m$ is decreased (Figure II.10, Panel B).

In non-competitive inhibition, the binding of the inhibitor to the enzyme reduces its activity but it does not affect the binding of substrate. In contrast to competitive inhibition the inhibitor cannot be driven from the enzyme by higher substrate concentrations. As a result, $V_{max}$ will decrease due to the inability for the reaction to proceed as efficiently, but $K_m$ will remain the same as the substrate can still bind to the enzyme (Figure II.10., Panel C).

In uncompetitive inhibition, the inhibitor cannot bind to the free enzyme, but only to the Enzyme-Substrate complex. The Enzyme-Inhibitor-Substrate complex thus formed is enzymatically inactive. This reduction in the effective concentration to the E-S complex decreases $K_m$ and $V_{max}$. This type of inhibition is rare (Figure II.10., Panel D).

Mixed type inhibition resembles the non-competitive inhibition, except that the Enzyme-Inhibitor-Substrate complex has residual enzymatic activity. Mixed type inhibitors can either increase or decrease $K_m$ and decrease $V_{max}$.

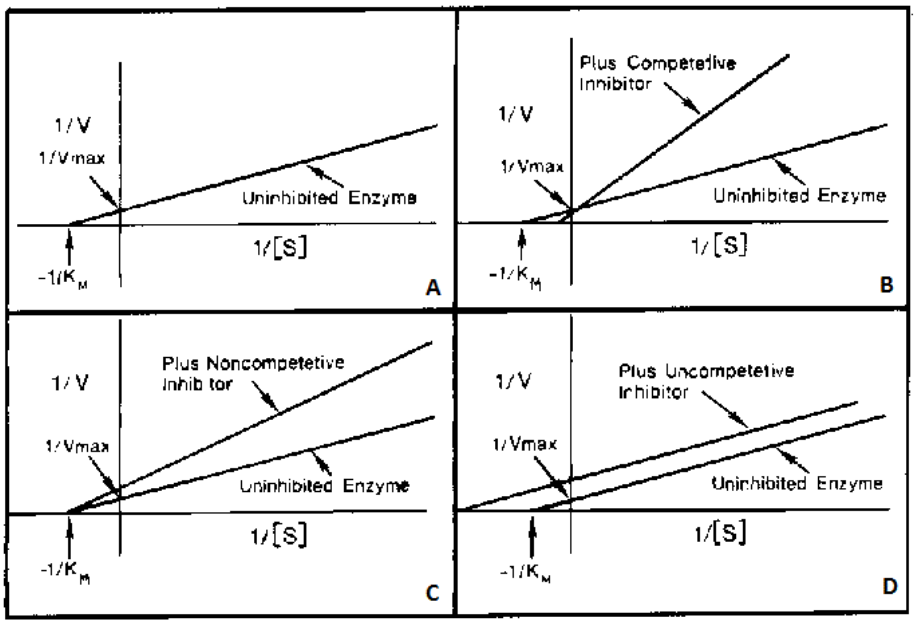


**Figure II.10.** Lineweaver-Burk Plots of Inhibited Enzymes

## II.6. CENTA : SUBSTRATE FOR DETECTION OF β-LACTAMASE ACTIVITY

CENTA (Figure II.11.) is a chromogenic cephalosporin reagent which structurally resembles nitrocefin and cephaloridine. It was shown that this reagent is sensitive to many β-lactamases. It changes color from light yellow to chrome yellow during the hydrolysis of the β-lactam ring (Jones *et al*., 1982). Such substrates cannot practically be used for the detection of β-lactamase-producing strains on agar plates but its hydrolysis can be directly monitored in the visible wavelength range. Consequently it becomes a particular interest for the kinetic characterization of β-lactamase and for the detection of the enzymes in periplasmic protein extract and chromatographic fractions. It is also used in high-throughput screening tests for the selection of new β-lactamase inactivators (Bebrone *et al*., 2001).

The kinetic parameters of the interaction between TEM-1 β-lactamase and CENTA (Bebrone *et al*., 2001) are given in Table II.1.

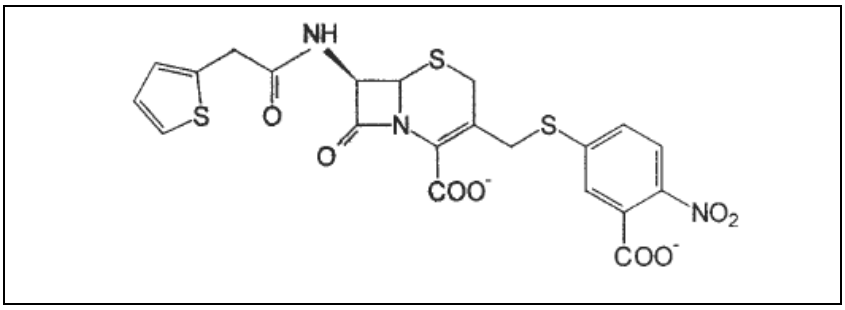


**Figure II.11.** The structure of CENTA.

**Table II.1.** Kinetic parameters for TEM-1 β-lactamase with CENTA.

| Enzyme | CENTA (mM) | Enzyme (nM) | Kcat (s-1) | Km (μM) | Kcat/Km ($\mu M^{-1}s^{-1}$) |
|---|---|---|---|---|---|
| TEM-1 | 0.025-0.3 | 11.5 | 110 | 70 | 1.6 |

## II.7. RECENT STUDIES ON INHIBITION OF B-LACTAMASE AND PEPTIDES

In the 80 years since their discovery, the β-lactam antibiotics have progressed through structural generations, each in response to the progressive evolution of bacterial resistance mechanisms. The most important mechanism of the β-lactam antibiotic resistance is the destruction of the antibiotics by the enzyme β-lactamase. The problem of resistance to β-lactam antibiotics is compounded by the transfer of β-lactamase genes both within and between bacteria species. TEM-1 is a major source of resistance in Gram-negative bacteria (Huang *et al*., 1996).

Use of β-lactamase inhibitors in combination with antibiotics is one of the successful antibacterial strategies (Vinod *et al*., 2009). β-lactamase inhibitors cannot inhibit β-lactamase enzyme with the same affinity. However, enzyme kinetic studies have shown that both clavulanic acid and sulbactam can inhibit many enzymes in a progressive manner (Cullmann *et al*., 1996).

Peptide based potential inhibitors of the TEM-1 β-lactamase can provide a basis for further development of potent inhibitors against β-lactamase activity (Sun *et al*, 2005). But a solution should be found when delivering peptides into the body (Kumar *et al*., 2006).

β-lactamase inhibitory protein, BLIP, competitively inhibits its targets with two binding loops that occlude the active site. In each loop, key side chains mimic interactions observed with the acyl-enzyme intermediate bound to its antibiotic

substrate (Hanes *et al*., 2009). In addition, in the TEM-1/BLIP system, the cooperativity of salt bridge networks (Albeck *et al*., 2000) suggested that salt bridges existing in networks contribute favorably to affinity and specificity. The BLIP-β-lactamase interaction has become a model system to investigate protein–protein interactions and has been the focus of several structural, thermodynamic and binding specificity studies (Palzkill *et al*., 2010). In order to understand the molecular basis of affinity, alanine scanning mutagenesis of BLIP against TEM-1, has been reported (Zhang *et al*., 2003, Zhang *et al*., 2004).

A high-resolution X-ray crystallographic structure of the complex between β-lactamase TEM-1 and BLIP has been solved (Strynadka *et al*., 1996) and it has been found that a significant degree of homology exits between the proteins (Schreiber *et al*., 2011). On the basis of the structure of BLIP, novel peptide inhibitors of β-lactamase have been constructed. The cocrystal structure of TEM-1 β-lactamase and BLIP indicates that residues 46 to 51 of BLIP make critical interactions with the active site of TEM-1 β-lactamase (Rudgers *et al*., 2001).

To determine which residues in TEM-1 β-lactamase are critical for binding BLIP, the method of phage display was employed as a powerful technique for studying protein-ligand interaction (Rudgers *et al*., 1999). This complex is shown to be very stable, with an affinity of 0.1 – 0.6 nM (Rudgers *et al*., 1999, Petrosino *et al*., 1999, Strynadka *et al*., 1994). Phage display method has been used to identify a small domain of BLIP that contains a cluster of residues that are sufficient for binding and inhibition of TEM-1 β-lactamase (Rudgers and Palzkill, 2001).

Protein-protein interactions have an important role in biological processes and are important targets for drug design. The two-hybrid system is used for the high-throughput screening of protein interactions, their affinity and sensitivity (Fields and Song, 1989, Sun *et al*. 2005). A gene sequence is designed to make interactions with the active site of TEM-1 β-lactamase because peptides are shown to inhibit TEM-1 β-lactamase. Therefore, these systems provide a model to study different aspects of protein-protein interactions and a better understanding of the affinity between TEM-1 and BLIP. Further study may help to design novel inhibitors which are common target in both Gram positive and Gram negative bacteria and may provide insights into novel means of β-lactamase inhibition (Gretes *et al*., 2009).

# CHAPTER III

## THE STUDY

### III.1 EXPERIMENTAL MATERIALS

**III.1.1.** Bacterial Strains and the Plasmid

In this study, *Escherichia coli* TB1 strains were used from our laboratory stocks. β-lactamase expression was obtained from pUC18 plasmid carrying the gene for RTEM-1 β-lactamase. These cells were abbreviated as *E. Coli* TB1 and *E. Coli* TB1 (pUC18).

*E. Coli* BL21(DE3) cells harboring pET-26SJ construct was used for periplasmic expression of BLIP. pET-26SJ vector carrying the BLIP gene with the native leader sequence was a generous gift from Susan Jensen (University of Alberta). These cells were abbreviated as *E. Coli* BL21 (pET26-SJ).

**III.1.2.** Chemicals and Enzymes

All chemicals and solutions used in this study were purchased from MERCK (Germany), MOLEKULA (Germany), APPLICHEM (Germany) and SIGMA (USA). Protein size markers were purchased from Fermentas.

**III.1.3.** Growth Media for *Escherichia coli*

**Table III.1.** LB Medium

| | |
|---|---|
| Yeast Extract | 5 g |
| Peptone | 10 g |
| NaCl | 10 g |

Per Liter of growth media completed to one liter with deionized and distilled water. For selective media (Yamamoto, 1982), 100 mg/ml ampicillin was added.

### III.1.4. Buffers and Solution

#### III.1.4.1 SDS-PAGE Buffers and Solutions

**Stain**

Commassie Brillant Blue-G250

**Table III.2.** Acrylamide-Bisacrylamide mixture (30:0.8)

| | |
|---|---|
| Acrylamide | 29.2 g |
| N'N'-bis-methylene-acrylamide | 0.8 g |

Per 100 ml of solution, completed with deionized and distilled water.

Stored in the dark, at 4 °C.

**Table III.3.** 4X Seperating Gel Buffer (1.5M Tris-HCl, pH 8.8)

| | |
|---|---|
| Tris base | 18.5 g |

Per 100 ml of solution, completed with deionized and distilled water.

pH was adjusted to 8.8 with 3N HCl and stored at 4°C.

**Table III.4.** 4X Stacking Gel Buffer (0.5M Tris-HCl, pH 6.8)

| | |
|---|---|
| Tris-base | 3 g |

Per 50 ml of solution, completed with deionized and distilled water.

pH was adjusted to 6.8 with 5N HCl and the solution was stored at 4°C.

**Table III.5.** 10% SDS

| | |
|---|---|
| SDS | 10 g |

Per 100 ml of solution, completed with deionized and distilled water.

Stored at room temperature.

**Table III.6.** 10% APS

| | |
|---|---|
| Amonnium persulfate | 0.5 g |

Per 5 ml of solution, completed with deionized and distilled water.

**Table III.7.** 2X SDS Gel Loading Buffer

| | |
|---|---|
| 0.5 M Tris-HCl, pH 6.8 | 1 ml |
| 10 % SDS | 1.6 ml |
| Glycerol | 0.8 ml |
| β-mercaptoethanol | 0.4 ml |
| Bromophenol blue | 0.4 ml |

Per 10 ml of solution, completed with deionized and distilled water.

Stored at -20$^{o}$C.

**Table III.8.** 10X SDS-PAGE Running Buffer (g/L)

| | |
|---|---|
| Glycine | 154 g |
| Tris-base | 30 g |
| SDS | 10 g |

**Table III.9.** Electrophoresis separating gel (10 %)

| | |
|---|---|
| Solution A | 4 ml |
| Solution B | 2.5 ml |
| 10% APS | 50 µl |
| TEMED | 5µl |
| $dH_2O$ | 3.5 ml |

**Table III.10.** Electrophoresis stacking gel (5 %)

| | |
|---|---|
| Solution A | 2.5 ml |
| Solution C | 0.67 ml |
| 10% APS | 30 µl |
| TEMED | 5 µl |
| $dH_2O$ | 2.5 ml |

**Table III.11.** Fixing Solution

| | |
|---|---|
| Ethanol | 50 % |
| Phosphoric acid | 2 % |

**Table III.12.**Washing Solution

| | |
|---|---|
| Methanol | 34 % |
| Ammoniumsulfate | 17 % |
| Phosphoric acid | 2 % |

**III.1.4.2.** SDS-PAGE Molecular Weight Markers

**Table III.13.** SDS-PAGE marker (Fermentas #SM0441)

| Protein (≈0.2mg/ml) | Source | Molecular Wight (kDa) |
|---|---|---|
| B- galactosidase | *E. Coli* | 117 |
| Bovine serum albumin | bovine plasma | 84 |
| Ovalbumin | chicken egg white | 49 |
| Carbonic anhydrase | Bovine erythrocytes | 34 |
| β- lactoglobulin | bovine milk | 25 |
| Lysozyme | chicken egg white | 19 |

**III.1.4.3.** Buffers Used in Enzyme Activity Measurements

**Table III.14.** $K^+PO_4$ Buffer (1M, pH 7.0) (g/L)

| | |
|---|---|
| 1M $K_2HPO_4$ | 450 ml |
| 1M $KH_2PO_4$ | 550 ml |

**III.1.4.4.** Preparation of Substrate Solution

25 mg CENTA was dissolved in 1 ml 50 mM $K^+PO_4$ buffer and aliquoted and maintained at -20°C. The concentration of the stock solution was 4.7 mM.

**III.1.5.** Enzyme and Peptide

Enzyme : *E. Coli* RTEM-1 (Invitrogen, PV3575)

Peptide : NH-HA AGD YYA Y-CONH2 (H45-Y53 residues of BLIP)

**III.1.6.** Laboratory Equipment

**Table III.15.** List of Laboratory Equipments

| **Purpose** | **Equipment** |
|---|---|
| Autoclaves | Systec 3870 ELV Steam Strilizer, Germany<br>Nüve OT 032, Türkiye |
| Balances ( 0.1, 1, 10mg ) | Ab204-S, Pg403-S, Pg4002-S, Metler Toledo, Sweden |
| Centrifuge | 3K30 Sigma, Germany |
| Deep Freezer (-20°C) | Hlf585, Heto Holten, Denmark |
| Deep Freezer (-86°C) | Uf4420, Heto Holten, Denmark |
| Ice Machine | Arttex Bar Line, Italia |
| Magnetic Stirrer | MSH 300 BIOSAN |
| Microcentrifuge | 1-15k, Sigma, Germany |
| Micropipets | Finnpipet, Finland |
| No Frost Refrigerator | Arçelik, Turkiye |
| Orbital Shaker | Certomat Bs1, B. Braun, Germany |
| pH meter | Mp220k, Metler Toledo, USA |
| Power Supply | Heidolph MR3001, Germany |
| Safety Cabinet | Labculture ESCO, USA |
| Safety Thermostat | Nüve FN500, Türkiye |
| SDS-PAGE Electrophoresis System Mini | Protean, BIORAD, Canada |
| Spectrophotometer | Lambda 35, Perkin Elmer, USA |
| UV Monitor | UV Lamb, (Biolab, USA) |
| Vortex | Heidolph, Germany |
| Water Purification Systems | MILLI-Q UF Plus (MILLIPORE, USA)<br>MILLI-RQ Plus (MILLIPORE, USA)<br>ELGA LabWater, England |

## III.2. METHODS

In all experiments, sterilized equipment was used. Glassware, pipetman tips, eppendorf tubes, petri dishes etc. were either bought sterile or were sterilized in an autoclave at 1.02 atm and 121°C for 15 minutes. Antibiotics and IPTG were filter sterilized using a membrane with a pore size of 45μm. Experimental work that required sterile environment was carried out under a laminar flow.

### **III.2.1.** Preparation of Frozen Stocks

Frozen stocks were prepared from a single colony from a master plate which was inoculated into LB media and grown overnight at 37°C and 180 rpm. 500 μl from the grown cells were mixed with 50 % sterile glycerol and stored at -80°C.

### **III.2.2.** Preparation of Preculture

5 ml of LB medium was inoculated from a frozen stock culture which was kept at -80°C and the cells were grown overnight at 37°C and 180 rpm. Growth was monitored by measuring the optical density at 600 nm using a spectrophotometer. LB media were supplemented with 100 μg/ml ampicillin for the selection of *E. Coli* TB1 (pUC18) cells and 50 μg/ml kanamycin for the selection of *E. Coli* BL21 (pET26-SJ).

### **III.2.3.** Culture Conditions

#### **III.2.3.1.** β-Lactamase Production

Cells were grown in 200 ml of medium in a 1 lt of flask at 37°C and 180 rpm. LB media were supplemented with 100 μg/ml ampicillin for the selection of pUC18 harboring cells. The cells were grown to an optical density of approximately 1.5 at 600 nm.

#### **III.2.3.2.** BLIP Production

For the selection of *E. Coli* BL21 (pET26-SJ), cells were grown in 200 ml of LB medium supplemented with 50 μg/ml kanamycin, in a 1 lt of flask at 37°C and 180 rpm. BLIP production was under the control of a T7 promoter, therefore the cells were induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for recombinant protein expression.

Cells were induced when optical density at 600 nm reached 0.5 and cells were allowed to grow 3 hours after induction.

**III.2.4.** Quantitation of Growth

Growth of *E. Coli* cultures was determined by measuring optical densities of the cells at 600 nm using a spectrophotometer. LB medium was used to dilute the samples to keep the spectroscopic readings within reliable limits which are between 0.2 and 0.7. Preculture of *E. Coli* cells were inoculated into fresh LB medium at a 1:100 dilution. 0.1-1 ml samples were taken at desired time intervals to monitor cell growth.

**III.2.5.** Extraction of Periplasmic Proteins and BLIP

Osmotic shock procedure was used to extract RTEM-1 β-lactamase (Nossal *et al*, 1966) and BLIP from *E. Coli*. LB broth inoculated with a preculture at a 1:100 dilution ($OD_{600}$ of preculture was adjusted to 0.7) was incubated at 37°C and 180 rpm. When cells expressing β-lactamase reached the optical density of 1.5 at 600 nm, they were centrifuged at 7.000 rpm for ten minutes at 4°C. For the extraction of BLIP, following induction, *E. Coli* BL21 (pET-26SJ) cells were grown for 3 hours and they were centrifuged at 7.000 rpm for ten minutes at 4°C. After discarding the supernatants, the cells were resuspended in 20 % sucrose in 30 mM Tris-HCl (pH 8.0) with 1 mM ethylenediaminetetraacetic acid (EDTA). The cells were incubated for 20 minutes at room temperature and were centrifuged at 9.000 rpm for 20 minutes; the cell pellet was resuspended in ice-cold 5 mM $MgSO_4$ which caused periplasmic proteins to be released. The cells were removed from the periplasmic extract by centrifugation at 9.000 rpm for 20 minutes. The supernatant contained periplasmic protein mixture including RTEM-1 β-lactamase or BLIP. Protein extracts were stored at -20°C for further use.

**III.2.6.** Determination of Protein Concentration

Protein concentration in the periplasmic protein extract was determined using the method described by Bradford (1976). Protein samples were mixed with Bradford solution and their optical densities at 595 nm were recorded. Bovine Serum Albumin (BSA) was used as the standard for the preparation of the calibration curve. Bradford samples with different BSA concentrations were

prepared and the optical densities of these samples at 595 nm were recorded. A standard curve of absorbance versus micrograms of protein was constructed using linear regression analysis. To determine the protein concentration of cell extracts, this plot was used. The calibration curve obtained is given in Appendix A.

**III.2.7.** SDS-PAGE Analysis of Periplasmic Proteins

SDS-PAGE is used to separate proteins according to their molecular sizes. For this purpose periplasmic protein extract was mixed with 2X Sample Buffer containing SDS at a 1:1 ratio and boiled for five minutes for denaturation of the proteins. The sample and the marker were then loaded to the SDS polyacrylamide gel and run at 110 V for approximately 75 minutes. When the electrophoretic separation was finished, the gels were removed from the plate and placed into the fixation solution overnight to fix proteins. The following day the gels were washed with solution 2 for an hour. Then the gels were stained with Commassie G-250 at least overnight and were dried. The protein bands of the samples were compared against the standard.

**III.2.8.** *In-vitro* Enzyme Activity Assay

In this study, as a source of β-lactamase periplasmic protein extract from *E. Coli* TB1 (pUC18) cells were used and periplasmic protein extracts from *E. Coli* BL21 (pET-26SJ) cells carrying the BLIP gene was used as the source of BLIP. The activity of RTEM-1 β-lactamase was monitored by the hydrolysis of CENTA (Calbiochem) as the substrate. CENTA hydrolysis caused a change in the absorbance at OD of 405 nm. The assay involved monitoring the rate of hydrolysis of the substrate by RTEM-1 β-lactamase.

Reactions were carried out in a total reaction volume of 1 ml. Reaction mixture contained 47 μM CENTA with a pre-determined amount of periplasmic protein extract. Periplasmic protein extract from cells not harboring pUC18 plasmid was used as control.

One Unit (U) of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at 25°C and pH 7.0 (Equation III.1):

$$\text{Activity (U)} = \frac{Vt \times dA/dt}{\varepsilon\lambda \times Vs \times d} \times 10^6 \qquad \textbf{(III.1)}$$

Vt = Total reaction volume of (ml)

dA/dt = Absorbance change per time ($min^{-1}$)

$\varepsilon\lambda$ = Extinction coefficient of the substrate ($cm^2.mol^{-1}$)

(The extinction coefficient of CENTA at 405 nm is 6400 $M^{-1}$ $cm^{-1}$)

Vs = Volume of enzyme (ml)

d = Light path (1 cm)

**III.2.9.** Purification of RTEM-1 β-lactamase

Different methods were investigated alone and with different combinations for the purification of the β-lactamase enzyme.

**III.2.9.1.** Dialysis

Periplasmic protein extract was dialyzed to remove salts and the low molecular weight impurities. To prepare tubing for dialysis, the tubing was boiled in 2 liters of 20 % EtOH for 1 hour. The tubing was cooled down in distilled water. Finally the tubing was stored submerged in 20 % EtOH at 4°C. The tubing was washed thoroughly with distilled water before to use.

Extract containing the proteins was dialyzed overnight against the desired buffer.

**III.2.9.2.** Ion-exchange chromatography

Ion-exchange column was prepared by slowly adding 5 g DEAE-cellulose (SIGMA) to 0.1 M 300 ml sodium hydroxide with gentle stirring for 30 min (final pH was 13). Sodium hydroxide solution was discarded and the resin was washed with double distilled water until pH reached 8.0. Then the solution was replaced with 0.1 M hydrochloric acid with gentle stirring for 30 min (final pH was 1.0). The resin was washed with double distilled water until pH reached 3.0. Distilled water was discarded and was replaced with 500 mM Tris-HCl (pH 8.0). The resin was stirred for 30 min. Tris-HCl buffer was discarded and following equilibration with

50 mM Tris-HCl (pH 8.0), the resin was degassed and transferred into a glass column.

The prepared resin was loaded onto the column (2.5 x 10 cm) at 4°C. Then the resin in the column was washed with 10 mM Tris-HCl buffer (pH 7.0) until the readings at $A_{280}$ was zero. Periplasmic proteins that were removed from $MgSO_4$ and place in 10 mM Tris-HCl buffer (pH 7.0) using dialysis were loaded onto the column. The column was washed with 10 mM Tris-HCl (pH 7.0). β-lactamase was then eluted with 100 mM Tris-HCl (pH 7.0). Protein concentration was determined from the absorbance at 280 nm and the enzyme activity was calculated from the absorbance at 405 nm (Figure IV.6.).

**III.2.9.3.** Concentration of the crude enzyme

Two different methods, ammonium sulfate precipitation and ultrafiltration were used to concentrate β-lactamase enzyme found in the periplasmic protein extract.

In this first method, 65 and 80 % ammonium sulfate were used to precipitate the enzyme. The precipitate was resuspended in 10 mM Tris-HCl buffer (pH 7.0) and dialyzed overnight against the same buffer to remove ammonium sulphate. Proteins were verified on SDS-PAGE to estimate their purity.

The second method was ultrafiltration. 20 ml of the periplasmic proteins containing β-lactamase were concentrated to 2 ml using ultrafiltration membranes (Amicon) with a 10.000, 30.000, and 50.000 molecular weight cut-off limits. Protein concentration was determined by recoding absorbance at 595 nm as described in section III.7.6. Enzyme activities of the protein extract over the membrane and lower phase ultrafiltration were measured at 405 nm as described in III.7.8.

**III.2.9.3** Purification of the Concentrated Enzyme by Ion-Exchange Chromatography

Two different columns 2.5 x 10 cm and 1 x 5 cm were used for the purification of the enzyme using ion-exchange chromatography. After the DEAE-cellulose resin was transferred into a glass column at 4°C, the column was washed with 10 mM Tris-HCl buffer (pH 7.0) until the readings at $A_{280}$ of washing buffer form the column was zero. Then the concentrated enzyme was loaded onto the column and allowed to flow through the column at a flow rate of 30ml/h and 12ml/h

for the big and the small column, respectively. After the column loaded with the proteins was washed with 10 mM Tris-HCl (pH 7.0), β-lactamase was eluted by Tris-HCl (pH 7.0) buffer; at a concentration of 100 mM, 150 mM or 200 mM. Protein concentration was determined measuring the absorbance at 280 nm. Enzyme activity of each fraction measured and calculated as explained in Section III.2.8. Purity of fractions was checked on SDS-PAGE.

**III.2.10.** Enzyme Inhibition Assay

Commercial RTEM-1 β-lactamase, periplasmic protein extract and purified periplasmic protein extract were used for inhibition assays. β-lactamase has been incubated with 7.4 μg/ml of periplasmic extract containing BLIP. As a control group, the same amount of enzyme has been incubated with 5 mM $MgSO_4$. 5 mM $MgSO_4$ was the final solution following osmotic shock to keep the periplasmic proteins. The mixtures placed in glass tubes and were incubated for 45 minutes, at 25°C (room temperature) and 37°C (human body temperature). Enzyme-BLIP interaction was monitored at 405 nm to examine the inhibitory effect of BLIP under the assay conditions. Kinetic parameters $K_m$ and $V_{max}$ for Enzyme-BLIP/Enzyme Control interactions were calculated. To calculate $K_m$ value, at least 5 substrate concentrations were chosen in the reaction course. These experiments were repeated in triplicate and the type of inhibition was determined.

# CHAPTER IV

## RESULTS AND DISCUSSION

### IV.1. GROWTH OF *E. COLI* TB1 CELLS

The growth of wild type *E. Coli* TB1 cells and *E. Coli* TB1 (pUC18) cells grown in LB medium were compared. LB medium has a rich content of vitamins and amino acids. *E. Coli* cells consumed ampicillin (100 μg/ml) supplied in the growth medium in the first ten hours (Utkur *et al*., 2006). Therefore, the appropriate time to harvest the cells for sufficient β-lactamase production was determined based on the growth profile of the cells. Optical readings of the samples were monitored at 600 nm and growth profiles of the cells are given in Figure IV.1.

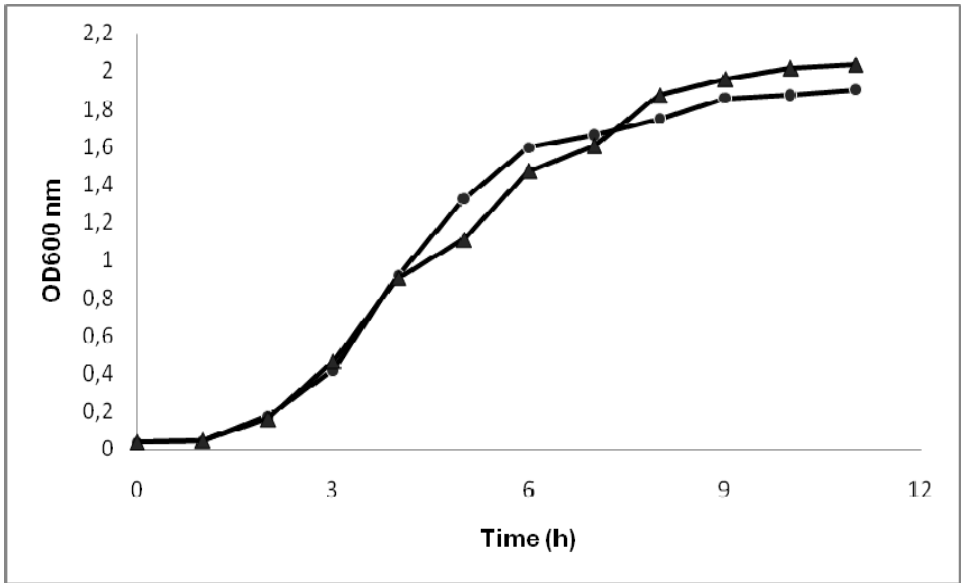


**Figure IV.1.** Growth profiles of *E. Coli* TB1 (●) and *E. Coli* TB1 (pUC18) cells (▲)

Both *E. Coli* strains entered exponential phase shortly after inoculation into the culture. During exponential phase, they showed a similar growth pattern and after 12 hours, both cultures entered stationary phase. The final OD at 600 nm was approximately 2.0 for both cultures.

## IV.2. EXTRACTION OF THE PERIPLASMIC PROTEINS AND ELECTROPHORETIC ANALYSIS

Since β-lactamase is a periplasmic protein, *E. Coli* TB1 (pUC18) cells were subjected to osmotic shock to extract periplasmic proteins containing β-lactamase. Periplasmic proteins from wild type *E. Coli* TB1 cells were extracted by the same method to be used as control in enzyme assays. Protein concentration of the extract with the periplasmic proteins was determined by Bradford method. Protein extracts were analyzed on SDS-polyacrylamide gels. Same amount of proteins from each sample were loaded to the polyacrylamide gels and the proteins were visualized by Commassie staining (Figure IV.2).

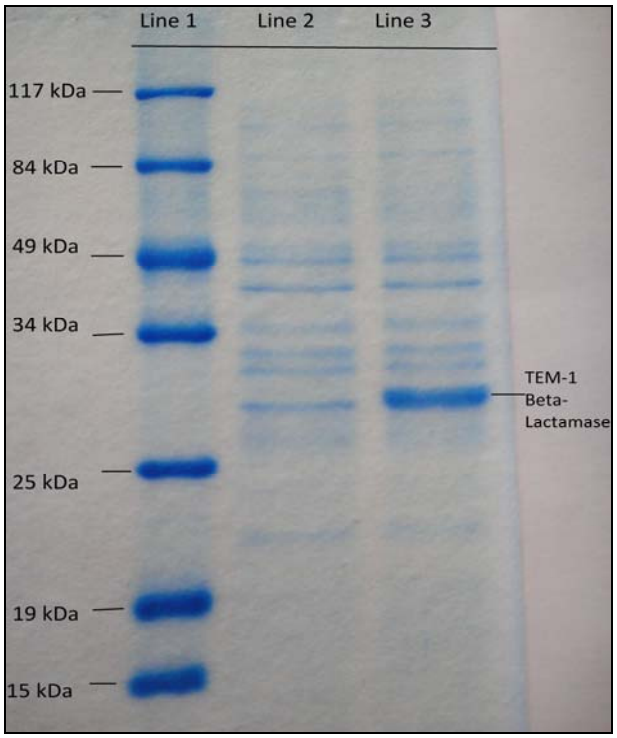


**Figure IV.2.** Electrophoretic analysis of the periplasmic protein extracts *E. Coli* TB1 cells. Lane 1: Molecular weight marker, Lane 2: *E. Coli* TB1 cells, Lane 3: *E. Coli* TB1 (pUC18) cells

In Figure IV.2. Lane 3, the protein band corresponding to β-lactamase appeared between 25 kDa and 34 kDa. Its intensity suggested that there should be significant β-lactamase present in the cells when they were subjected to osmotic shock at an optical density of 1.5 at 600 nm.

## IV.3. β-LACTAMASE ACTIVITY IN THE PERIPLASMIC PROTEIN EXTRACT

In the periplasmic protein extract, the exact amount of β-lactamase is unknown and the total amount of protein and hence β-lactamase amount may vary due to the slight differences in growth conditions. Therefore β-lactamase activity in various volumes of periplasmic protein extract has been measured. Initial experiments were carried out using 50 µl periplasmic protein extract with a total of 3.7 µg protein. CENTA hydrolysis, monitored as the change in absorbance at 405 nm for 1 minute, is given in Figure IV.3.

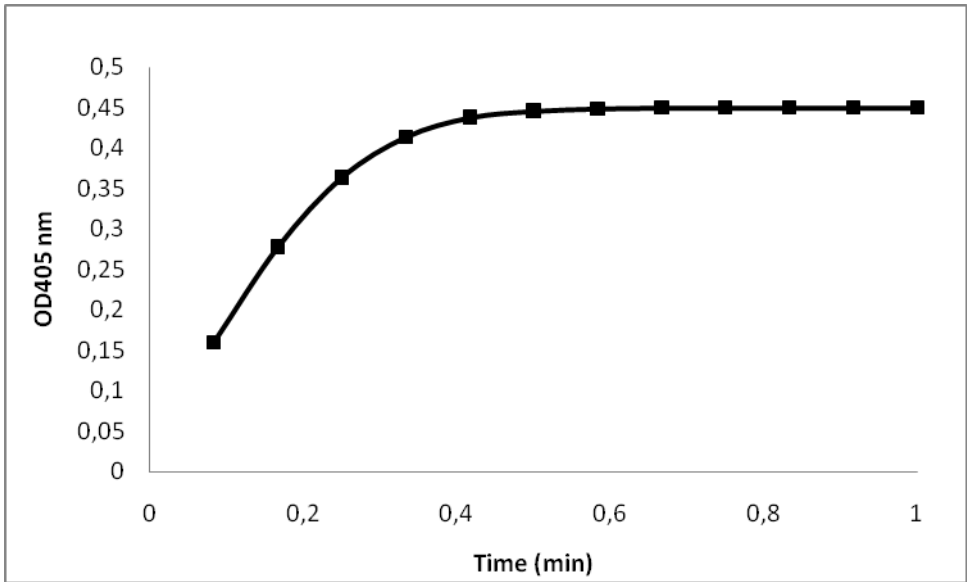


**Figure IV.3.** Hydrolysis of CENTA by β-lactamase found in 50 µl periplasmic protein extract

The differences in the absorbance readings recorded every 5 seconds were not enough for activity calculations (Figure IV.3.) since the reaction finished within a minute. It was not possible to obtain the linear initial rate from the curve obtained. This was due to the high amount of β-lactamase in the sample used for the assay. Therefore the amount of periplasmic protein extract to be used in the assay was reduced and 5 µl, 10 µl, 20 µl, and 25 µl of periplasmic protein extract with 0.37 µg, 0.74 µg, 1.48 µg, and 1.85 µg proteins, respectively were used. CENTA hydrolysis was monitored at 405 nm for one minute. The recorded absorbance readings are given in Figure IV.4.

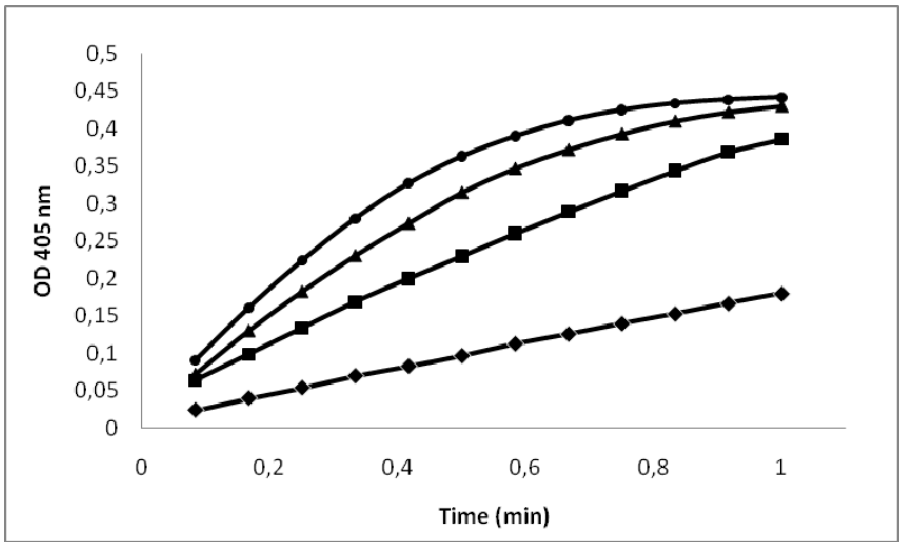


**Figure IV.4.** CENTA hydrolysis using 5 µl (♦), 10 µl (■), 20 µl (▲), and 25 µl (●) periplasmic protein extract

Based on the results presented in Figure IV.4, by using 5 or 10 µl periplasmic protein extract with 0.37 and 0.74 µg proteins, it was possible to get the linear initial rate before the rate of the reaction started to level off. Unfortunately, as already stated, protein concentration in the periplasmic protein extract may vary slightly in each experiment. Therefore total protein amount found in the sample was taken as the standard in further experiments.

## IV.4. PURIFICATION OF β-LACTAMASE ENZYME

Periplasmic proteins are found in 5 mM $MgSO_4$ following extraction by osmotic shock. This necessitates the removal of the salts and possible low molecular weight impurities, before the periplasmic protein extract could be applied to the ion-exchange column. For this reason the extract was dialyzed. Dialysis also enabled us to change the buffer composition proteins were found in.

### **IV.4.1.** Purification using Ion-Exchange Chromatography

15 ml periplasmic protein extract has been removed from salts and low molecular weight impurities by dialyzing it against 10 mM Tris-HCl buffer (pH 7.0). Periplasmic proteins found in 10 mM Tris-HCl buffer were loaded to the DEAE-cellulose column equilibrated with 10 mM Tris-HCl buffer (pH 7.0).

Following elution, protein concentration and β-lactamase activity in each fraction was determined. The results were plotted (Figure IV.5).

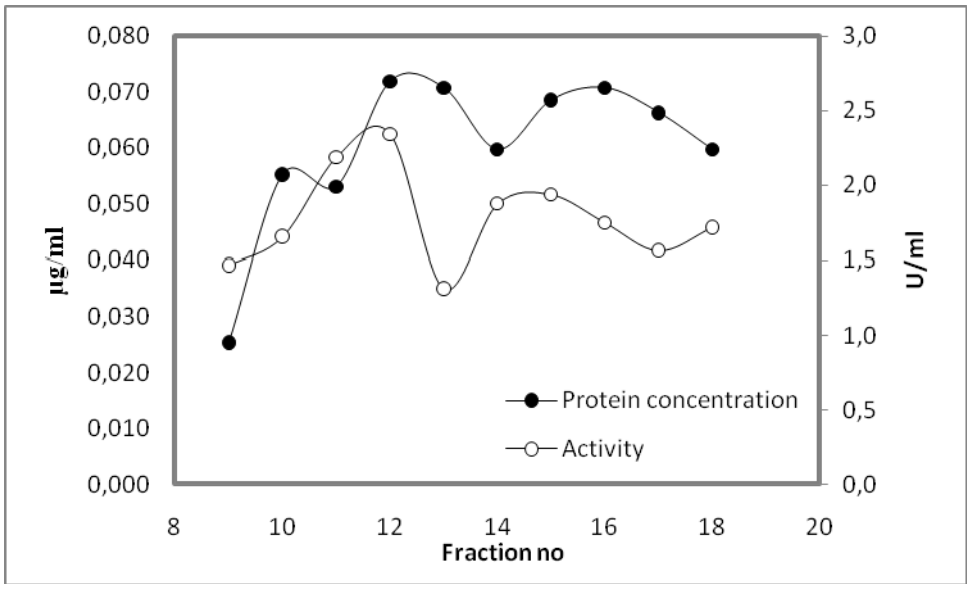


**Figure IV.5.** Activity and protein concentration in each fraction of partially purified β-lactamase after dialysis

β-lactamase which is negatively charged, was expected to bind to immobilized functional groups in the anion exchange chromatography, which are positively charged. Following elution, it was expected to pool β-lactamase within a couple of fractions. Unfortunately results presented in Figure IV.5 show that the enzyme was dispersed to many fractions. It was not possible to collect β-lactamase in successive fractions. Highest enzyme activity was measured in the 12$^{th}$ fraction but comparable activity was obtained in fractions 13, 15 and 16, as can be seen in Figure IV.5.

**Table IV.1.** Recovery of β-lactamase during purification

| Step | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|
| Periplasmic Protein Extract | 0.302 | 167.2 | 554.2 | 100.0 | 1 |
| Dialysis | 0.223 | 79.7 | 357.0 | 64.4 | 0.64 |
| Anion-exchange chromatography (Peak 12) | 0.072 | 1.5 | 20.4 | 3.7 | 0.04 |

In Table IV.1. purification of β-lactamase using ion-exchange chromatography has been summarized. Purification fold of 0.04 indicated that this method was not useful in purifying the enzyme. The concentration of β-lactamase in

the periplasmic protein extract was too low. Therefore the enzyme was distributed to too many fractions hence it was lost. In addition to these, it is also possible that proteins were lost due to non-specific binding onto the dialysis membrane, due to protein aggregation and instability during the intermediate stages of buffer exchange (McPhie, 1971).

**IV.4.2.** Concentration of the Protein Extract

Proteins found in the periplasmic extract were too dilute for purification directly with ion-exchange chromatography. In an effort to concentrate β-lactamase enzyme, precipitation with ammonium sulfate and ultrafiltration have been used.

**IV.4.2.1.** Ammonium Sulfate Precipitation

Periplasmic protein extract was subjected to ammonium sulfate precipitation and the fraction that precipitated at 65 and the 80 % ammonium sulfate was retained. Solubility of proteins changes with salt concentration. In the presence of 65 % ammonium sulfate, some proteins were expected to precipitate, some to remain in the solution. In the presence of 80 % ammonium sulfate, sufficiently high ionic strength, basically all proteins were expected to precipitate. Based on reports found in literature, at % 65 ammonium sulfate concentrations, the solubility of β-lactamase is higher. As this value is increased, solubility of β-lactamase was expected to decrease and the protein should precipitate out from the solution.

Results obtained from ammonium sulfate precipitation are summarized in Tables IV.2 and IV.3.

**Table IV.2.** Summary of protein concentrations using 65 % ammonium sulfate

| | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|
| Crude extract | 1.87 | 27468.8 | 14709.6 | 100 | 1.0 |
| Supernatant | 0.55 | 8375.0 | 15158.8 | 30.5 | 1.0 |
| Precipitate | 0.14 | 573.4 | 3992.0 | 2.1 | 0.3 |

The results in Table IV.2 show that with 65 % ammonium sulfate, most of the β-lactamase was retained in the solution whereas it was not possible to concentrate it. There was no change in purification fold.

**Table IV.3.** Summary of protein concentrations using 80 % ammonium sulfate

| | Total Protein (mg) | Total Activity (U) | Spesific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|
| Crude extract | 2.83 | 41203.1 | 14537.6 | 100 | 1 |
| Supernatant | 0.55 | 3028.1 | 5536.3 | 7.3 | 0.4 |
| Precipitate | 0.91 | 17334.4 | 19084.7 | 42.1 | 1.3 |

The results in Table IV.3. show that, with 80 % ammonium sulfate, most of the β-lactamase precipitated from the solution. The specific activity increased from 14537.6 U/mg protein to 19084.7 U/mg of protein. Hence purification fold was 1.3. However this purification fold was still not high enough to conclude that the protein has been concentrated successfully.

In addition to this, in either case, protein yield was very low. At the end of precipitation, more than half of the enzyme was lost. Even with 80 % ammonium sulfate, only 42.1 % of the initial enzyme was retained in the precipitated fraction.

**IV.4.2.2** Ultrafiltration

As a second approach to concentrate β-lactamase enzyme found in the periplasmic extract, ultrafiltration was used. Therefore the periplasmic protein extract was concentrated by polyethersulfone (PES) 10.000, regenerated cellulose 30.000 and regenerated cellulose 50.000 MWCO ultrafiltration membranes. Since β-lactamase is a 31.5 kDa protein, it was either expected to remain over the membrane or to flow through the membrane depending on the molecular weight cut-off of the ultrafiltration membrane. The protein extracts over the membrane and in the flow through were collected and the enzyme activity was calculated.

With the 10.000 MWCO membrane, β-lactamase was expected to remain over the membrane. Following ultrafiltration using 10.000 MWCO membrane, specific activity increased from 19190.5 to 1661786 U/mg. Hence 87 fold purification was obtained (Table IV.4.).

**Table IV.4.** Summary of the ultrafiltration step by 10.000 MWCO membrane

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 6.0 | 1.92 | 36750 | 19190.5 | 100 | 1,0 |
| Crude extractover the Membrane | 1.0 | 0.02 | 36072.9 | 1661786 | 98.2 | 86.6 |
| Lower phase | 5.0 | 1.01 | 867.2 | 862.4 | 2.4 | 0.001 |

With 30.000 MWCO membrane, β-lactamase was expected to be divided between the solutions that flows through the membrane and that remains over the membrane. In Table IV.5. it can be seen that the specific activity of the protein extract that remained over the membrane was only 158.6 U/mg and that found in the flow through was 635.4 U/mg protein whereas the specific activity found in the periplasmic protein extract was 1637.3 U/mg protein which meant that the protein was further diluted.

**Table IV.5.** Summary of the ultrafiltration step by 30.000 MWCO membrane

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 6.0 | 0.396 | 648 | 1637.3 | 100 | 1.0 |
| Crude extract over the Membrane | 1.0 | 0.242 | 38.4 | 158.6 | 9.7 | 0.09 |
| Lower phase | 5.0 | 0.028 | 89 | 635.4 | 38.8 | 0.38 |

With 50.000 MWCO membrane, all β-lactamase found in the periplasmic protein extract was expected to flow through the membrane. Results presented in Table IV.6. show the total yield was less than 20 % and that most of the protein or enzyme activity was lost. It was not possible to get a high purification fold with a 50.000 MWCO membrane.

**Table IV.6.** Summary of the ultrafiltration step by 50.000 MWCO membrane

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 6.0 | 0.396 | 648 | 1637.3 | 100 | 1.0 |
| Crude extract over the Membrane | 1.0 | 0.137 | 30 | 218.4 | 13.3 | 0.13 |
| Lower phase | 5.0 | 0.886 | 69.5 | 78.5 | 4.8 | 0.05 |

Based on the purification fold of the results presented in Tables IV.5. and IV.6., the enzyme was not concentrated with regenerated cellulose 30.000 and 50.000 MWCO membranes. PES 10.000 MWCO membranes can be successfully used for the concentration of the enzyme.

**IV.4.3.** Purification of the Concentrated Enzyme

DEAE-cellulose anion-exchange chromatography was performed with either 2.5 x 10 cm or 1 x 5 cm columns as described in section IV.4.2.

**IV.4.3.1.** Purification of β-lactamase Concentrated with Ammonium Sulfate Precipitation

β-lactamase has slightly been concentrated using 80 % ammonium sulfate (Table IV.3.), hence the precipitate has been applied to the anion-exchange column (2.5 x 10 cm) after it has been dialyzed (Figure IV.6.).

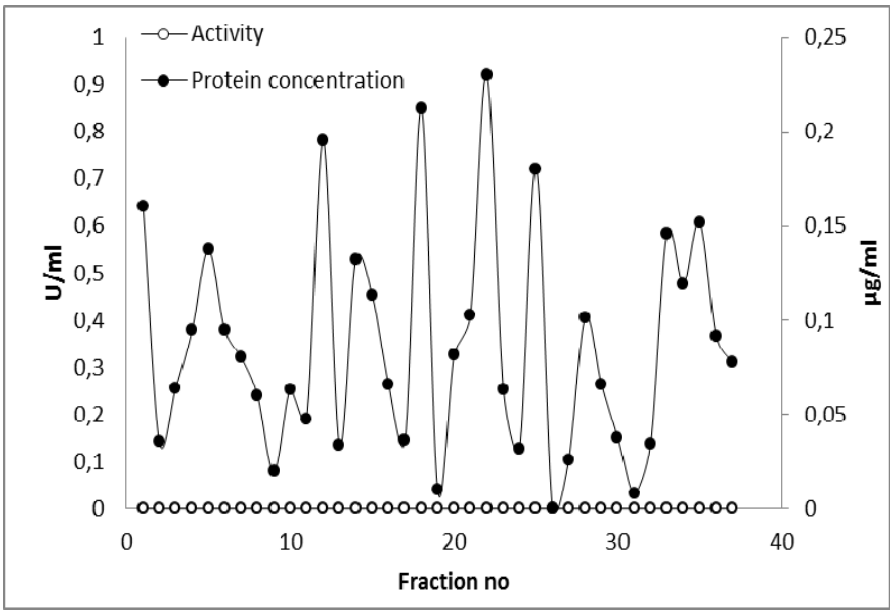


**Figure IV.6.** Elution of RTEM-1 β-lactamase with 100 mM Tris-HCl (pH 7.0).

The enzyme was expected to bind to the column and then to be eluted from the column by adjusting the ionic concentration of the mobile phase. As Figure IV.6. shows, none of the elution fractions retained β-lactamase activity. It is possible that ammonium sulfate precipitation followed by the dialysis to remove the $MgSO_4$ salt diluted the enzyme or the enzyme has lost its activity.

**IV.4.3.2.** Purification of β-lactamase using the Ultrafiltrated Enzyme

After dialysis, the protein extract over the membrane concentrated by PES 10.000 MWCO membrane was loaded onto the column. After elution, active fractions were combined and the activity of the combined enzyme was measured.

Analysis of Figure IV.7, showed that when β-lactamase hase been eluted with 100 mM Tris-HCl, fractions between 32 and 40 contained significant amount of the enzyme thus they were combined. Table IV.7 gives the characteristics of purification.

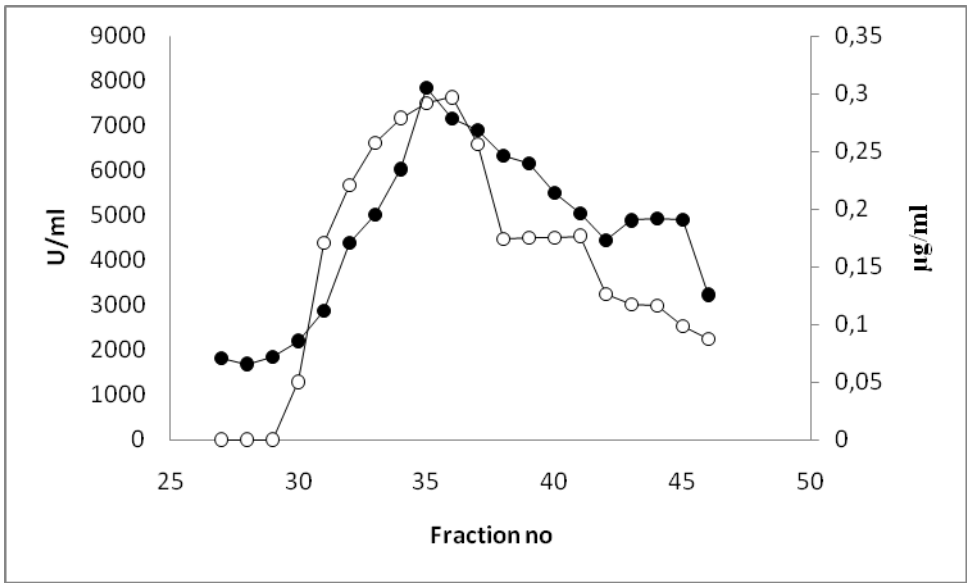


**Figure IV.7.** Elution of RTEM-1 β-lactamase with 100 mM Tris-HCl (pH 7.0).

**Table IV.7.** Summary of the purification of the β-lactamase concentrated with PES 10.000 MWCO membrane and eluted with 100 mM Tris-HCl

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 20.0 | 3.123 | 43718.8 | 14000.5 | 100 | 1.0 |
| Concentrated and dialyzed extract | 2.0 | 1.540 | 36875.0 | 23950.0 | 84.3 | 1.7 |
| Combined fractions | 4.5 | 0.847 | 29432.8 | 34757.9 | 67.3 | 2.5 |

Approximately 67 % of the total activity as yield means that even if 33 % of the enzyme was distributed among the other fractions, and the fold which was 2.5 times of the crude extract means that the enzyme was partially purified (Table IV.7.).

Analysis of Figure IV.8, showed that when β-lactamase hase been eluted with 150 mM Tris-HCl,fractions between 16 and 21 contained significant amount of the enzyme thus they were combined. Table IV.8 gives the characteristics of purification.

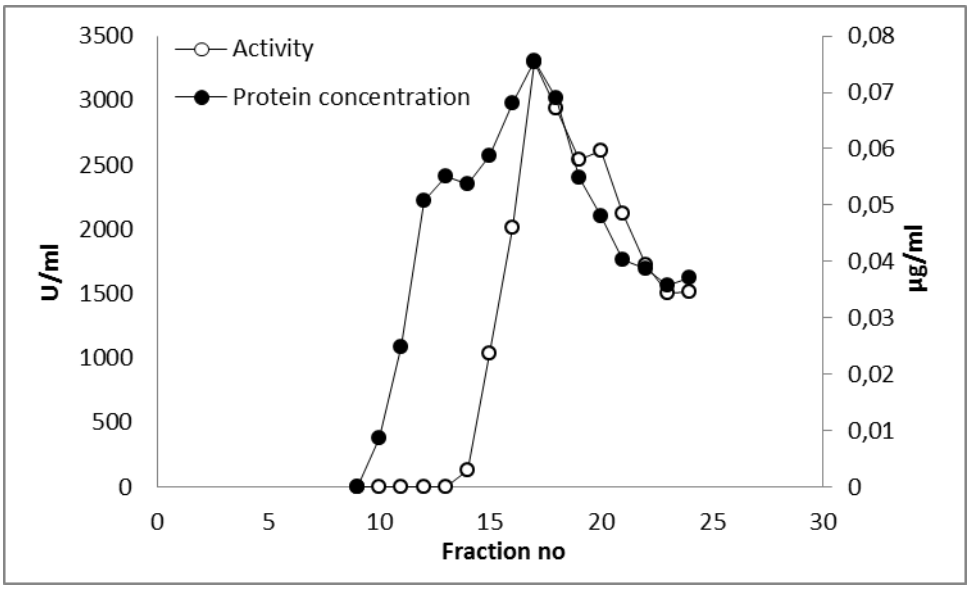


**Figure IV.8.** Elution of RTEM-1 β-lactamase with 150 mM Tris-HCl (pH 7.0).

**Table IV.8.** Summary of the purification of the β-lactamase concentrated with PES 10.000 MWCO membrane and eluted with 150 mM Tris-HCl

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 20.0 | 1.949 | 99062.5 | 50822.9 | 100 | 1.0 |
| Concentrated and dialyzed extract | 2.0 | 1.058 | 60718.8 | 57383.5 | 61.3 | 1.1 |
| Combined fractions | 6.6 | 0.080 | 20439.4 | 256911.6 | 20.6 | 5.1 |

The purification fold of the combination of the fractions was 5.1. The purification of the enzyme was slightly better than when the enzyme was eluted with 100 mM Tris-HCl (Table IV.7).

Analysis of Figure IV.9, showed that when β-lactamase has been eluted with 200 mM Tris-HCl, fractions between 9 and 15 contained significant amount of the enzyme thus they were combined. Table IV.9. gives the characteristics of purification.

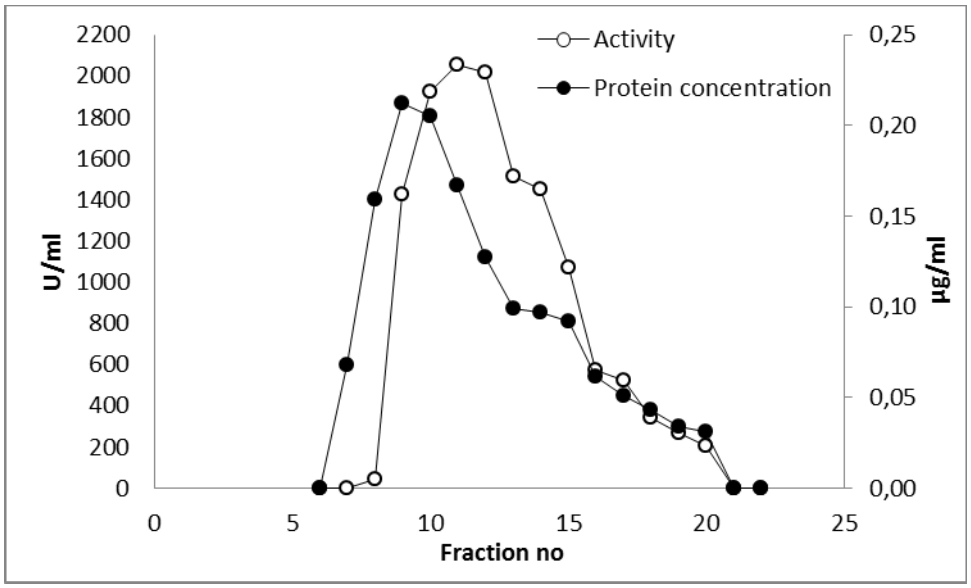


**Figure IV.9.** Elution of RTEM-1 β-lactamase with 200 mM Tris-HCl (pH 7.0).

The yield was approximately 40 % and the fold has been improved in each step of the purification (Table IV.9.).

**Table IV.9.** Summary of the purification of the β-lactamase concentrated with PES 10.000 MWCO membrane and eluted with 200 mM Tris-HCl

| | V (ml) | Total Protein (mg) | Total Activity (U) | Specific Activity (U/mg protein) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude extract | 7.0 | 1.875 | 49809.4 | 26566.2 | 100 | 1.0 |
| Concentrated and dialyzed extract | 1.0 | 0.686 | 47031.3 | 68562.0 | 94.4 | 2.6 |
| Combined fractions | 7.7 | 0,.180 | 19659.1 | 109506.1 | 39.5 | 4.1 |

As a result, Figures IV.7., IV.8., and IV.9. indicate that when the molarity of the buffer was increased, proteins were eluted in early fractions.

The presence of β-lactamase in the combined fractions has been confirmed using SDS-page analysis (Figure IV.10).

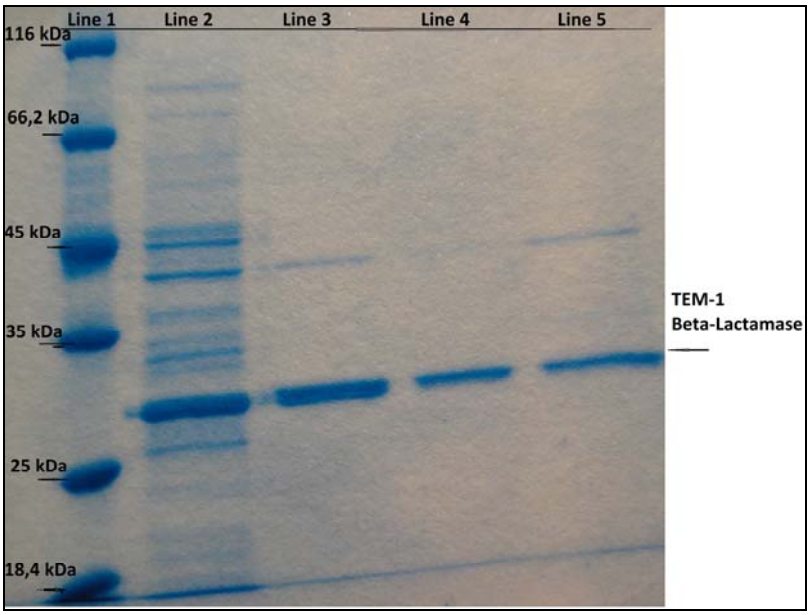


**Figure IV.10.** Analysis of the purified β-lactamase on SDS-PAGE. Lane 1: Molecular weight marker, Lane 2: Crude periplasmic protein extract, Lane 3: Protein eluted with 100 mM Tris-HCl (pH 7.0), Lane 4: Protein eluted with 150 mM Tris-HCl (pH 7.0), Lane 5: Protein eluted with 200 mM Tris-HCl (pH 7.0)

SDS-polyacrylamide gel electrophoresis of protein fractions after successive purification steps demonstrated that the β-lactamase protein was successfully purified.

## IV.5. *IN-VITRO* β-LACTAMASE-LIGAND RECOGNITION

Spectrophotometric method was used for the *in-vitro* β-lactamase activity measurements for which the initial concentrations of the substrate and the enzyme were adapted from published data found in the literature (Rudgers *et al,* 2001). In this study, CENTA, the chromogenic substrate for β-lactamase, was used. The CENTA product formed a significant change in the absorbance value at 405 nm when initial CENTA concentration was 47 μM (Nilay Budeyri, 2009). Therefore, further activity measurements were carried out with this substrate concentration.

### IV.5.1. *In-vitro* β-lactamase Inhibition by BLIP

Initially, β-lactamase activity in the protein extract from *E. Coli* TB1 cells was investigated. *E. Coli* TB1 (pUC18) cells were used to determine β-lactamase expression with respect to time. Four separate cultures were grown and harvested at different $OD_{600}$ values (0.6, 0.8, 1.0 and 1.5). Proteins were extracted by osmotic shock method and β-lactamase activity from these extracts was measured (Figure IV.11). Total enzyme activity of cells harvested at different times was calculated and the results are summarized in Table IV.10.

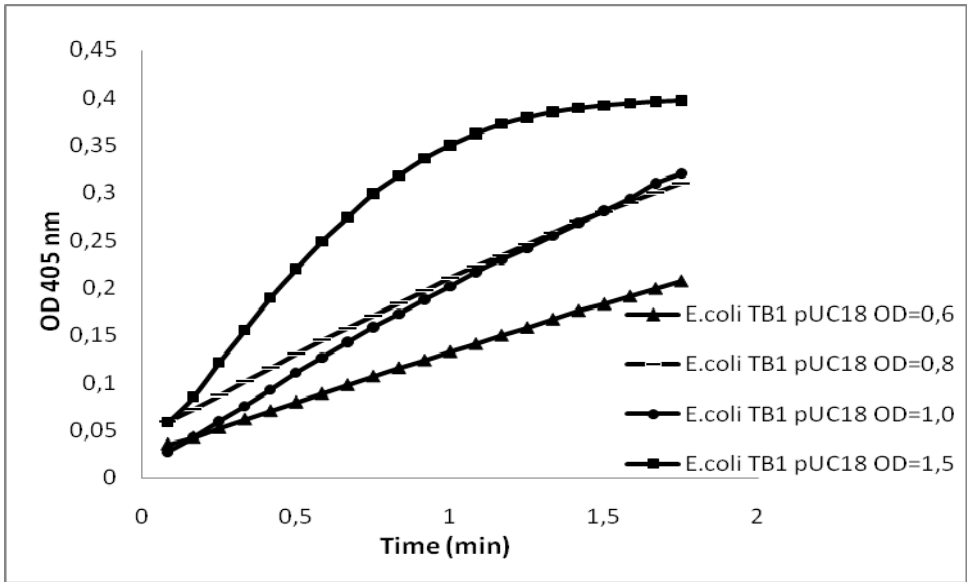


**Figure IV.11.** *In-vitro* β-lactamase activity of periplasmic extracts of *E. Coli* TB1 (pUC18) cells with different OD600 values

In Table IV.10, as the results indicate, β-lactamase activity increases as the optical density measurements taken just before cells are harvested increases.

Maximum enzyme activity was obtained when optical density at 600 nm was 1.610. Thus it can be concluded that the amount of β-lactamase produced within the cell is proportional to the number of cells present.

**Table IV.10.** β-lactamase activity from *E. Coli* TB1 (pUC18) cells harvested at various times during growth

| Expression system | $OD_{600}$ of cell culture (nm) | Activity (U/L) |
|---|---|---|
| *E. Coli* TB1 pUC18 | 0.598 | 1673.4 |
| | 0.801 | 2584.4 |
| | 1.010 | 3009.4 |
| | 1.610 | 6067.2 |

The X-ray structure of BLIP in complex with the TEM-1 β-lactamase is known and indicates that several BLIP residues make direct contact with the TEM-1 β-lactamase (Strynadka *et al*., 1996). It was well-known that RTEM-1 β-lactamase has two amino acids substitutions different than TEM-1; I84A and VI84A and both of which alter neither activity nor structure of the enzyme. Experiments were designed to test if β-lactamase is inhibited under these conditions.

Previous studies have been conducted only with purified proteins. If BLIP was used as a commercial inhibitor, it is expected to inhibit β-lactamase in the cells. In order to mimic intracellular environment, we have decided to use periplasmic protein extract containing β-lactamase and BLIP.

To investigate whether BLIP inhibited the activity of β-lactamase *in-vitro*, β-lactamase was mixed with increasing concentration of inhibitor. CENTA was used as the substrate at a concentration of 47 μM to monitor the hydrolysis of β-lactamase at 405 nm. Periplasmic protein extract with 5 mM $MgSO_4$ was used as the control. The results are given in Figures IV.12. and IV.13.

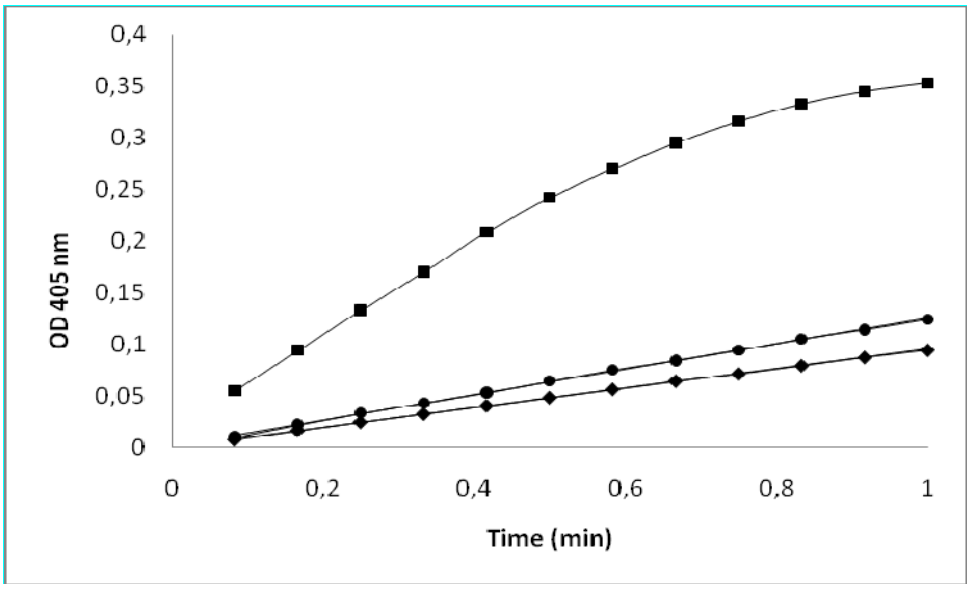


**Figure IV.12.** β-lactamase incubated with 74 ml (●), 147 ml (♦) and 294 ml (■) of $MgSO_4$

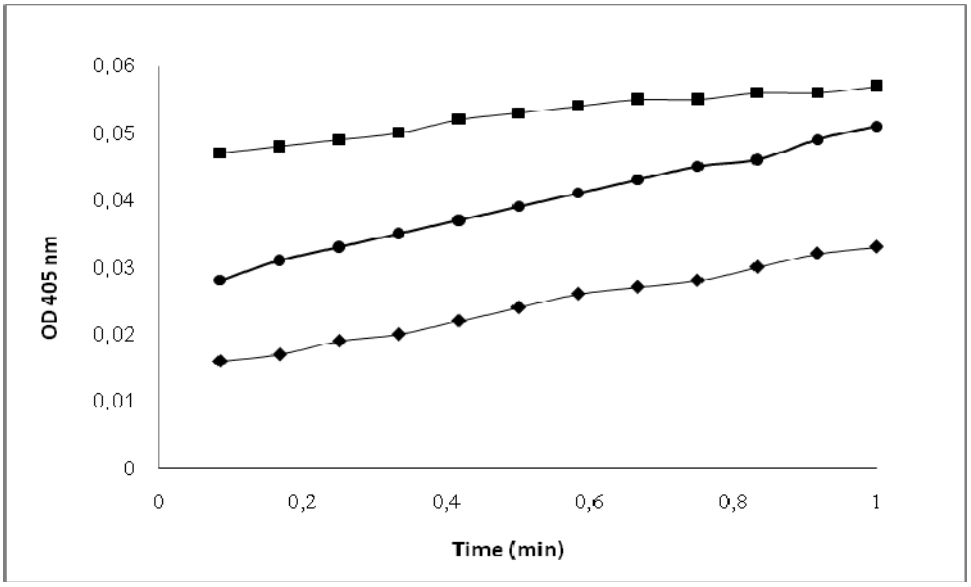


**Figure IV.13.** β-lactamase incubated with BLIP at concentrations of 1.8 µg/ml (●), 7.4 µg/ml (♦) and 29.4 µg/ml (■)

*In-vitro* RTEM-1 β-lactamase activity was calculated as described in Section III.7.9. Table IV.11. summarizes β-lactamase activity in units per liter periplasmic protein extract from *E. Coli* TB1 (pUC18) cells incubated with BLIP. Incubation with $MgSO_4$ was used as control.

**Table IV.11.** Activity of β-lactamase enzyme incubated with different concentrations of BLIP (U/L protein extract)

| Type of β-lactamase | Amount of protein in the periplasmic extract contaning BLIP (μg) | Activity of Control Group (U/L) | Activity of Sample incubated with Periplasmic Protein with BLIP (U/L) |
|---|---|---|---|
| Periplasmic Protein Extract | 1.8 | 1931.3 | 378.1 |
| | 7.4 | 3000.0 | 298.4 |
| | 29.4 | 7054.7 | 175.0 |

The rate of CENTA hydrolysis is given by the slope of absorbance readings. If BLIP inhibits β-lactamase, β-lactamase activity should be lower. The decrease in the slope (Figure IV.13.) indicates that in the presence of BLIP, there is reduction in β-lactamase activity. β-lactamase was inhibited very slightly with BLIP when the amount of protein in the periplasmic extract containing BLIP was 1.8 μg. As the amount of periplasmic extract containing BLIP in the reaction mixture was increased, hydrolysis rate decreased significantly. From these results, it can be concluded that periplasmic extract from *E. Coli* BL21 (pET-26SJ) cells containing 7.4 μg or higher protein amounts can effectively inhibit β-lactamase. As the amount of periplamic protein extract with BLIP is raised to 29.4 μg, inhibition is very clear. More than half of the total enzyme was inhibited. Nevertheless, periplamic protein extract containing 7.4 μg of protein was adequate to inhibit β-lactamase. Hence further experiments were done with this amount.

**IV.5.2** Influence of the Temperature on the Enzyme Inhibition

The optimum temperature for human enzymes is usually between 35°C and 40°C. It is showed that an *E. Coli* strain isolated from the human and growing optimally *in-vitro* at 37°C has evolved to function at that temperature (Daniel *et al*., 1996). Human enzymes start to denature quickly at temperatures above 40°C. Kinetic properties of enzymes vary with temperature, it is thus important to keep it constant, by using incubators. In order to characterize peptide-based drug behavior in the cell, the effect of the temperature on the enzymatic activity of β-lactamase was investigated.

By incubating the assay mixture for 45 minutes at room temperature (25°C) and 37°C, the possibility of RTEM-1 β-lactamase inhibition by BLIP at 37°C has been investigated. As seen in Table IV.12, at 37°C, even though β-lactamase activity was reduced by about 15 %, it can be concluded that the increase in the temperature to 37°C did not considerably affect β-lactamase activity.

**Table IV.12.** Activity of RTEM-1 β-lactamase at 25°C and 37°C (U/L)

| Type of β-lactamase | Control Group | | Sample incubated with BLIP | |
|---|---|---|---|---|
| | At 25°C | At 37°C | At 25°C | At 37°C |
| Periplasmic Protein Extract | 3603.9 | 3018.8 | 272.7 | 224.8 |

Based on the results presented in Table IV.12., there were no significant differences in activity of RTEM-1 β-lactamase over the studied temperatures. This result is an important step forward in future studies in which the behavior of peptide-based drugs will be studied.

**IV.5.3** Enzyme Kinetics

From Lineweaver-Burk plot and equation II.2 (Section II.6), kinetic parameters of β-lactamase enzyme were determined. $K_m$ and $V_{max}$ values for β-lactamase were assessed using commercial pure RTEM-1 β-lactamase, periplasmic protein extract and purified RTEM-1 β-lactamase. In enzyme kinetic experiments found in literature, the substrate concentration commonly lies between 0.025 and 0.3 mM for the calculation of $K_m$. With this information, the experiments to calculate $K_m$ were designed to have CENTA concentrations between 0.05 and 0.2 mM.

The kinetic parameters calculated with the commercial enzyme, crude extract and purified β-lactamase are summarized in Tables IV.13., IV.14. and IV.15.

**Table IV.13.** Kinetic parameters of RTEM-1 β-lactamase

| SAMPLE | Periplasmic Protein with BLIP (7.4 μg/ml) | $K_m$ (μM) | Vmax |
|---|---|---|---|
| Commercial Pure β-lactamase | - | 25.2 | 52.1 |
| | + | 55.6 | 52.1 |
| Crude enzyme | - | 69.0 | 10000.0 |
| | + | 31.2 | 666.6 |

In Table IV.13., comparative analysis of the obtained $K_m$ values demonstrated that $K_m$ value was lower when the enzyme was inhibited with BLIP. This showed that specificity of the commercial pure TEM-1 for the CENTA substrate was higher in the absence of BLIP. On the other hand $K_m$ for the crude enzyme was lower in the presence of the inhibitor, BLIP.

The $K_m$ values obtained with the crude and the commercial enzyme were comparable to the $K_m$ value of 70 μM reported in literature

Of the commercial pure RTEM, as $K_m$ increases and $V_{max}$ decreases in the presence of BLIP. This is known as competitive inhibition. The inhibitor BLIP competes with the substrate CENTA for the β-lactamase enzyme.

In the presence of BLIP, β-Lactamase found in the periplasmic protein extract had a higher affinity for CENTA than pure commercial TEM-1 since $K_m$ of the crude extract was higher. In this case, both $K_m$ and $V_{max}$ decreased, therefore BLIP behaved as an uncompetitive inhibitor ($K_m$ = 31.2 mM). Uncompetitive inhibitors do not combine with the free enzyme, but only with the enzyme-substrate complex. This observation would suggest that the inhibitor binds to the enzyme-substrate complex, as in Figure IV.14. where S is the substrate (CENTA) and I is the inhibitor (BLIP). BLIP showed very strong inhibitory activity toward commercial pure RTEM-1 β-lactamase but not towards β-lactamase in the periplasmic protein extract.

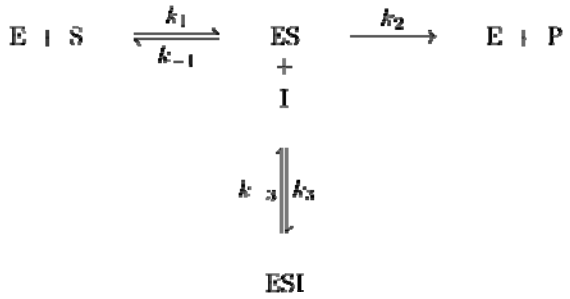


**Figure IV.14.** Uncompetitive inhibition scheme

The activity of the purified enzyme in the presence and absence of BLIP with various CENTA concentrations were measured and the kinetic parameters were calculated. The results obtained were presented in Table IV.14

**Table IV.14.** Kinetic parameters of Purified RTEM-1 β-lactamase

| SAMPLE | Periplasmic Protein with BLIP (7.4 µg/ml) | $K_m$ (µM) | $V_{max}$ |
|---|---|---|---|
| Purified β-lactamase (with 100 mM elution) | - | 750.0 | 100000.0 |
| | + | 431.0 | 10000.0 |
| Purified β-lactamase (with 150 mM elution) | - | 148.3 | 16666.7 |
| | + | 26.8 | 714.3 |
| Purified β-lactamase (with 200 mM elution) | - | 205.6 | 11111.1 |
| | + | 116.3 | 909.1 |

Both $K_m$ and $V_{max}$ decreased in the presence of BLIP. Therefore this is again uncompetitive inhibition.

Kinetic parameters of the purified enzyme following dialysis were also determined. Values obtained are summarized in Table IV.15.

**Table IV.15.** Kinetic parameters of purified and dialyzed RTEM-1 β-lactamase

| SAMPLE | Periplasmic Protein with BLIP (7.4 μg/ml) | $K_m$ (μM) | $V_{max}$ |
|---|---|---|---|
| Purified and Dialyzed RTEM-1 after 200 mM Tris-HCl elution | - | 116.1 | 9.8 |
| | + | 42.7 | 47.2 |

In contrast to the periplasmic protein extract, purified enzyme after dialysis was not uncompetitely inhibited by BLIP (Table IV.15). A mixed type inhibition was found for the enzyme which was dialyzed against 50 mM $K^+PO_4$ after elution with 200 mM Tris-HCl. Hypothetically, purification of enzyme could result in a decrease in $K_m$ value if an inhibitor is in interaction with the enzyme. As $K_m$ decreased, the increased affinity of the β-lactamase to BLIP may be due to the purity of the enzyme.

**IV.5.4.** *In-vitro* β-lactamase Inhibition by Peptide

Peptides derived from BLIP have been shown to inhibit β-lactamase (Rudgers *et al*, 2001). The peptide of this study was very similar in sequence to the already investigated peptides. However since β-lactamase in the crude extract is found together with many other proteins and metabolites (possible low molecular weight compounds), the inhibition potential of the peptide was unclear.

The reaction mixture contained 100 μM peptide in addition to the enzyme and the substrate, CENTA. Periplasmic protein extract incubated without the peptide was used as control. The result is given in Figure IV.14.

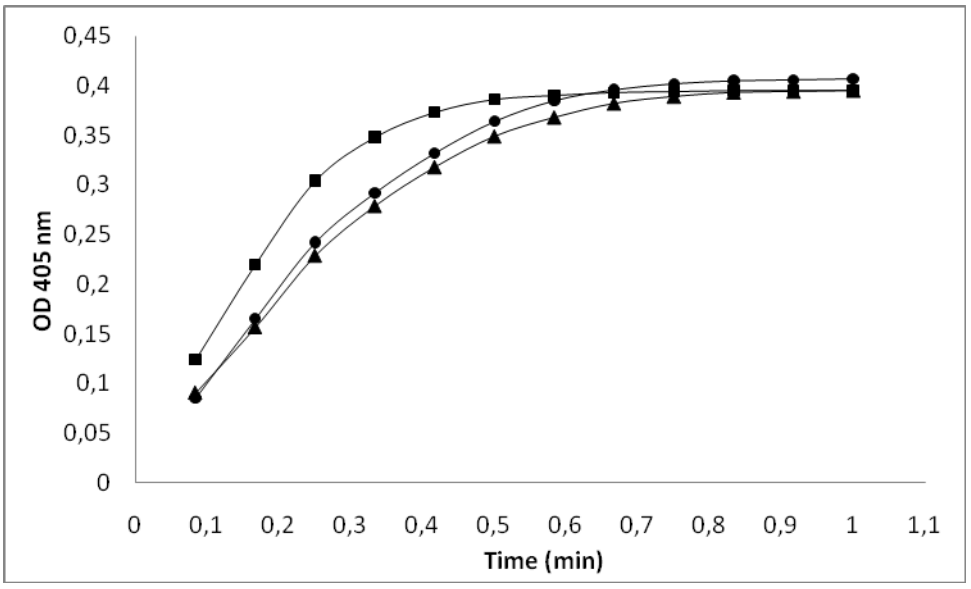


**Figure IV.15.** *In-vitro* β-lactamase inhibition by the peptide with inhibitor concentration of 100 μM. 10 μl periplasmic protein extract with peptide without incubation (■), 10 μl periplasmic protein extract without peptide with incubation (●), 10 μl periplasmic protein extract with peptide with incubation (▲).

**Table IV.16.** Activity of β-lactamase enzyme incubated with 100 μM Peptide (U/L)

| Sample | Peptide (100 μM) | Incubation | Enzyme Activity (U/L) |
|---|---|---|---|
| Periplasmic Protein Extract | + | - | 16875 |
| | - | + | 13087 |
| | + | + | 11925 |

A decrease in the initial rate (the slope) is expected in case the inhibitor is effective against β-lactamase. In accordance to this, in the presence the peptide, 9 % decrease in total activity was measured. Total activity dropped from 13087 to 11925 U/L crude extract.

Unfortunately, it should be noted that incubation also causes inactivation of the enzyme. Enzyme mixed with the peptide but not incubated had a total activity of 16875 U L crude extract.

The results show that this peptide derived from BLIP can indeed be effective in the cell as well.

# CHAPTER V

## CONCLUDING REMARKS and RECOMMENDATIONS

### V.1. CONCLUDING REMARKS

The aim of this research was to investigate the *in-vitro* binding properties of BLIP and BLIP based peptide to RTEM-1 β-lactamase using experimental procedures. Accordingly, *E. Coli* TB1 (pUC18) cells was used for the expression of RTEM-1 β-lactamase and *E. Coli* pET-26SJ vector carrying the BLIP gene with the native leader sequence was used for periplasmic expression of BLIP.

*In-vitro* studies were conducted with commercially pure RTEM-1 β-lactamase, periplasmic protein extract and purified RTEM-1 β-lactamase. Two temperatures 25ºC and 37ºC were examined to determine whether the enzyme was affected by the temperature or not. Osmotic shock fluid contains all periplasmic proteins therefore purification of the β-lactamase was a preliminary step toward examining *in-vitro* inhibitory properties of the BLIP.

Three different methods were investigated to purify RTEM-1 β-lactamase; first was the anion-exchange chromatography method, the second one was the concentration of the enzyme with ammonium sulfate precipitation and ultrafiltration of the periplasmic protein extract with ultrafiltration membranes and the third method was the loading of the concentrated enzyme onto an anion-exchange chromatography column. When anion-exchange chromatography was applied alone, the enzyme was not purified successfully. However, PES 10.000 MWCO membrane was used to successfully ultrafiltrate the periplasmic protein extract (98.2 % as yield) and the protein extract over the membrane was loaded onto the column. After purification the fractions with higher enzyme activity were pooled. It was observed that using 150 mM Tris-HCl buffer during the elution step, increased the protein yield 5.1 times more when compared to the periplasmic protein extract. This experiment was performed by incubating commercially pure RTEM-1 β-lactamase, periplasmic protein extract and purified RTEM-1 cells with BLIP and $MgSO_4$ as a control group. The kinetic parameters of the β-lactamase/BLIP interaction were calculated to define the type of the inhibition.

The influence of the temperature on the enzyme inhibition was investigated. At 25ºC, the activity of the enzyme was 3603.9 U and at 37ºC, it was 3018.8 U. The result suggests that there is no large difference of enzymatic activity after the incubation. This result suggests that BLIP can inhibit RTEM-1 β-lactamase in the cell.

To verify the expression and presence of BLIP and BLIP based peptide in the cells, *in-vitro* β-lactamase activity was measured. The incubation of BLIP with RTEM-1 cells suggested that BLIP inhibits RTEM-1 β-lactamase at a concentration of 1.8 μg/ml, under *in-vitro* conditions where the total activity units decreased 5 times than the control group. However, the BLIP concentration should be increased to at least 7.4 μg/ml to see effective *in-vitro* β-lactamase inhibition. β-lactamase inhibition was performed by BLIP based peptide with 100 μM concentrations of the peptide; 6 amino acids (H45-Y53 residues of BLIP). When the periplasmic protein extract was incubated with BLIP based peptides, the rate was reduced by 1.41 fold comparing to periplasmic protein extract with BLIP based peptide without incubation. From these result, it was observed that BLIP based peptide slightly affected the enzyme activity. It can be concluded that the BLIP based peptide clearly needs further optimization to be a viable inhibitor.

The kinetic parameters; $K_m$ and $V_{max}$ for *in-vitro* BLIP and β-lactamase inhibition was determined. The results indicate that the type of the inhibition was competitive inhibition, as it was described in the literature with increasing $K_m$ and decreasing $V_{max}$, for the commercial pure RTEM-1 β-lactamase. However, BLIP was found to be an uncompetitive inhibitor of this periplasmic protein extract and purified enzyme since both $K_m$ and $V_{max}$ were decreased. It may be due to the fact that the detection of this type of inhibition may indeed require correct identification of both enzyme and substrate. The kinetic parameter, $K_{i,}$ was not determined since the amount of BLIP in the periplasmic protein extract was unknown.

### V.2. RECOMMENDATIONS

Further experiments should be carried out to purify the β-lactamase enzyme. Once β-lactamase is purified via affinity chromatography, the kinetic parameter, $K_i$ for *in-vitro* BLIP and β-lactamase inhibition should be determined and the reason of the change of the inhibition type should be determined.

In this study, *in-vitro* inhibition effect of BLIP based peptides could not be observed due to the low concentration of peptides. The reason for this should further be investigated by gradually increasing the peptide concentration. Different BLIP based peptides should be investigated to find the best β-lactamase inhibitors.

BLIP based peptide is actually taken up by the cell or not still remains to be answered. The location of the peptide can be determined by chromatography methods for further experiments.

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APPENDIX A

**BSA Calibration Curve**

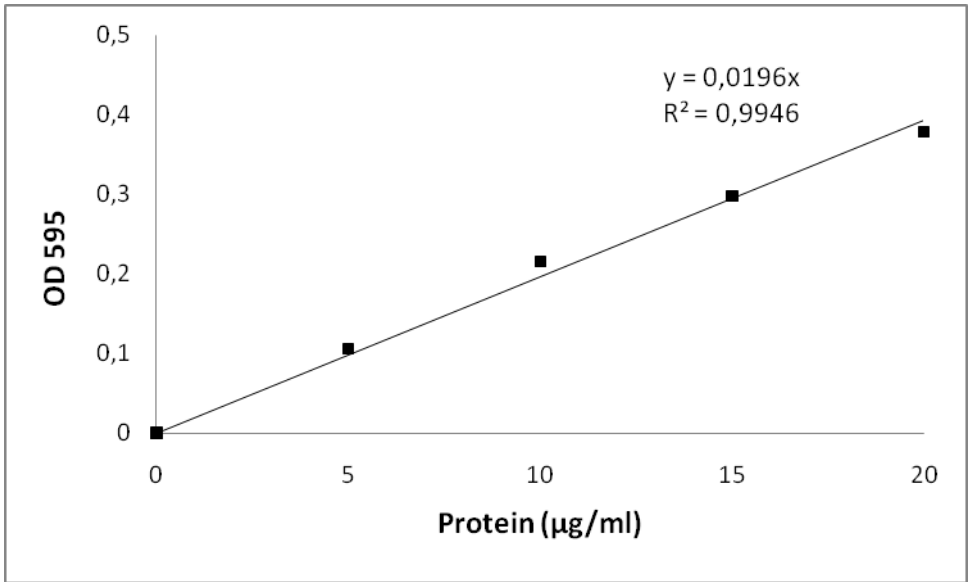


**Figure A.1.** BSA Calibration Curve

APPENDIX B

## CENTA CALIBRATION CURVE

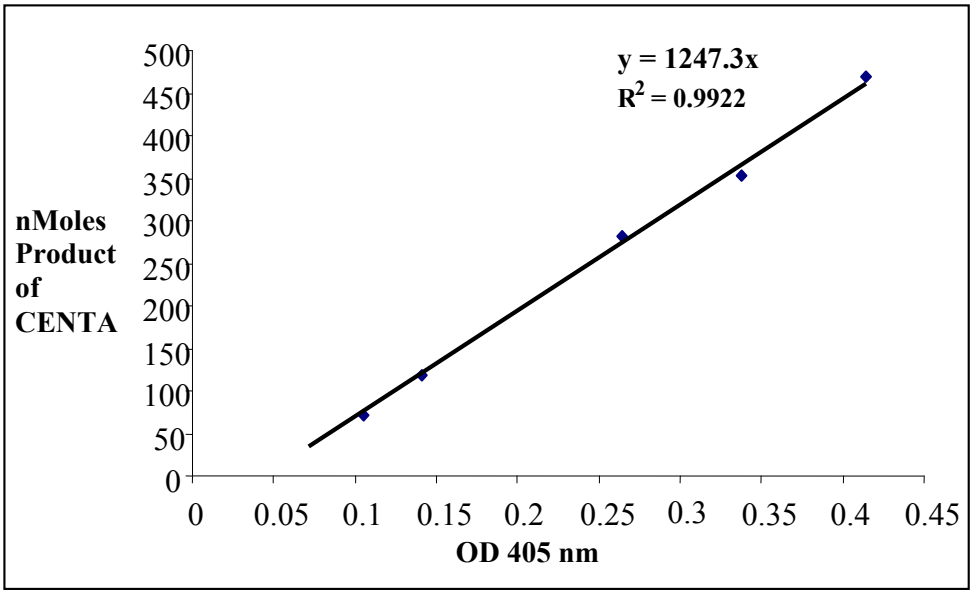


**Figure B.1**. CENTA Calibration Curve

# AUTOBIOGRAPHY

I graduated from the Molecular Biology and Genetics Department from Haliç University, Istanbul. I enrolled in the Bioengineering MSc Program at Marmara University in 2008.

**Projects**

"Production of Industrial Enzymes for the Feed Industry", Scientific and Technological Research Council of the Republic of Turkey (TUBITAK) as a scholarship.

**Selected Posters**

**Naze Gül Avcı**, Nilay Budeyri, Berna Sarıyar Akbulut, Elif Özkırımlı Ölmez. '*In-vitro* Inhibitory Effect of BLIP Derived Peptides upon TEM-1 Beta-Lactamase.' 35th FEBS Congress Molecules of Life, June 26 – July 1 2010, Gothenburg, Sweden.

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Nilay Budeyri, A. Ezgi Akkaya, Celal Ceylan, **Naze Gül Avcı**, Gizem Buldum, Özlem Kocaman, Natali Yüzari, Elif Özkırımlı Ölmez, Berna Sarıyar Akbulut. 'Investigation of TEM-1 Beta-Lactamase Ligand Interaction.' 9th National Chemical Engineering Conference, June 22-25 2010, Gazi University, Ankara, Turkey.

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