

T.C.
MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN
PURE AND APPLIED SCIENCES

INVESTIGATION OF PENETRATION OF INHIBITOR INTO
CELLS AND THEIR INTERACTIONS WITH INTRACELLULAR
PROTEINS/METABOLITES

Gizem BULDUM

THESIS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
BIOENGINEERING

SUPERVISOR
Assoc. Prof. Berna SARIYAR AKBULUT
Co-ADVISOR
Assist. Prof. Elif ÖZKIRIMLI ÖLMEZ

İSTANBUL 2011

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Gizem BULDUM

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ÖZET

İNHİBİTÖR HÜCRE İÇİNE GİRİŞİ VE HÜCREİÇİ PROTEİNLERLE / METABOLİTLERLE ETKİLEŞİMLERİNİN ARAŞTIRILMASI

β -laktam antibiyotikleri, yüksek etkinlik ve düşük maliyet gibi özellikleri sebebiyle çok yaygın şekilde kullanılan antimikrobiyal maddelerdir. Yaygın kullanımları, bakterilerin antibiyotiklere karşı direnç kazanmasına ve giderek büyüyen bir halk sağlığı problemi haline gelmesine sebep olmuştur. Direnç mekanizmalarından en sık görüleni bakterilerin β -laktamaz enzimi üreterek antibiyotiği etkisiz hale getirmesidir. Bu nedenle, β -laktam antibiyotiklerinin tedavi edici etkisinin sürdürülebilmesi için β -laktamaz inhibitörleri önemli bir araştırma konusu haline gelmiştir. *Streptomyces clavuligerus*, tarafından üretilen β -laktamaz inhibitör proteini (BLIP) ve BLIP' in yapısı temel alınarak oluşturulan peptitler β -laktamaz enzimini inhibisyon özelliğine sahiptirler. Bu bilgiler ışığında, bu çalışmada *in-vitro* ve *in-vivo* metotlar kullanılarak β -laktamazın BLIP ve BLIP yapısı temel alınarak oluşturulan peptitler tarafından inhibisyonu incelenmiştir. *In-vivo* inhibisyon deneylerinde R-TEM-1 β -laktamaz ve inhibitörü olan BLIP' in berabere ekspresyonu pUC18 ve pET-26SJ vektörleri ile *E. coli* BL21(DE3) hücrelerinde sağlanmıştır. BLIP'in varlığında, periplazmadaki toplam β -laktamaz aktivitesi %95 azalmıştır. BLIP' in üretimi canlı hücre sayısı başına düşen β -laktamaz aktivitesinde 35 kat düşüşe sebep olmuştur. BLIP ve BLIP- β -laktamaz kompleksinin varlıkları sırasıyla SDS ve Native-PAGE analizleriyle doğrulanmıştır. BLIP'in yapısı temel alınarak oluşturulan peptitle ile *in-vivo* inhibisyon çalışmaları yapmak amacıyla, pUC18 plazmidi taşıyan *E. coli* hücreleri inkübe edilmiş, fakat peptitlerin inhibe edici özelliği gözlenememiştir. *In-vitro* inhibisyon çalışmalarında BLIP' in periplazmik sıvıdaki β -laktamazı %69 ve ticari β -laktamazı %80 oranında inhibe ettiği saptanırken, peptitlerin güçlü bir *in-vitro* inhibisyon etkisi gözlenememiştir.

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ABSTRACT

INVESTIGATION OF PENETRATION OF INHIBITOR INTO CELLS AND THEIR INTERACTIONS WITH INTRACELLULAR PROTEINS/METABOLITES

β -lactam antibiotics are the most widely utilized antimicrobials by virtue of their high efficacy and low cost. Owing to the widespread usage, bacteria acquire resistance to these antibiotics and cause a growing public health problem. The most prevalent bacterial resistance mechanism to β -lactam antibiotics is the production of β -lactamases to render the antibiotic inactive. In this context, β -lactamase inhibitors emerge as an important research field to continue the therapeutic value of antibiotics. β -lactamase inhibitor protein (BLIP), which is produced by *Streptomyces clavuligerus*, and BLIP derived peptides have inhibition potential against β -lactamase enzymes. In this study, *in-vitro* and *in-vivo* methods have been used to investigate β -lactamase inhibition by BLIP and BLIP derived peptides. *In-vivo* binding experiments have been performed with *E. coli* BL21(DE3) cells harboring both pUC18 and pET-26SJ vector for simultaneous expression of R-TEM-1 β -lactamase and BLIP. In the presence of BLIP, β -lactamase activity found in periplasm decreased 95%. Induction of BLIP production led to a 35 fold decrease in β -lactamase activity per viable cell. The presence of BLIP and BLIP- β -lactamase complex in the periplasmic fraction were verified by SDS-PAGE and Native-PAGE analysis respectively. In order to investigate *in-vivo* enzyme inhibition by BLIP derived peptides, *E. coli* K12 cells harboring pUC18 were incubated with the peptides, but no growth inhibition has been observed. While BLIP inhibited the crude β -lactamase and commercial β -lactamase 69% and 80% respectively, no strong *in-vitro* inhibition has been observed by peptides.

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ABBREVIATIONS

APS	: Ammonium persulfate
BLIP	: β -Lactamase Inhibitor Protein
CFU	: Colony Forming Unit
EDTA	: Ethylenediaminetetraacetic acid
Da	: Dalton
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
kDa	: Kilodalton
L	: Liter
LB	: Luria-bertani broth
μg	: Microgram
μL	: Microliter
μM	: Micromolar
ml	: Mililiter
mM	: Milimolar
M	: Molar
MW	: Molecular Weight
nm	: Nanometer
nM	: Nanomolar
OD	: Optical Density
PAGE	: Polyacrylamide gel electrophoresis
rpm	: Rotation per minute
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	: N,N,N,N-tetramethylethylendiamine
U	: Unit

SYMBOLS

A	: Ala, Alanine
D	: Asp, Aspartic acid
E	: Glu, Glutamine acid
F	: Phe, Phenylalanine
G	: Gly, Glycine
H	: His, Histidine
K	: Lys, Lysine
K_i	: Inhibition Constant
L	: Leu, Leucine
M	: Met, Methionine
N	: Asn, Asparagine
P	: Pro, Proline
R	: Arg, Arginine
S	: Ser, Serine
T	: Thr, Threonine
U	: Enzyme Units
V	: Val, Valine
W	: Trp, Tryptophan
Y	: Tyr, Tyrosine

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CHAPTER I

INTRODUCTION and AIM

The introduction of β -lactam antibiotics into the health care system represents one of the most important contributions to medical science in recent history. Today, β -lactams remain the most widely utilized antibiotics owing to their comparatively high effectiveness, low cost, ease of delivery and minimal side effects (Gretes *et al.*, 2009; Wilke *et al.*, 2005; Doran *et al.*, 1990).

β -lactam antibiotics target transpeptidase enzymes that synthesize the bacterial cell wall and cause cell death by inhibiting the synthesis of the bacterial peptidoglycan layer (Wang *et al.*, 2007; Wilke *et al.*, 2005; Kang *et al.*, 2000).

β -lactam antibiotics have been continually threatened by the development of bacterial resistance. As early as in 1940, a bacterial enzyme capable of neutralizing the antibiotic drug effect was described. This enzyme was later designated as β -lactamase (Frere, 1995). Production of β -lactamases, enzymes which cleave the β -lactam ring, is the most common mechanism of resistance among both gram-positive and gram-negative bacteria (Doran *et al.*, 1990).

The β -lactamase enzymes inactivate β -lactam antibiotics by hydrolyzing the peptide bond of the characteristic four-membered β -lactam ring rendering the antibiotic ineffective. The inactivation of the antibiotic provides resistance to the bacterium. A method to combat this β -lactam resistance has been the use of small molecule β -lactamase inhibitors, such as clavulanic acid and sulbactam (Page and Laws, 1998). These mechanism-based inhibitors protect the β -lactam drug from hydrolysis by β -lactamases and restore the therapeutic potential of the antibiotic (Therrien and Levesque, 2000). The inhibitors are used in combination with various β -lactam antibiotics since they do not possess antimicrobial activity. β -lactamases often exhibit high affinity for the inhibitor molecules and are inactivated by irreversibly binding to the inhibitor (Rudgers and Palzkill, 2001).

In 2002, the global market for antibiotics was estimated at 25 billion US\$ (Coates *et al.*, 2002), of which about 50 % was for β -lactam antibiotics. Because β -lactam antibiotics have been heavily used since the 1940s, many strains of bacteria resistant to β -lactam antibiotics have emerged in clinical care settings. As the frequency of microbial resistance to β -lactams continues to increase, the usefulness of the currently available antibiotics diminishes and the willingness of pharmaceutical companies to invest in the discovery and development of new antimicrobials wanes (Hall and Barlow, 2004).

Several β -lactamase inhibitors have been identified in species of the soil bacterium *Streptomyces*. Clavulanic acid was initially purified from the soil bacterium *Streptomyces clavuligerus*, which also produces a protein inhibitor of β -lactamases called β -lactamase inhibitory protein (BLIP) (Doran *et al.*, 1990). BLIP has become a model for a broad range of structural, mutagenic, biophysical, and computational studies. It is a 165 amino acid protein composed of two-domains of ~78 residues each and is a potent inhibitor of TEM-1 β -lactamase ($K_i = 0.1\text{--}0.6$ nM) (Strynadka *et al.*, 1994; Petrosino *et al.*, 1999; Rudgers and Palzkill, 1999).

Previous studies have shown that peptides designed from key interacting residues of BLIP have inhibitory effects on β -lactamase activity (Gretes *et al.*, 2009; Rudgers *et al.*, 2001). Since protein-protein interactions are important events in most biological processes and are important targets for drug design, understanding BLIP and β -lactamase interaction will enable the design of peptide based drugs to fight with β -lactamase mediated antibiotic resistance (Rudgers and Palzkill, 2001; Albeck and Schreiber, 1999).

The aim of this study was to investigate *in-vivo* and *in-vitro* β -lactamase inhibition by BLIP and BLIP derived peptides. Inhibition has been investigated by monitoring growth and viability of *E. coli* cells co-expressing β -lactamase and BLIP. The complex that was expected to form between β -lactamase and BLIP was investigated by electrophoretic methods. Inhibitory potential of peptides has been examined by incubating *E. coli* cells with the peptides. Finally, both commercial β -lactamase and crude β -lactamase found in the periplasmic protein extract were used to investigate inhibition of BLIP and BLIP derived peptides under *in-vitro* conditions.

CHAPTER II

GENERAL BACKGROUND

II.1. β -LACTAM ANTIBIOTICS AND β -LACTAMASE MEDIATED RESISTANCE TO β -LACTAM ANTIBIOTICS

Antibiotics are important therapeutic agents used for treating infections caused by various pathogenic Gram-negative and Gram-positive bacteria. Today, about 100 different β -lactam antibiotics are used clinically in the antibacterial treatment of humans (Kasier, 2004). The β -lactam group of antibiotics is by far the largest group of antibacterial agents used in clinical medicine. (Wilke *et al.*, 2005; Schroeder *et al.*, 2002; Rudgers *et al.*, 2001; Doran *et al.*, 1990).

Penicillin, penicillin derivatives, cephalosporins, cephamycins, carbapenems, clavams, monobactams, and monocarbams are classified as β -lactam antibiotics. They all possess an essential four-membered lactam ring that may be fused to form bicyclic ring structures or may exist as isolated rings (Figure II.1). Several natural and synthetic β -lactams have been described since the discovery of penicillin in 1928 by Fleming (Essack, 2001).

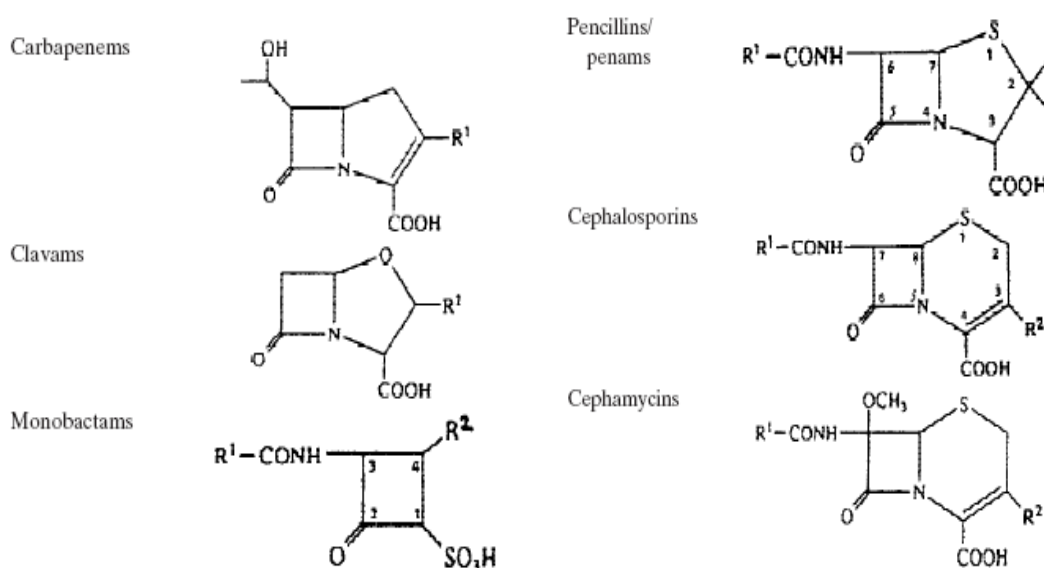


Figure II.1. Chemical structures of β -lactam antibiotics (Essack, 2001).

β -lactam compounds account for approximately 50 % of worldwide consumption of antibiotics as a result of the high selectivity, low toxicity, and versatility of these compounds (Livermore, 1998; Essack, 2001; Poole, 2004). The global market for antibiotics was estimated at US\$ 25 billion in 2002, and nearly 50 % of this market belonged to β -lactam antibiotics (Hall and Barlow, 2004).

Bacteria of all species rely on a heavily cross-linked peptidoglycan layer (cell wall) for the preservation of cell shape and rigidity. Peptidoglycan is an essential component of both Gram-negative and Gram-positive bacterial cell wall (Figure II.2). It protects the organism from osmotic rupture, determines cell shape, and is integral to cell growth and division. Its net-like structure, composed of saccharide chains cross-linked by peptides, maintains cell integrity and viability. It must thus remain physically continuous during the bacterial cell cycle (Essack, 2001). β -lactam antibiotics interfere with the final stage of cell wall (peptidoglycan) synthesis by inhibiting the bacterial enzymes, transpeptidases, and carboxypeptidases that catalyze the reactions of peptidoglycan synthesis. These enzymes commonly called penicillin-binding-proteins (PBPs), crosslink the peptidoglycan polymers. Inhibition of PBPs causes bacteriolysis by yielding a wall unable to withstand osmotic forces (Essack, 2001). This results in weakly cross-linked peptidoglycan, which makes the growing bacteria highly susceptible to cell lysis and death (Wilke *et al.*, 2005).

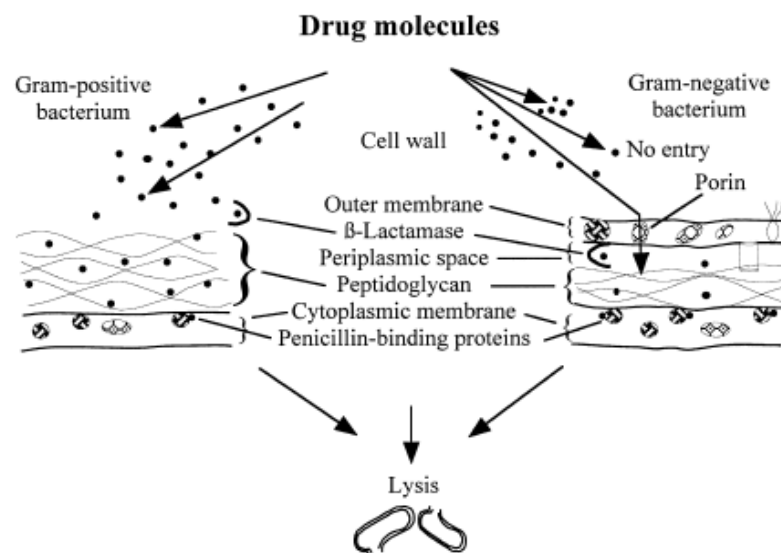


Figure II.2. Structures of the outer wall in Gram-positive and in Gram-negative bacteria.

Bacterial resistance to antibiotics is an important and growing concern in the treatment of infectious diseases (Schroeder, 2002). Resistance to β -lactam antibiotics is an especially severe threat because β -lactams kill a broad spectrum of bacteria and have very low toxicity to humans (Livermore, 1996).

β -lactams are rendered inactive against bacteria by way of three primary mechanisms of resistance. The most common mechanism is the production of enzymes that degrade or modify the antibiotic before it can reach the appropriate target site. In this case, β -lactamases, a family of enzymes that degrade β -lactam antibiotics, are found widely disseminated amongst Gram-positive and Gram-negative bacteria. The second mechanism is alteration of the antibiotic target site. In this case, the β -lactam-resistant cell-wall transpeptidases perform this role; this is now a major cause of resistance in several pathogens including the problematic Gram-positive Staphylococcal and Streptococcal species. The final mechanism is prevention of access of the antibiotic to the target by way of altered permeability or forced efflux (Wilke *et al.*, 2005).

A collection of over 400 enterobacterial strains isolated between 1954 and 1971 included no antibiotic resistant strains (Datta and Hughes, 1983), until 2003 over 20 % are resistant to ampicillin or amoxicillin (Bruinsma *et al.*, 2003). Antibiotic resistance arises quickly and spreads rapidly, especially when resistance genes are horizontally transferred via plasmids and integrons among individuals, among species, and even among bacterial kingdoms (Hall and Barlow, 2004).

II.2. β -LACTAMASES

β -lactamases are bacterial enzymes that confer resistance to β -lactam antibiotics, which include the penicillins and cephalosporins. These drugs act by inhibiting bacterial penicillin binding proteins (PBPs) that are essential for the synthesis of the bacterial peptidoglycan layer (Petrosino *et al.*, 1998).

The production of β -lactamase enzymes is the most common mechanism of bacterial resistance to β -lactam antibiotics such as the penicillins and cephalosporins. These enzymes catalyze the hydrolysis of the β -lactam ring to create ineffective antimicrobials. β -lactamases were first identified in *Staphylococcus aureus* strains in

the late 1940s, prior to the introduction of penicillin into the clinical setting (Abraham and Chain, 1988).

To date, over 530 β -lactamase enzymes have been reported (K. Bush, 9th International Congress on β -lactamases, Leonessa, Italy). These enzymes are divided into four classes based on the primary sequence similarity and catalytic mechanism. Classes A, C and D utilize an active-site serine in their mechanism of action, whereas class B β -lactamases require divalent metal cations (Zn^{++}) to catalyze β -lactam hydrolysis and break the amide bond in the β -lactam ring. (Ambler, 1980; Babic, 2006). As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis (Williams, 1999). When they are expressed, they are secreted into the periplasmic space in gram-negative bacteria or they are either bound to the cytoplasmic membrane or excreted in gram-positive bacteria (Wilke *et al.*, 2005; Rudgers *et al.*, 2001; Rudgers and Palzkill, 1999).

β -lactamase-mediated resistance has increased because of selective pressure from widespread use of β -lactam antibiotics and is now a serious threat to antibiotic therapy (Petrosino *et al.*, 1998).

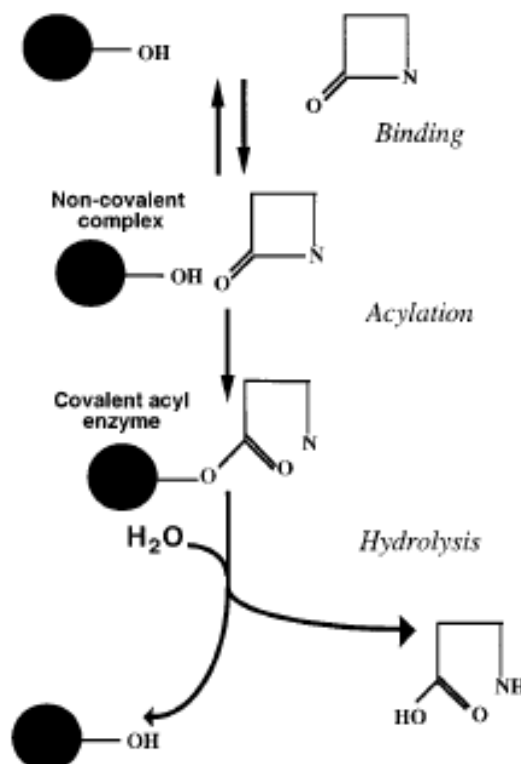


Figure II.3. Action of serine β -lactamase (Livermore, 1995).

II.3. TEM-1 β -LACTAMASES

TEM-1 is the most commonly encountered β -lactamase in gram-negative bacteria. Up to 90 % of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Bradford, 2001). The first plasmid-mediated β -lactamase in gram-negatives, TEM-1, was described in the early 1960s. The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM. Being plasmid and transposon mediated has facilitated the spread of TEM-1 to other species of bacteria. Within a few years after its first isolation, the TEM-1 β -lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* (Bradford, 2001).

TEM-1 β -lactamase is a class A enzyme encoded by *bla*TEM-1. Epidemiological studies have shown that TEM-1 is the most common plasmid-encoded β -lactamase in gram-negative bacteria. pUC18 plasmid, shown in Figure II.5, carries the gene *bla*TEM-1 producing TEM-1 β -lactamase. The largest component of pUC18 is the region containing the 861 bp TEM-1 β -lactamase gene, which confers resistance to the antibiotic ampicillin. TEM-1 is naturally found in the periplasm of gram-negative bacteria such as *E. coli* (Lim *et al.*, 2001). Plasmid-mediated β -lactamases can transfer rapidly between bacterial genera and consequently pose a major threat to the successful use of β -lactam agents. More than 60 different types of plasmid-encoded β -lactamases have been characterized (Payne *et al.*, 1994).

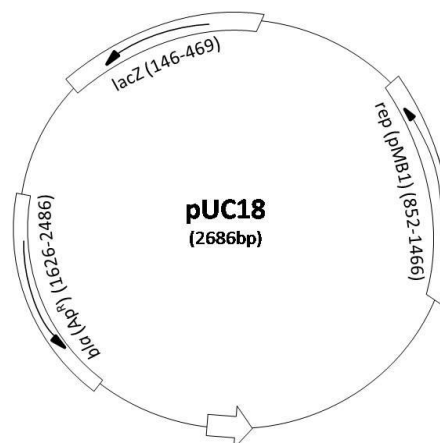


Figure II.4. pUC18 vector map

β -lactamases are able to efficiently hydrolyze penicillins and many cephalosporins; therefore, they are important sources of bacterial resistance to the β -lactam antibiotics. (Rudgers *et al.*, 2009)

TEM-1 β -lactamase has 263 amino-acid residues in the crystallographic structure with a molecular weight of 28.9 kDa (Savard *et al.*, 2004).

TEM-1	HPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSDFKVLCCGAVLSRID	60
TEM-1	AGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTIGGP	120
TEM-1	KELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQ	180
TEM-1	QLIDWMEADKVAGPLLRSLPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTG	240
TEM-1	SQATMDERNRQIAEIGASLIKHW	263

Figure II.5. The sequence of TEM-1 β -lactamase (Reynolds *et al.*, 2006)

There are two principal ways to overcome the hydrolytic action of β -lactamases. The first strategy involves finding inhibitors (or inactivators) of β -lactamases. The second strategy involves finding a new β -lactam antibiotic that demonstrates great affinity for the target PBP and is not hydrolyzed, or is hydrolyzed poorly, by β -lactamases. However, the advent of each new control strategy was met with the emergence of mutant forms of TEM that were resistant to the new agent (Babic *et al.*, 2006).

While chemical modification has helped to improve the resistance of the naturally occurring β -lactam antibiotics to cleavage, the need for further improvement resulted in an effort to find useful β -lactamase inhibitors (Doran *et al.*, 1990).

II.4. β -LACTAMASE INHIBITORS

Currently, there are three β -lactamase inhibitors used in the clinic: clavulanic acid, sulbactam, and tazobactam. These compounds structurally resemble penicillin (Figure II.5). β -lactamase inhibitors are combined with a partner β -lactam antibiotic, the former protecting the latter from enzymatic hydrolysis.

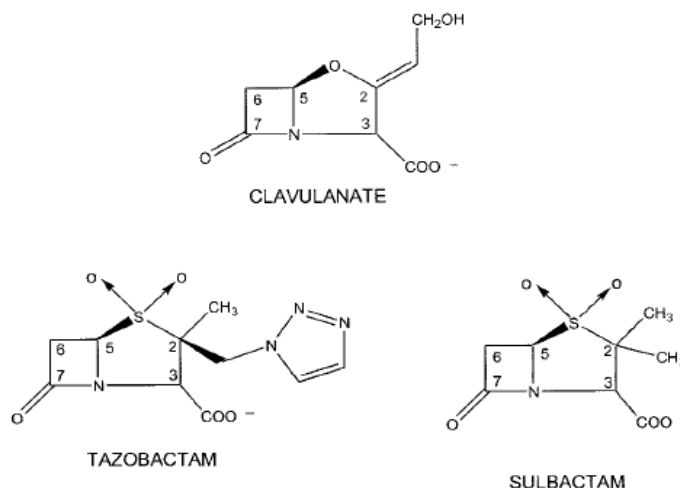


Figure II.6. Chemical Structures of class A β -lactamase inhibitors
(Helfand *et al.*, 2003)

The three β -lactamase inhibitors, acting like β -lactams, bind to the active site of β -lactamases but fail to be efficiently hydrolyzed. Each demonstrates high affinity (nM– μ M) for β -lactamases, is slowly or poorly hydrolyzed by the enzyme, undergoes different reaction chemistry than β -lactams and occupies the active site significantly longer (Helfand *et al.*, 2003; Kuzin *et al.*, 2004; Padayatti *et al.*, 2004, 2005; Pagan-Rodriguez *et al.*, 2004). Hence, β -lactamase inactivators are also referred to as “suicide inhibitors” since they are “trapped” by the β -lactamase. This approach has been the subject of intense research efforts by pharmaceutical companies and many academic laboratories (Brenk *et al.*, 2005; Buynak *et al.*, 2000; Kuzin *et al.*, 2001; Minasov *et al.*, 2002; Morandi *et al.*, 2003; Nukaga *et al.*, 2003; Pagan-Rodriguez *et al.*, 2004; Trehan *et al.*, 2001; Venkatesan *et al.*, 2004; Wang *et al.*, 2003, Babic *et al.*, 2006)

II.4.1. β -lactamase Inhibitor Protein (BLIP)

Gram positive bacterium *Streptomyces clavuligerus* produces a protein inhibitor of β -lactamase called BLIP which is the first protein based inhibitor of β -lactamases. β -lactamase inhibitory protein (BLIP) is a 165 amino acid protein with a molecular weight of 17.523 kDa and consists of two tandemly linked 76 amino acid domains (Doran *et al.*, 1990). The BLIP– β -lactamase complex has become a protein–protein interaction model system with increasingly more detailed information on structure–activity relationships. (Rudgers *et al.*, 2001)

BLIP inhibits several class A β -lactamases such as TEM-1 and SHV-1 with a wide range of affinities. (Yuan *et al.*, 2009; Gretes *et al.*, 2009; Zhang and Palzkill, 1999; Strynadka *et al.*, 1994). Strynadka *et al.* (1994) reported that the plasmid-mediated TEM-1 β -lactamase from the gram-negative *E. coli* was strongly inhibited by BLIP with a K_i value of 0.6 nM.

Albeck and Schreiber (1999) studied the *in-vitro* binding of TEM-1 β -lactamase- BLIP complex and determined the kinetic parameters. The binding affinity was calculated as 0.4 nM which was very similar to that reported by Strynadka *et al.*(1994) indicating that the activity of the recombinant protein is identical to that of the secreted protein.

II.5. PEPTIDE INHIBITORS OF β -LACTAMASE

In response to the environmental selective pressure resulting from decades of use of β -lactam ring-based biomolecules such as penicillin, β -lactamases have evolved as a highly efficient bacterial defense mechanism. While chemical modification has helped to improve the resistance of the naturally occurring β -lactam antibiotics to cleavage, the need for further improvement resulted in an effort to find useful β -lactamase inhibitors (Phichith *et al.*, 2010). Recent advances in structural biology have revealed that residues 46-51 (AAGDYY) of BLIP are crucial for the interaction between BLIP and TEM-1 β -lactamase, a major type of class A β -lactamase (Rudgers *et al.*, 2001).

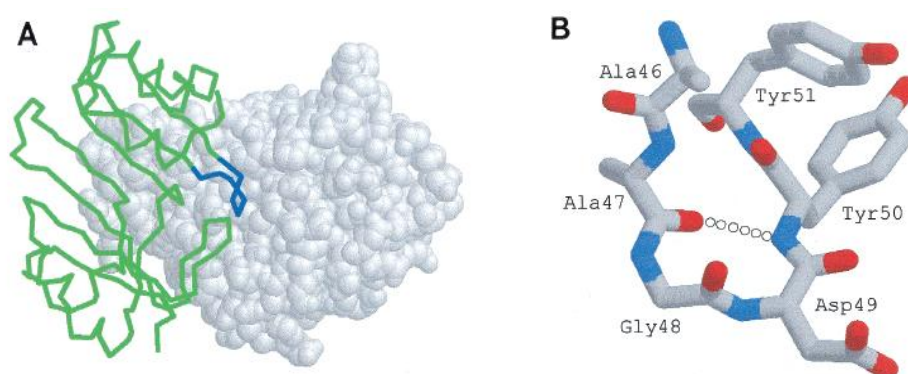


Figure II.7. Representation of BLIP (green) binding to TEM-1 β -lactamase (white, space-fill model)(The region of BLIP from residues 45 to 52 is shown in blue)(A).

Structure of the Ala-46 to Tyr-51 peptide extracted from the BLIP structure (B).

(Phichith *et al.*, 2010)

Crystallographic analyses of BLIP and TEM in apo form and in the complex gave insight into the nature of the inhibitory interaction and defined a peptide loop exposed on the surface of BLIP which interacts with the active site of the β -lactamase (Strynadka *et al.*, 1996). 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 (Petrosino *et al.*, 1999; Rudgers and Palzkill, 2001; Rudgers *et al.*, 2001).

The reduction of proteins to short peptides that retain binding function is an important intermediate step toward the development of novel small-molecule drugs that disrupt protein-protein interactions. Over the past decade, several protein inhibitors have been converted into small-molecule inhibitors that bind to their target substrates with high affinities (Cunningham, B. C., and J. A. Wells, 1997).

Various studies have shown that TEM-1 β -lactamase was inhibited by peptides derived from BLIP with different K_i values changing from 446 μ M to 0.01 nM (Rudgers and Palzkill, 2001; Rudgers *et al.*, 2001; Huang *et al.*, 2000).

Rudgers *et al.* (2001) reported the inhibition potential of peptides based on the 46-51 region of BLIP. The cyclic peptide with cysteine residues at both ends inhibits TEM-1 with a K_i of 603, while in reduced form, the peptide binds to TEM-1 with a K_i of 488 (Rudgers *et al.*, 2001).

Sun *et al.* (2005) performed experiments using yeast two-hybrid system with the intention of finding a novel peptide inhibitor. Two peptides were shown to inhibit TEM-1 β -lactamase *in-vivo* while *in-vitro* studies indicated that only one of these inhibited TEM-1 β -lactamase. Xie *et al.* (2010) proved this same peptide was also active against SHV β -lactamase *in-vitro*. By introducing various point mutations into this peptide, it was found that residue 15 on the peptide, equivalent to Y50 in BLIP, was very important for the binding of β -lactamase (Sun *et al.*, 2005).

Huang *et al.* (2003) found a linear 6-mer peptide which was 50 % identical with a type II β -turn sequence of BLIP. This peptide could inhibit TEM-1 β -lactamase with a K_i of 136 μ M. This peptide was also shown to inhibit different classes of β -lactamases (Huang *et al.*, 2003).

Phichith *et al.* (2010) studied Pep90 which is a cyclic heptapeptide enclosed between two cysteine residues. Pep90 could inhibit both TEM-1 β -lactamase ($K_i = 333 \text{ l M}$) and several penicillin-binding proteins. It was determined that the tryptophan residue of Pep90 is of crucial importance for its inhibitory activity. Using Pep90 as a scaffold, a new class of peptidomimetics that retained inhibitory activity towards TEM-1 β -lactamase was generated (Phichith *et al.*, 2010).

CHAPTER III

THE STUDY

III.1. MATERIALS

III.1.1. Bacterial Strains and Plasmids

In this study, *Escherichia coli* BL21(DE3) and K12 strains were used from our laboratory stocks. β -lactamase expression was obtained from pUC18 plasmid carrying the gene for R-TEM-1. pET-26b(+) plasmid vector was purchased from Novagen. pET-26b(+) vector carrying the BLIP gene with its native leader sequence was a generous gift from Susan Jensen (University of Alberta) and was retained in *E. coli* BL21(DE3) cells. This plasmid was named as pET-26SJ and used for periplasmic expression of BLIP.

III.1.2. Chemicals

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

III.1.3. Growth Media for *Escherichia coli*

Table III.1 LB medium

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Add dH ₂ O up to 1 liter	

Table III.2 LB agar medium

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Add dH ₂ O up to 1 liter	

Table III.3 M9 minimal medium

Chemical	Amount
5X M9 Salts	200 ml
1M MgSO ₄	2 ml
1M CaCl ₂	100 µL
20 % Glucose	40 ml
Add dH ₂ O up to 1 liter	

Table III.4 M9 salts

Chemical	Amount
Na ₂ HPO ₄	33.9 g
KH ₂ PO ₄	15 g
NaCl	2.5 g
NH ₄ Cl	5 g
Add dH ₂ O up to 1 liter	

III.1.4. Buffers and Solutions

III.1.4.1. SDS-PAGE Buffers and Solutions

Acrylamide-bisacrylamide solution (30:0.8)

Per 100 ml of solution, completed with deionized and distilled water and stored in dark at 4°C.

Acrylamide 29.2

Bisacrylamide 0.8 g

Electrophoresis separating gel buffer (1.5M Tris, pH 8.8)

Per 100 ml of solution completed with deionized and distilled water. pH was adjusted to 8.8 with HCl and stored at 4°C.

Tris 18.15 g

Electrophoresis stacking gel buffer (0.5M Tris, pH 6.8)

Per 50 ml of solution completed with deionized and distilled water. pH was adjusted to 6.8 with HCl and stored at 4°C.

Tris 3.0 g

10 % SDS Solution:

Per 100 ml of solution completed with deionized and distilled water and stored at room temperature.

SDS 10 g

10 % APS Solution:

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

Ammonium persulfate 0.5 g

Table III.5 SDS-PAGE separating gel (12 % w/v, pH 8.8)

Chemical	Amount
Acrylamide-Bisacrylamide Solution (30:0.8)	4 ml
Electrophoresis separating gel buffer	2.5 ml
dH ₂ O	3.5
10% SDS	100 µL
10% APS	100 µL
TEMED	5 µL

Table III.6 SDS-PAGE stacking gel (5 % w/v, pH 6.8)

Chemical	Amount
Acrylamide-Bisacrylamide Solution (30:0.8)	0.67 ml
Electrophoresis separating gel buffer	1 ml
dH ₂ O	2.5
10% SDS	42 µL
10% APS	60 µL
TEMED	5 µL

2X Sample Buffer

Per 10 ml of solution, completed with deionized water and stored at -20 °C.

Electrophoresis separating gel buffer	2.5 ml
10% SDS	4 ml
Glycerol	2 ml
2-Mercaptoethanol	1 ml
Bromphenol blue	0.02 g

5X Running Buffer

Per 1 liter of solution, completed with deionized and distilled water:

Tris	15 g
Glycine	72 g
SDS	5 g

Table III.7 SDS-PAGE Fixing Solution

Chemical	Amount (%)
Ethanol	50
Phosphoric acid	2

Table III.8 SDS-PAGE Washing Solution

Chemical	Amount (%)
Methanol	34
Ammonium sulfate	17
Phosphoric acid	2

Table III.9 SDS-PAGE Staining Solution

Chemical	Amount (%)
Methanol	34
Ammonium sulfate	17
Phosphoric acid	2
Commassie Brilliant Blue -G250	0.066

III.1.4.2. Native-PAGE Buffers and Solutions

40 % Acrylamide-bisacrylamide solution (30:0.8)

Added deionized H₂O to make a final volume of 250 ml and stored in a brown glass bottle at 4°C:

Acrylamide 100 g

Bisacrylamide 2.65 g

4X seperating gel buffer (pH 8.8)

Per 100 ml of solution completed with deionized and distilled water. pH was adjusted to 8.8 with HCl and stored at 4°C.

Tris 18.15 g

4X Stacking gel buffer (pH 6.8)

Per 50 ml of solution completed with deionized and distilled water. pH was adjusted to 6.8 with HCl and stored at 4°C.

Tris 15.1 g

2X Sample Buffer

Per 10 ml of solution completed with deionized and distilled water and stored at - 20°C.

4X stacking gel buffer (pH 6.8) 2.5 ml

Glycerol 2 ml

Bromphenol blue 0.02 g

Running Buffer (pH 8.3)

It is added deionized H₂O to make a final volume of 2 liter. The solution is at about pH 8.3 without adjustment. This solution is readily made fresh each time.

Tris 6 g

Glycine 28.8 g

Table III.10 Native-PAGE Staining Solution

Chemical	Amount
Commassie Brilliant Blue -R250	0.25 g
Methanol	125 ml
Acetic acid	25 ml
dH ₂ O	100 ml

Table III.11 Native-PAGE Destaining Solution

Chemical	Amount
Methanol	100 ml
Acetic acid	100 ml
Add dH ₂ O up to 1 liter	

Table III.12 Native-PAGE Separating Gel Mixture (pH 8.8)

Chemical	Amount
40% Acrylamide-Bisacrylamide Solution (37.5:1)	2.5 ml
4X Separating gel buffer	2.5 ml
dH ₂ O	1ml
10 % APS	100 µL
TEMED	10 µL

Table III.13 Native-PAGE Stacking Gel Mixture (pH 8.8)

Chemical	Amount
40% Acrylamide-Bisacrylamide Solution (37.5:1)	0.4 ml
4X Stacking gel buffer	1.0 ml
dH ₂ O	2.6 ml
10 % APS	50 µL
TEMED	5 µL

III.1.4.3. Enzyme Activity Measurement Buffers and Solutions

K⁺PO₄ buffer (1M, pH 7.0)

1 M K₂HPO₄ 450 ml

1M KH₂PO₄ 550 ml

CENTA Stock Solution (4.7 mM)

25 mg CENTA was dissolved in 10 ml 50 mM K⁺PO₄ buffer. The buffer was aliquoted and maintained at -20°C.

III.1.4.4. Other Stocks and Solutions

1 M IPTG 238.302 g / 100 ml (1M)

Ampicillin 100 mg/ml

Kanamycin 50 mg/ml

III.1.5. Enzyme

E. coli R-TEM-1 (Sigma, P-3553)

III.1.6. Peptides

B45-53: NH-HA AGD YYA Y-CONH₂ 10 mg

B45-53-biotin: Biotin-NH-HA AGD YYA Y-CONH₂ 5,6mg

B45-53- fluor: 5(6)-Fluoresceine-NH-LL IIL HAA GDY YAY- CONH₂ 5.5 mg

The peptides containing 45-53 residues of BLIP were used in experiments. All of them were dissolved 50 mM K⁺PO₄ buffer and stock solutions were aliquoted and maintained at -20 °C. DMSO was also used to dissolve B45-53-biotin and B45-53-fluor in addition to 50 mM K⁺PO₄ buffer. The final concentration of DMSO in solutions was %15 and %20 for each peptide, respectively.

III.1.7. Laboratory Equipment

Autoclaves	Systec 3870 ELV Steam Sterilizer, Germany Nüve OT 032, Türkiye
Balances (0.1, 1, 10 mg)	Ab204-S, Pg403-S, Pg4002-S, Mettler 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, Turkey

Orbital Shaker	Certomat Bs1, B. Braun, Germany
Safety Thermostat	Nüve FN500, Türkiye
pH Meter	Mp220k, Mettler Toledo, USA
Power Supply	Heidolph MR3001, Germany
SDS-PAGE Electrophoresis System	Mini Protean, BIORAD, Canada
Spectrophotometer	Lambda 35, Perkin Elmer, USA
Safety Cabinet	Labculture ESCO, USA
Vortex	Heidolph, Germany
Water Purification Systems	ELGA LabWater, England

III.2. METHODS

III.2.1. Sterilization

In this study, sterilized equipments were used and experimental work that required sterile environment was carried out under laminar flow. Equipment including glassware, tips, tubes, solutions and culture media were sterilized in autoclave at 1.02 atm and 121°C for 15 minutes. Antibiotics, glucose and IPTG were filter sterilized using sterilized membranes with a pore size of 45 µm.

III.2.2. Preparation of Bacterial Stocks

A single colony from a master plate was inoculated with a sterile tip into fresh 5 ml LB medium supplemented with appropriate antibiotic and grown overnight at 37 °C and 180 rpm. 500 µL grown cells were added to sterile tubes containing 30 % sterile glycerol and stocks were stored at -80°C.

III.2.3. Preparation of Preculture

5 ml of sterile liquid medium supplemented with the appropriate antibiotic, was inoculated with 50 µL frozen stock culture.

III.2.4. Growth Conditions

Cells were grown in orbital shakers at 37°C and 180 rpm. Culture volume was kept at one fifth of the flask volume to provide efficient aeration. LB or M9 medium

was used as growth media in experiments. Media were supplemented with 100 µg/ml ampicillin for pUC18 harboring cells and with 50 µg/ml kanamycin for pET-26b(+) or pET-26SJ harboring cells. IPTG (isopropyl β-D-1-thiogalactopyranoside) was used for the induction of the expression of the target BLIP gene. IPTG was added to the growing culture to a final concentration of 1 mM or 0.2 mM when OD₆₀₀ of cell culture reached 0.5.

III.2.5. Monitoring Cell Growth

Optical densities of *E. coli* cells were measured at 600 nm using a spectrophotometer. Samples were diluted to keep the spectroscopic readings between reliable limits (0.2-0.8).

III.2.6. Viable Cell Count

Bacterial samples were plated to determine the change in the number of viable cells following induction with IPTG or incubation with the peptides. For a ten-fold dilution, 100 µL of the bacterial sample taken from the growing cell suspension at the desired time interval was transferred into a glass tube containing 0.9 ml of fresh liquid medium and mixed by vortex to provide homogenization. For another ten-fold dilution, 100 µL from this mixture was taken and deposited in a new glass tube containing 0.9 ml fresh liquid medium. This process continued until the desired dilution was reached. Finally, 1 ml of the dilution was deposited in a sterile Petri dish and agar containing appropriate antibiotic was mixed gently by moving the plates through an eight-shaped path on the ground without lifting. The plates were left to cool to harden and then incubated overnight at 37°C in a sterilizator. The colonies were counted to determine the colony forming units on each Petri dish. Average values from triplicates plates were taken.

III.2.7. Plasmid Transformation into *E. coli* BL21(DE3) (pUC18) Cells

CaCl₂ protocol was used to transform *E. coli* BL21(DE3) (pUC18) cells with the pET-26b(+) plasmid. Preculture of *E. coli* BL21(DE3) (pUC18) cells, were grown overnight in LB broth. This culture was used for inoculation into fresh LB broth at a 1:100 dilution (OD₆₀₀ was kept below 0.05). Cells were grown at 37 °C with vigorous shaking in flasks to facilitate aeration. When the cells reached the optical density of 0.5 at 600 nm, they were centrifuged at 3,000 rpm for ten minutes

at 4 °C in sterile tubes. The supernatant was discarded and the cells were resuspended in 100 µL of sterile, ice-cold 0.1 M CaCl₂. Cells were incubated on ice for 30 minutes. The tube was mixed gently every ten minutes to allow a homogenous mixture. 1-2 ng pET-26b(+) was added to the cells. The cells were then incubated on ice for one hour to allow the plasmid DNA to enter the cells. Following a heat shock at 42 °C for 60 seconds, the cells were further incubated on ice for ten more minutes. Following the addition of 800 µL of LB broth without any antibiotics, the cells were incubated at 37 °C for one hour. The transformed cells were centrifuged at 3000 rpm for one to five minutes and then plated on selective plates containing 100 mg/ml ampicillin and 50 mg/ml kanamycin. The plates were incubated overnight at 37 °C.

III.2.8. Determination of Dry Cell Weight

Cells were grown overnight at 180 rpm and 37 °C in 100 ml of medium in a 500 ml of flask. Cells were centrifuged at 9000 rpm and washed with distilled water for two to three times. 50 ml of the cells, resuspended in 100 ml of distilled water, were dried at 80 °C and the dry cell weight was determined. The remaining 50 ml, with the optical density 0.4-0.6 was used to prepare diluted samples containing 0.125, 0.250, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 ml original cell culture. All volumes were completed to 5 ml by distilled water. OD 600 nm values were read in a spectrophotometer. Cell dry weight (mg/ml) for each sample was determined using data from the dried cell sample. Calibration chart was prepared by plotting optical density vs. cell dry weight. The slope of this graph was used to determine the dry cell weight of a known OD at 600 nm.

III.2.9. Extraction of Periplasmic Proteins

Periplasmic proteins were extracted from cells by osmotic shock procedure which was described by Nossal *et al.*, at 1966. 100 ml of sterile LB medium was inoculated with a preculture at a final OD of 0.7 and placed in an orbital shaker at 37°C, 180 rpm. In cases where induction with IPTG was needed, *E. coli* BL21(DE3) cells harboring pUC18 and pET-26b(+) or pET-26SJ were grown until the optical density of the cell culture reached 0.5 at 600 nm and 0.2 or 1 mM IPTG was added. Cells were allowed to grow three hours after induction. In cases where induction was not desired, *E. coli* BL21(DE3) cells harboring pUC18 and pET-26b(+) or pET-26SJ

were grown until to a final OD of 0.8-1.0. Grown cells were centrifuged for 10 min at 7,000 rpm, +4°C and the supernatant was discarded. After harvesting, cells were resuspended in 20 % sucrose solution in 30 mM Tris-HCl (pH 8.0) with 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were gently incubated for 20 min at room temperature. Following centrifugation at 9,000 rpm for 20 min, the pellet was rapidly resuspended in ice-cold 5 mM MgCl₂ and gently incubated for 20 min. Cells were removed from the periplasmic protein extract by centrifugation at 9,000 rpm, +4°C for 20 min. Supernatant contained periplasmic proteins including R-TEM-1 β -lactamase.

III.2.10. Determination of Protein Concentration

Protein concentration in the periplasmic protein extract was determined using the method described by Bradford (1976). Samples containing protein were mixed with Bradford solution and their absorbance at 595 nm was recorded. Bovine Serum Albumin (BSA) was used as the standard for the preparation of the calibration curve. Samples with different BSA concentrations were and mixed with Bradford solution. The absorbance of these samples at 595 nm was measured to construct the plot that relates protein concentration to the absorbance at 595 nm. The calibration curve is given in Apppendix B.

III.2.11. SDS-PAGE Analysis of Periplasmic Proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique that involves the separation of proteins based on their sizes in an electric field. For this purpose, protein samples were mixed with 2X Sample Buffer containing SDS at a 1:1 ratio and boiled for 5 minutes to denature the proteins. The marker and the samples were loaded into the wells of 12 % SDS polyacrylamide gels. Proteins were allowed to run at 120 V for approximately 90 minutes. Then gels were gently removed from glass plates and placed in fixing solution overnight to fix the proteins into the gel. The following day, gels were washed with washing solution for an hour. Finally, gels were stained with Commasie G-250 at least overnight and destaining solution was used to remove excess unbound stain. The gels were dried for 40 min at 80 °C. The protein bands of the samples were compared against the Standard and the presence of β -lactamase, BLIP and their complex were verified.

III.2.12. Native-PAGE Analysis of Periplasmic Proteins

"Native" or "non-denaturing" gel electrophoresis is performed in the absence of SDS which is the protein denaturant. 10 ml separating gel mixture was prepared containing 10 % acrylamide. Osmotic shock samples were dissolved in 2X Sample Buffer without SDS. Samples were loaded under native conditions. Proteins were allowed to run at 120 V for approximately 75 minutes. The gel was stained in the Staining Solution for 2-3 hours. After staining, the dye that was not bound to the proteins was removed in the Destaining Solution.

III.2.12.1. Investigation of Protein Bands of Native-PAGE Gels

In order to verify the presence of β -lactamase on the Native gel, activity measurement was performed by using sliced gel pieces expected to correspond to β -lactamase. A piece corresponding to β -lactamase and a control gel piece without β -lactamase were excised from an unstained gel and mixed with 470 μ M CENTA substrate. The mixtures were incubated for two days at 25°C and 30 rpm in the presence of running buffer. The change in color of mixtures was monitored.

III.2.13. *In-vitro* Enzyme Activity Measurement

In-vitro β -lactamase-BLIP interaction was determined by β -lactamase activity measurement. Periplasmic protein extract from *E. coli* BL21(DE3) (pUC18) cells were used as the source of β -lactamase. All activity measurements were performed in a total reaction volume of 1 ml. CENTA (Calbiochem) was used as the substrate and in all experiments, 47 μ M CENTA was mixed with pre-determined amounts of periplasmic protein extract. Hydrolysis of CENTA was measured by monitoring the change in the absorbance at 405 nm. Periplasmic protein extract from *E. coli* (pUC18) or *E. coli* (pUC18 + pET-26) cells was used as control to calculate total activity before inhibition.

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

$$U = \frac{V_t \times (d_A / d_t) \times 1000 \times DF}{\epsilon \lambda \times V_s \times d} \quad (\text{III.1})$$

V_t = Total reaction volume of (ml)

$\varepsilon\lambda$ = Extinction coefficient of the substrate (cm².mol⁻¹)

V_s = Volume of enzyme (ml)

d_A / d_t = Absorbance change per time (min⁻¹)

DF = Dilution Factor

d = Light path (1 cm)

III.2.14. Interaction of β -lactamase and BLIP under *in-vivo* Conditions

In-vivo β -lactamase-BLIP interaction was monitored by measuring β -lactamase activity in cells co-expressing β -lactamase and BLIP. For this purpose, periplasmic protein extract from *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells was used. These cells expressed β -lactamase constitutively and BLIP under IPTG induction. Periplasmic protein extract from *E. coli* BL21(DE3) (pUC18+pET-26b(+)) cells was used as control. Both cells were grown until their OD reached 0.5 at 600 nm and induced with 0.2 or 1 mM IPTG. Cells were allowed to grow for one half or three hours before extraction of their periplasmic proteins. β -lactamase activity was measured in each extract and compared.

III.2.15. *In-vivo* β -lactamase Inhibition by Peptides

E. coli K12 (pUC18) cells were grown in M9 Minimal Medium at 37 °C for monitoring growth inhibition by peptides. The cells were grown in a total volume of 2 ml and were incubated with 100 μ M peptide (B45-53 B45-53-biotin, and B45-53-fluor) when they were in early logarithmic phase (OD_{600 nm} ~ 0.2). Cell growth was monitored every hour at OD_{600 nm} and bacterial cell samples were plated to determine the change in viable cell number. Bacterial samples grown in the absence of the peptide were also plated on LB agar plates as a control.

III.2.16. *In-vitro* β -lactamase Inhibition by BLIP

Periplasmic protein extract from *E. coli* BL21(DE3) (pUC18) cells were used as the source of β -lactamase and periplasmic protein extracts from *E. coli* BL21(DE3) (pET-26SJ) was used as the source of BLIP. The extracts were mixed with each other based on their protein concentrations. This mixture was incubated for

1 hour at 30 rpm and 25 °C in the presence of phosphate buffer to achieve binding. After incubation, CENTA was added to the mixture and its hydrolysis CENTA was by monitored by recording the change in the absorbance at 405 nm.

III.2.17. *In-vitro* β -lactamase Inhibition by Peptides

In-vitro β -lactamase-peptide interaction was determined by β -lactamase activity measurement. Periplasmic protein extract from *E. coli* BL21(DE3) (pUC18) cells were used as the source of β -lactamase. Pre-determined amount of periplasmic protein extract containing β -lactamase was mixed with various concentrations of peptides. The mixture was incubated for 1 hour at 30 rpm and 25 °C in the presence of phosphate buffer to achieve binding. After incubation, CENTA was added to the mixture and its hydrolysis was monitored by recording the change in the absorbance at 405 nm. Periplasmic protein extract incubated with the buffer was used as control to calculate total activity before the inhibition.

CHAPTER IV

RESULTS AND DISCUSSION

IV.1. EXPRESSION SYSTEMS

In this study, pUC18 plasmid carrying the gene for R-TEM-1 β -lactamase vector was used for β -lactamase production. pET-26b(+) vector carrying the BLIP gene with its native leader sequence (pET-26SJ) was used for periplasmic expression of BLIP. This vector is highly efficient for expression of BLIP under the control of the strong T7 promoter. The experiments were performed with the *E. coli* BL21(DE3) host strain in combination with plasmids containing T7 promoter constructs (pET26b(+)). The expression of BLIP was easily and efficiently achieved by inducing the promoter with IPTG.

In an effort to investigate simultaneous expression of β -lactamase and BLIP, *E. coli* BL21(DE3) (pET-26SJ + pUC18) cells were used. *E. coli* BL21(DE3) (pET-26b(+) + pUC18) cells were used as control. Expression systems used in experiments and the proteins expressed using these systems are summarized in Table IV.1.

Table IV.1. Expression systems and expressed proteins.

Cells	Plasmids	Proteins Expressed
<i>E. coli</i> BL21(DE3)	pUC18	B-lactamase
<i>E. coli</i> BL21(DE3)	pET-26SJ	BLIP
<i>E. coli</i> BL21(DE3)	pUC18 + pET-26b(+)	B-lactamase
<i>E. coli</i> BL21(DE3)	pUC18 + pET-26SJ	β -lactamase + BLIP
<i>E. coli</i> K12	pUC18	B-lactamase

β -lactamase is a periplasmic protein in gram negative bacteria such as *E. coli*. BLIP is originally a periplasmic protein in its host *S. clavuligerus*. For β -lactamase to

bind BLIP this protein should be expressed as a periplasmic protein in *E. coli* as well. With this intension, BLIP with its native signal sequence was expressed in *E. coli* cells. If both proteins, β -lactamase and BLIP, could be expressed as periplasmic proteins, there is the possibility of BLIP inhibiting β -lactamase action.

IV.2. ELECTROPHORETIC ANALYSIS OF β -LACTAMASE AND BLIP

Periplasmic extracts from recombinant *E. coli* cells were electrophoretically analyzed to confirm the co-expression of β -lactamase and BLIP by *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells. Samples with equal amounts of protein were loaded to the polyacrylamide gels. Both denaturing and native polyacrylamide gels were used for analysis..

IV.2.1. SDS-PAGE Analysis

The basic principle behind electrophoretic separation of proteins in denaturing gels, is the difference between their sizes. By comparing the sizes of β -lactamase and BLIP with reference to the standard proteins present in the marker, the expression of β -lactamase and BLIP has been confirmed. For this purpose *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were used. Both cultures were induced with 0.2 mM IPTG. Under these conditions constitutive β -lactamase expression was expected from *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells. Since pET-26b(+) plasmid did not possess any insert, even upon IPTG induction, no recombinant protein production was expected in this culture. Under the same conditions, both β -lactamase and BLIP expression was expected from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells.

Following periplasmic protein extraction, extracts from *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells have been analyzed on SDS-PAGE (Figure IV.1).

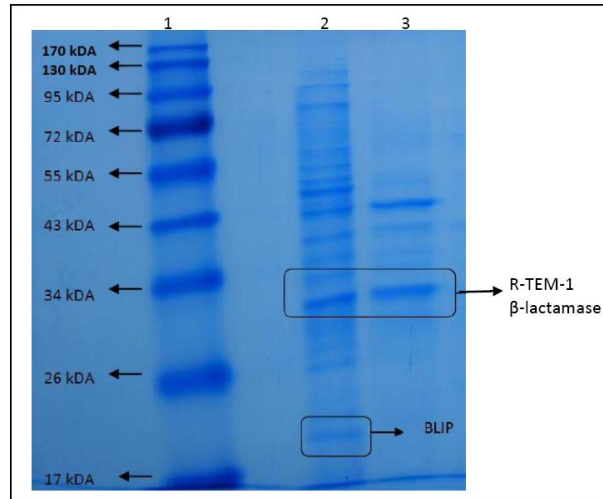


Figure IV.1. Electrophoretic analysis of periplasmic protein extracts from cells induced with 0.2 mM IPTG. Lane 1: Molecular weight marker; Lane 2: extract from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells; Lane 3: extract from *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells

In Figure IV.1, it can be seen that even after induction, in the periplasmic protein extract from *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells, there was a band of 28.9 kDa corresponding to β -lactamase (Lane 3). Following induction, the analysis of the periplasmic protein extract from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells analyzed on SDS-polyacrylamide gels indicated the presence of a 17.5 kDa band corresponding to BLIP in addition to the band corresponding to the 28.9 kDa β -lactamase band (Lane 2).

In order to check if there was leaky BLIP expression, the co-expression of β -lactamase and BLIP was investigated in *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced and uninduced conditions. Induction was performed with 0.2 mM IPTG. Uninduced cells were used as control. In the absence of IPTG induction, if there is no leaky expression, BLIP expression should not be observed.

The analysis of the periplasmic proteins from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells is given in Figure IV.2.

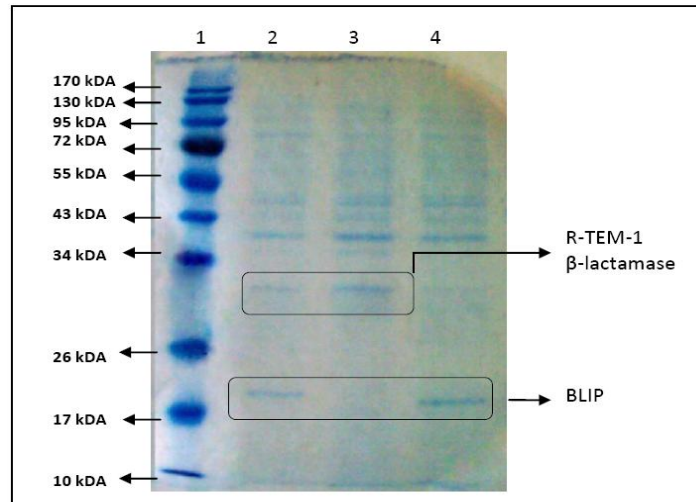


Figure IV.2. Electrophoretic analysis of periplasmic protein extracts. Lane 1: Molecular weight marker; Lane 2: extract from *E. coli* (DE3) (pUC18 + pET-26SJ) cells induced with 0.2 mM IPTG; Lane 3: extract from uninduced *E. coli* (DE3) (pUC18 + pET-26SJ) cells; Lane 4: extract from *E. coli* (DE3) (pET-26SJ) induced with 0.2 mM IPTG.

Both 28.9 kDa band of β -lactamase and 17.5 kDa band of BLIP were visible in the extracts of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced conditions (Lane 2 of Figure IV.2). Under uninduced conditions, only the 28.9 kDa band of β -lactamase was visible on the gel (Lane 3 of Figure IV.2).

In order to verify that the 17.5 kDa indeed corresponds to BLIP, the periplasmic extracts from *E. coli* BL21(DE3) (pET-26SJ) cells induced with 0.2 mM IPTG was also investigated on the SDS-polyacrylamide gel (Lane 4 of Figure IV.2). The visualization of the band of 17.5 kDa in the extracts of *E. coli* BL21(DE3) (pET-26SJ) cells, supported the finding that this is BLIP.

IV.2.2. Native-PAGE Analysis

Native PAGE is one of the most powerful techniques for studying the composition and structure of native proteins, since both the conformation and biological activity of proteins remain intact during this technique.

"Native" or "non-denaturing" gel electrophoresis are run in the absence of SDS. While in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass, in native PAGE the mobility depends on both the protein's

charge and its hydrodynamic size. The electric charge driving the electrophoresis is governed by the intrinsic charge on the protein at the pH of the running buffer. Gradient gels for native PAGE sharpen the protein bands and allow complex mixtures of proteins to be separated on a single gel.

Complexes remain associated for the most part and folded as they would be in the cell. One downside, however, is that complexes may not separate cleanly or predictably, since they cannot move through the polyacrylamide gel as quickly as individual, denatured proteins. Therefore, Native-PAGE gels are not as clear as SDS-PAGE gels.

Under denaturing conditions, the visualization of separate bands for β -lactamase and BLIP does not give any clue on the formation of a complex between these two proteins. This only proved that these proteins are expressed simultaneously. In order to investigate if BLIP binds β -lactamase, the periplasmic protein extract from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells has been analyzed under native conditions (Figure IV.3). Induction was achieved with 0.2 mM IPTG.

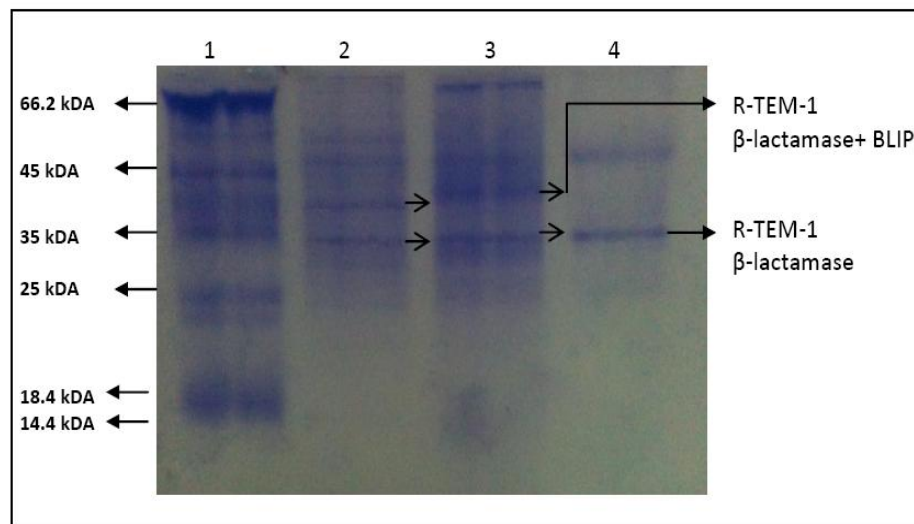


Figure IV.3. Electrophoretic analysis of periplasmic protein extracts. Lane 1: Molecular weight marker; Lane 2 and 3: extract from *E. coli* (pUC18 + pET-26SJ) induced with 0.2 mM IPTG; Lane 4: uninduced *E. coli* (pUC18 + pET-26SJ).

A band probably corresponding to the size of BLIP- β -lactamase complex was observed in the protein extract from *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells induced with 0.2 mM IPTG. A band probably corresponding to the size of β -lactamase alone was observed in the uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells. Unfortunately, protein bands of molecular weight marker were not clear; hence there was ambiguity in the presence of BLIP and β -lactamase. In order to prove accuracy of tagging the protein weight bands, pieces of bands corresponding to the size of β -lactamase were cut from Native-PAGE gel. Activity measurement was performed with gel pieces as described in Section III.2.12.1. In this case, an unstained gel was used in order to prevent β -lactamase activity loss during staining. A prestained molecular weight marker was used to estimate the part of gel corresponding to the size of β -lactamase.

The pieces of unstained gel were cut and mixed with 470 μ M CENTA substrate. A mixture which contained pieces of different gel bands were used as control. The mixtures were incubated for two days at 25 °C and 30 rpm in the presence of running buffer. The change in color of mixtures was monitored. The color of the mixture containing β -lactamase bands turned to yellow that showed the presence of β -lactamase (Figure IV.4).

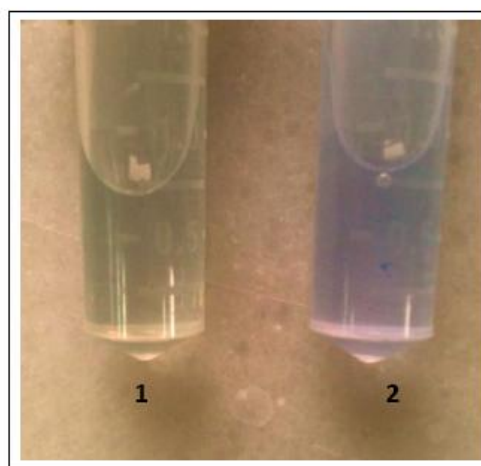


Figure IV.4. Change in color of the mixtures containing gel pieces. Sample 1: the mixture containing β -lactamase bands; Sample 2: the control mixture.

IV.3. DETERMINATION OF THE EFFECT OF BLIP EXPRESSION ON *E. COLI* GROWTH

Experiments have shown that both β -lactamase and BLIP were expressed by *E. coli* (pUC18 + pET-26SJ) cells and they were found as periplasmic proteins. If a complex between these two proteins could form, cells would be expected to die since the enzyme that confers resistance to the cells would be inactive. With that intension growth of *E. coli* (pUC18 + pET-26SJ) cells was monitored and their viability was measured following induction with IPTG for BLIP expression. β -lactamase was expressed constitutively but BLIP was expressed only after IPTG induction.

IV.3.1. Analysis of Cell Growth

E. coli BL21(DE3) (pUC18+pET-26b(+)) and *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells were grown under uninduced condition to investigate the presence of the recombinant plasmid on the cells. Growth profiles have been plotted in Figure IV.5.

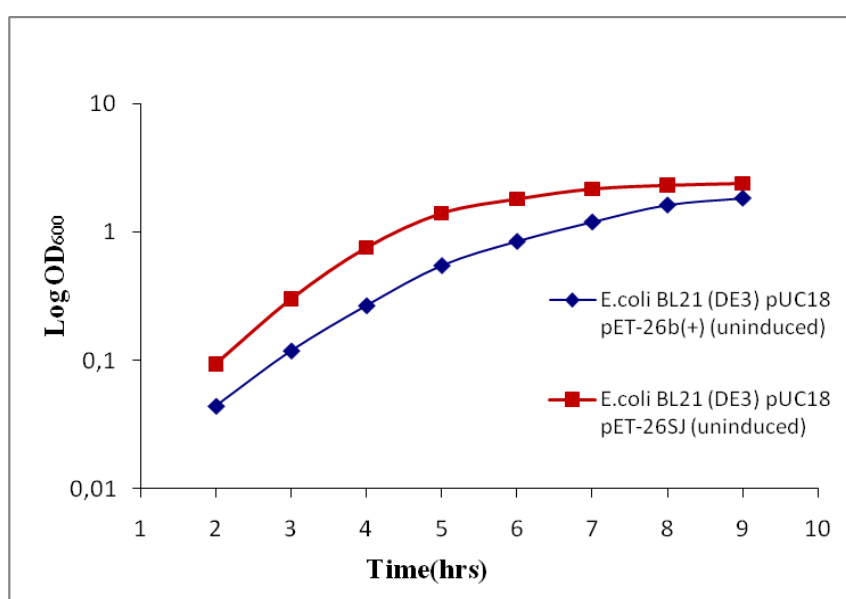


Figure IV.5. Growth of *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells (◆) and *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells (■) under uninduced conditions.

This experiment has been performed twice and the bars represent the error from the average of two samples. As can be seen in Figure IV.5, both *E. coli* BL21(DE3) cells entered exponential growth phase immediately after inoculation into the culture. The maximum specific growth rates, $\mu_{\max}$, were calculated for both *E. coli* BL21(DE3) cells. During exponential phase, *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells had a slightly higher maximum specific growth rate as 0.61 h^{-1} . The maximum specific growth rate for *E. coli* BL21(DE3) (pUC18+pET-26b(+)) cells was calculated as 0.57 h^{-1} . Both cells had similar growth profiles. This showed that pET-26SJ construct did not retard cell growth and there probably was no leaky BLIP expression.

In order to investigate the effect of simultaneous β -lactamase and BLIP expression, *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells were induced with 0.2 mM IPTG to initiate BLIP expression. Uninduced *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells were used as control. The latter cells expressed only β -lactamase. Optical densities of the cultures were monitored at 600 nm and the obtained growth profiles are shown in Figure IV.6. These experiments were performed in triplicate and the error bars represent. Since the optical density values were very close to each other, error bars were not visual in Figure IV.6.

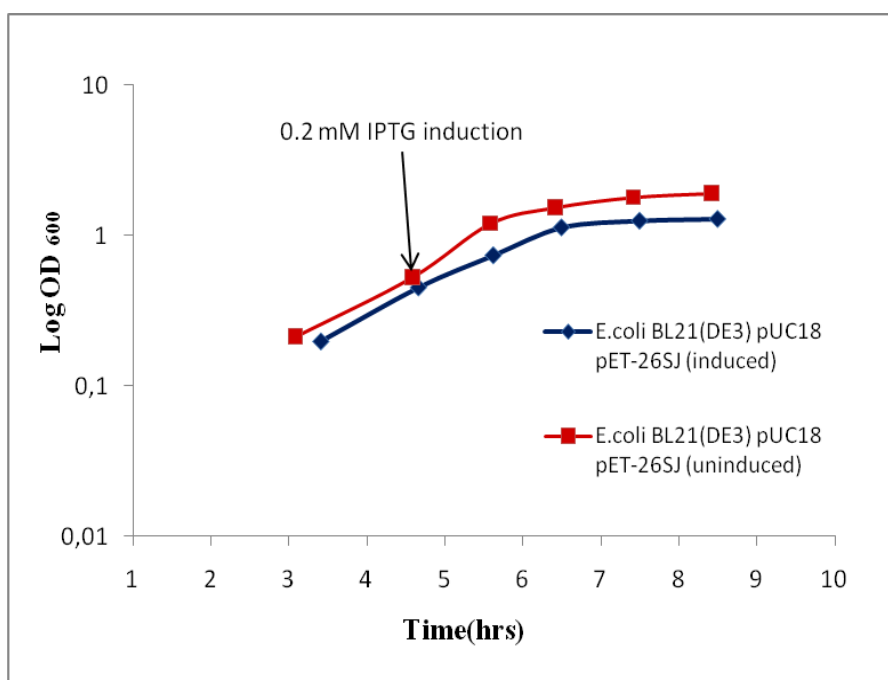


Figure IV.6. Growth profiles of induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells(◆) and uninduced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells(■).

Until IPTG induction, growth rates of the cells were similar, as expected. However, after induction with IPTG growth rate declined to 0.50 h^{-1} . This was either due to inhibition of β -lactamase by BLIP or due to metabolic burden from BLIP expression. If BLIP has bound β -lactamase, the enzyme would be inhibited. On the other hand, the expression of the foreign protein BLIP upon induction with IPTG could have caused metabolic burden.

IV.3.2. Measurement of Viable Cells

Measurement of growth by monitoring optical density of growing cultures may be misleading, because spectrophotometer readings can not differentiate between dead and live cells. BLIP expression is the reason for reduction in β -lactamase resistance hence cell death, the number of viable cells was counted before and after BLIP expression.

For this purpose, *E. coli* BL21(DE3) (pUC18+pET-26SJ) cells were induced with 0.2 or 1.0 mM IPTG to start BLIP expression. BLIP expression was allowed to continue for 3 hours. Same cells without IPTG induction and *E. coli* BL21(DE3) (pET-26SJ) cells with induction were used as control. Colony forming units on agar plates were counted and the numbers obtained were plotted against time (Figures IV.7 and IV.8). These experiments were performed in triplicate and error bars represent the range of error.

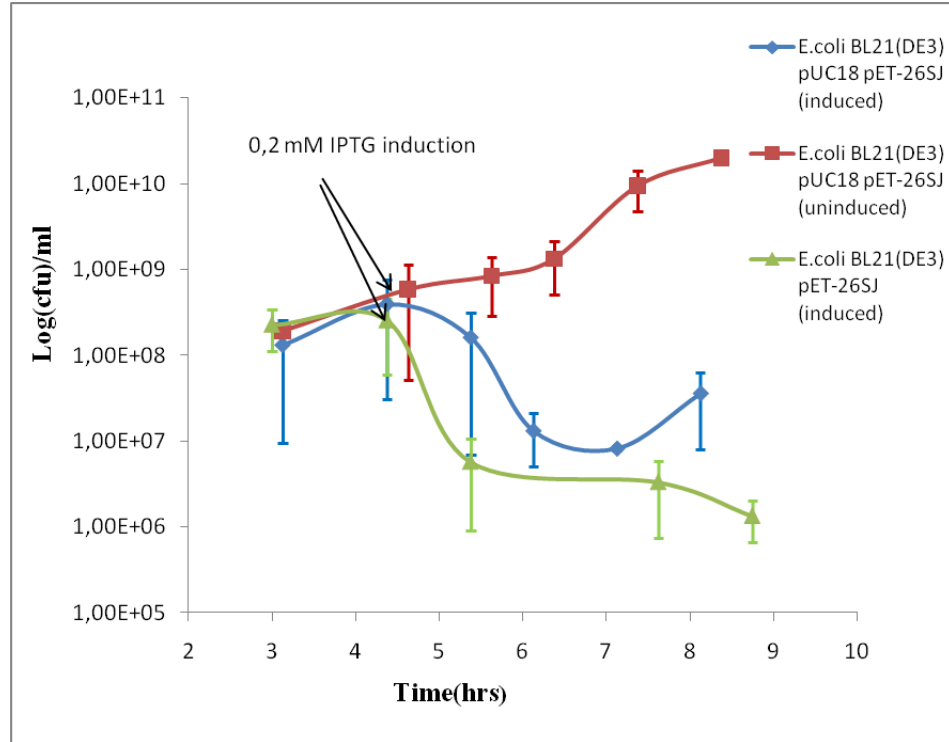


Figure IV.7. Viability of cells induced with 0.2 mM IPTG

Based on Figure IV.7, it was concluded that the number of viable cells of cultures were nearly the same until induction with 0.2 mM IPTG. After induction, cell viability dropped significantly as compared to uninduced *E. coli* BL21(DE3) cells. One hour following induction of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells, CFU/L dropped by 59.3 %. However, CFU of the same cells under uninduced conditions increased 43.4 % in the same time interval.

CFU/L of *E. coli* BL21(DE3) (pET-26SJ) cells decreased by 97.8 % one hour after induction and it continued to decrease. CFU/L results obtained for *E. coli* BL21(DE3) (pET-26SJ) cells showed that, BLIP production alone caused cell death as a result of metabolic burden.

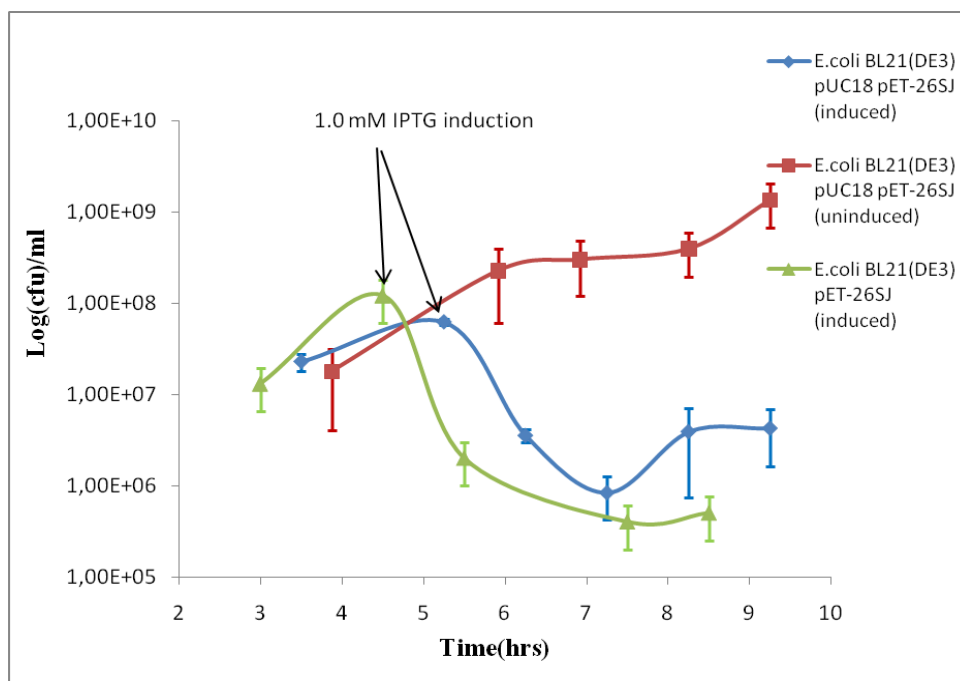


Figure IV.8. Viability of cells induced with 1.0 mM IPTG

Cell viability dropped after induction with IPTG. In the presence of higher IPTG concentration, the decrease in the number of viable cells was more. CFU/L of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells expressing both β -lactamase and BLIP decreased immediately after induction by 94.3 % (Figure IV.8). This showed that, as the concentration of IPTG increased from 0.2 mM to 1.0 mM, decrease in viable cell number was even more severe. 1 hour after induction, 94.3 % of the initial number of cells were dead. CFU/L of the *E. coli* BL21(DE3) (pET-26SJ) cells expressing only BLIP decreased 98.3 % when induced with 1 mM IPTG. According to this result, cells were not very sensitive to IPTG concentration. Significant cell death occurred with both IPTG concentrations

IV.3.3. Determination of Dry Cell Weight

In order to correlate OD readings at 600 nm to dry cell weight of *E. coli* cells, the method given in Section III.2.8 has been used. Cells were grown until their OD reached 1.76 at 600 nm. Calibration chart was prepared by plotting optical density vs. cell dry weight as shown in Appendix A. The equation as $y = 2.2079x + 0.0231$ was obtained and the slope of this line was used to determine the dry cell weight of a known OD_{600nm} value.

IV.3.4. Determination of the Cell Viability versus Optical Density

Optical density should be linearly correlated with the viable cell number. Unfortunately, after late exponential phase, the number of dead cells starts to increase but the spectrophotometer cannot differentiate these cells from live cells. In other words optical density measurements of growing cultures is insensitive to live cells after that point. For this reason, the graph of viable cell number vs. optical readings was plotted in order to see the range of linear relation between optical readings and live cells (Figure IV.4.). *E. coli* BL21(DE3) (pUC18+pET-26b(+)) and *E. coli* BL21(DE3) (pUC18+and pET-26SJ) grown under uninduced conditions were compared. They were allowed to express only β -lactamase.

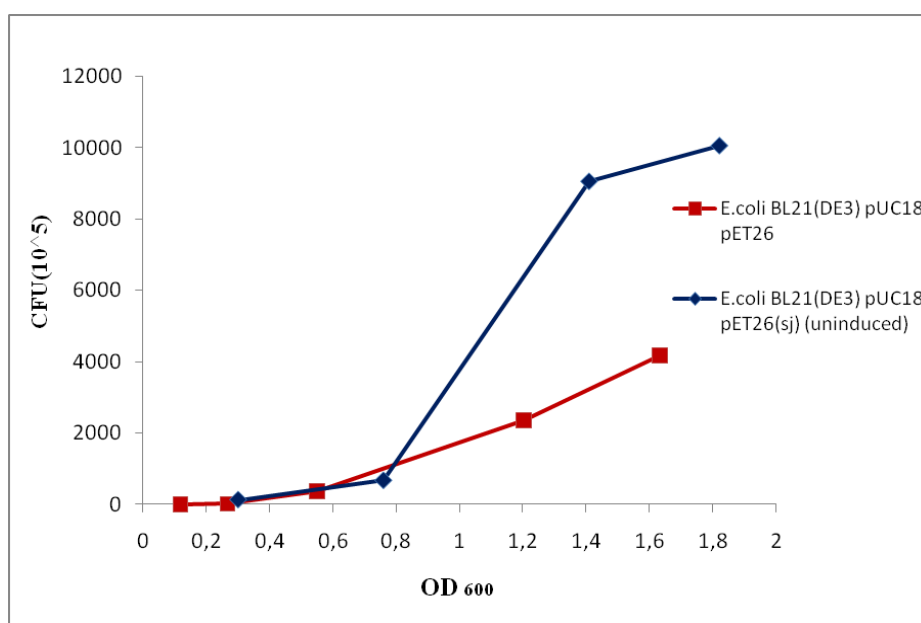


Figure IV.9. Cell viability versus optical density of *E. coli* BL21(DE3) (pUC18 + pET-26SJ) and *E. coli* BL21(DE3) (pUC18 + pET-26)

The number of viable cells of both cultures were similar until the optical density reading of 0.8. After OD₆₀₀ reached 0.8, the expected linear dependence was disrupted. This showed that for cultures with optical density below 0.8, the readings can reliably be correlated to viable cell number. Above that value, dead cells also are measured in the readings.

IV.4. *IN-VIVO* β -LACTAMASE INHIBITION BY BLIP

Examining cell growth either spectrophotometrically or by counting the number of viable cells has not provided strong clues on *in-vivo* β -lactamase-BLIP interaction. It has been proposed that β -lactamase-BLIP complex may form inside the cells which could reduce total β -lactamase activity. For this reason, β -lactamase activities in *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under induced and uninduced conditions were compared. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells induced with IPTG were used as a second control.

In order to compare total β -lactamase activity under induced and uninduced conditions, two *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cultures were grown until the optical density reached 0.5 at 600 nm. One of these cultures was induced with 0.2 mM IPTG. The cells were allowed to grow for 1.5 or 3 hours after BLIP expression started and the change in total β -lactamase activity was measured.

Total β -lactamase activity in U/L periplasmic extract found in the periplasmic cell extracts was calculated. Since the amount of total protein and hence β -lactamase varies slightly from one experiment to another, activity of β -lactamase was normalized by dividing total activity by total protein found in the extract and expressed as U/mg protein. The results are given in Table IV.2.

Table IV.2. *In-vivo* β -lactamase inhibition by BLIP for 1.5 hours.

Expression System	Proteins expressed	1,5 hours after induction	
		β -lactamase Activity (U/L periplasmic extract)	β -lactamase specific activity (U/mg protein)
<i>E.coli</i> BL21(DE3) (pUC18 + pET-26SJ) uninduced	β -lactamase	201.1	3.44
<i>E.coli</i> BL21(DE3) (pUC18 + pET-26SJ) induced	β -lactamase + BLIP	7.4	0.13
Inhibition		96 %	

Table IV.3. *In-vivo* β -lactamase inhibition by BLIP for 3 hours.

Expression System	Proteins expressed	3 hours after induction	
		β -lactamase Activity (U/L periplasmic extract)	β -lactamase specific activity (U/mg protein)
<i>E.coli</i> BL21(DE3) pUC18 pET-26SJ uninduced	β -lactamase	406.0	3.30
<i>E.coli</i> BL21(DE3) pUC18 pET-26SJ induced	β -lactamase + BLIP	24.7	0.17
Inhibition		95 %	

In the absence of BLIP, total β -lactamase activity found in the periplasmic cells extracts were 201.1 and 406.0 U/L after 1.5 and 3.0 hours, respectively. However, expression of BLIP resulted in about 95 % β -lactamase inhibition after BLIP expression started. This showed that when β -lactamase and BLIP were coexpressed, the complex between the two proteins formed and β -lactamase was. Interestingly, although β -lactamase activity in either culture, expressed in U/L periplasmic protein, nearly increased 2-fold, activities expressed in U/mg protein remained almost constant as induction period increased from 1.5 to 3 hours.

IV.5. INVESTIGATION OF RELATIONSHIP BETWEEN β -LACTAMASE PRODUCTION AND NUMBER OF VIABLE *E. COLI* CELLS

The amount of β -lactamase produced in each batch slightly varies. For this reason, there was the requirement for normalizing β -lactamase production. The most feasible way could be to relate total β -lactamase activity to viable cell number found in the culture. For simultaneous expression of β -lactamase and BLIP *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells and as control *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used.

The numbers of viable cell in the culture were used to determine β -lactamase produced per cell.

β -lactamase activity in cells grown without induction were measured first to investigate the difference in activity between *E. coli* BL21(DE3) (pUC18 + pET-26SJ) and *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells under the same conditions. Before centrifugation, OD readings at 600nm were recorded and the cells were plated to obtain colony forming units. The results of these experiments from the analysis of triplicate samples were summarized in Table IV.4. The raw data of the experiments were given in Appendix C.

Table IV.4. Minimum and maximum activity, OD₆₀₀ nm just before cells were harvested, CFU, Activity/OD, and Activity/CFU values of uninduced cells.

	Data Ranges of Uninduced Experiments	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26b(+)
	min - max	min – max
Activity(U/L periplasmic extract)	27.80 - 143.75	18.02 - 45.79
Harvest OD ₆₀₀	0.44 - 0.52	0.43 – 0.52
CFU(10 ⁶)	61 - 135	23 – 30
Activity/OD ₆₀₀	56.83 - 276.76	41.72 - 107.29
Activity/CFU	0.33 - 1.06	0.78 – 1.54

The results showed that β -lactamase activity per viable cell from *E. coli* BL21(DE3) pUC18 pET-26SJ cells (coexpressing β -lactamase and BLIP) and *E. coli* BL21(DE3) pUC18 pET-26b(+) cells (expressing β -lactamase) were comparable in the absence of BLIP expression. Activity per CFU values found for *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells varied between 0.33-01.06. Activity per CFU values of the control set varied between 0.78 – 1.54. The optical density just before the cells were harvested laid between 0.4-0.5 to be able to easily correlate optical density to viable cell number. As the numbers indicate, the number of viable *E. coli* BL21(DE3)

(pUC18 + pET-26SJ) cells was slightly higher than the control set. Activity found in *E. coli* BL21(DE3) (pUC18 + pET-26SJ) was also slightly higher than the control set. Overall the normalized activity values remained in the same range.

Comparison of activity data summarized in Table IV.4 showed only a slight difference in growth of cells and total obtainable enzyme activity in the absence of BLIP production. Consequently, it is reasonable to use induced *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells as the control set under induced conditions.

In order to compare and normalize enzyme activity in the presence of BLIP expression, cells were grown until OD reached 0.5 at 600 nm and induced with 0.2 mM IPTG and allowed to grow for three hours after induction. Before centrifugation, OD readings at 600nm were recorded and the cells were plated to obtain colony forming units.

Table IV.5. Minimum and maximum activity, harvesting OD₆₀₀, CFU, Activity/OD, and Activity/CFU values of induced cells.

	Data Ranges of Induced Experiments	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26b(+)
	min – max	min – max
Activity (U/L periplasmic extract)	2.41 - 4.35	27.47 - 63.74
Induction OD₆₀₀	0.46 - 0.51	0.48 – 0.49
Harvest OD₆₀₀	1.46 - 1.72	1.62 – 1.70
CFU(10⁶)	62 – 73	16 – 37
Activity/OD₆₀₀	1.65 - 2.53	17.01 - 37.52
Activity/CFU	0.04 - 0.06	1.72 – 1.72

The results obtained from cells induced expressing BLIP were summarized in Table IV.5 and the raw data were given in Appendix C. The results showed that induction of BLIP expression has led to a very important decrease, almost 35 fold in β -lactamase activity per viable cell compared to the control set. Activity per CFU values of the cells expressing both β -lactamase and BLIP varied between 0.04 -0.06. These values were too low when compared to the values obtained for both the control set (activity/CFU =1.72) and for the same culture grown under uninduced conditions. Even though harvesting OD₆₀₀ values were close to each other, the β -lactamase activity of data set lower 11.4- 14.7 fold than control set. This difference could have occurred as a result of β -lactamase inhibition by BLIP after induction and this is an important evidence of the *in-vivo* inhibition of β -lactamase.

Even though the number of viable cells from the culture expressing both proteins was higher as 2.0-3.9 fold than the control set, the number of viable cells from the induced control culture expressing β -lactamase alone slightly decreased compared to the *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells under uninduced conditions.

IV.6. β -LACTAMASE INHIBITION BY BLIP IN THE PRESENCE OF HIGH ANTIBIOTIC CONCENTRATION

The aim of this experiment was to investigate the effect of higher ampicillin concentration on β -lactamase production. Cells produced β -lactamase against ampicillin. The production of β -lactamase could change as a result of changing ampicillin in the growth medium. For this purpose, the ampicillin concentration was increased from 100 μ M/ml to 200 μ M/ml in the growth medium. *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were induced with 0.2 mM IPTG to express both β -lactamase and BLIP. *E. coli* BL21(DE3) (pUC18 + pET-26b(+)) cells were used as control. The results of these experiments are summarized in Table IV.4. The raw data were given in Appendix D.

Table IV.6. Minimum and maximum activity, harvesting OD₆₀₀, CFU, Activity/OD, and Activity/CFU values of the induced cells in the presence of high antibiotic concentration.

	Data Range of Experiments	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26b(+)
	min - max	min – max
Activity (U/L periplasmic extract)	5.12 - 79.22	76.86 - 145.74
Induction OD₆₀₀	0.53 - 0.61	0.42 – 0.45
Harvest OD₆₀₀	1.07 - 1.64	1.13 – 1.80
CFU(10⁶)	16 - 540	116 – 770
Activity/OD₆₀₀	3.12 - 49.39	42.70 - 110.80
Activity/CFU	0.10 - 3.01	0.19-0.91

Based on the results summarized in Table IV.6, the growth characteristics of cells were very different and unstable in the presence of higher ampicillin concentrations. The activity per viable cell was closer to each other at high antibiotic concentrations than other induced experiments. The previous results showed that induction of BLIP production in a growth medium with 100 µM/ml ampicillin concentration led to a very important decrease of 35 fold in β-lactamase activity per viable cell as compared to the control set. However, induction of BLIP production in a growth medium with 200 µM/ml ampicillin concentration led to a about 6.8 fold increase in β-lactamase activity per viable cell when compared to the control. Thus an increased ampicillin concentration could have enhanced the production of β-lactamase. As a result of the increased amount of β-lactamase in the periplasm, amount of uninhibited β-lactamase also increased. The total β-lactamase activities both of the cells were higher in the presence of 200 µM/ml ampicillin than in the presence of 100 µM/ml ampicillin.

IV.7. *IN-VITRO* β -LACTAMASE INHIBITION BY BLIP

In an effort to investigate the *in-vitro* inhibitory potential of BLIP, β -lactamase and BLIP each expressed in different cell cultures were used. *E. coli* BL21(DE3) cells harboring pUC18 plasmid were used to produce β -lactamase and *E. coli* BL21(DE3) (pET-26SJ) cells were used to produce BLIP. On the other hand, in order to investigate whether additional BLIP decreased β -lactamase activity further, induced *E. coli* BL21(DE3) (pUC18 + pET-26SJ) cells were used to produce β -lactamase and BLIP simultaneously.

The periplasmic extract containing BLIP and the periplasmic extract containing β -lactamase were mixed based on their protein concentrations because the amount of β -lactamase protein in a periplasmic protein extract is unknown and it is assumed that its ratio in the total protein extract remains relatively constant. The amount of total protein and the β -lactamase protein may vary in each periplasmic extract. Under these conditions, the amount of protein in the first sample was taken as the standard in further experiments.

During incubation of the reaction mixture, β -lactamase could lose activity due to the effect of incubation conditions such as temperature except inhibition effect of BLIP. The activity of β -lactamase was measured before and after incubation to determine whether its activity changed during incubation. Phosphate buffer-periplasmic extract mixture was incubated at 30 rpm and 25 °C for one hour.

Table IV.7. Effect of incubation on β -lactamase Activity (U/L)

Expression System for β -lactamase production	β -lactamase Activity(U/L)	
	before incubation	after incubation
<i>E. coli</i> BL21(DE3) pUC18	205	198
<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ (uninduced)	70	65

The results in Table IV.7 showed that incubation conditions did not cause a considerable effect on activity. Average decrease in β -lactamase activity was 5.3 per cent after incubation.

The protein concentration of a periplasmic protein extract containing β -lactamase was set as 7.8 mg in each reaction medium. The periplasmic extract containing BLIP was used at 7.8 mg and 31.2 mg protein concentrations. The mixture was incubated at 30 rpm and 25 °C for one hour.

Table IV.8. *in-vitro* β -lactamase inhibition in the presence of BLIP

Expression System for β -lactamase and BLIP production	Total Periplasmic Protein in Reaction Mixture (mg)		β -lactamase Activity(U/L)	Inhibition (%)
	β -lactamase	BLIP		
<i>E. coli</i> BL21(DE3) pUC18 (β -lactamase) <i>E. coli</i> BL21(DE3) pET-26SJ (BLIP)	7.8	-	1186	-
	7.8	7.8	365	69
	7.8	31.2	120	90
<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ (β -lactamase and BLIP) <i>E. coli</i> BL21(DE3) pET-26SJ (BLIP)	7.8	-	71	-
	7.8	7.8	37	48
	7.8	31.2	no activity	100

Based on the results summarized in Table IV.8, the incubation of equal amounts of periplasmic protein extract containing BLIP with periplasmic protein extract containing β -lactamase resulted in a 69 % decrease in the activity of β -lactamase obtained from *E. coli* (pUC18) cells. Even though the induced pUC18 pET-26SJ construct had a low activity because of *in-vivo* BLIP inhibition, the activity decrease 48 per cent with additional BLIP.

The same experiments were repeated with a 4-fold higher BLIP concentration (31.2 mg). As expected, the inhibition increased to 90 per cent for pUC18 construct in the presence of higher concentration of BLIP. Similarly, uninhibited β -lactamase produced by the induced pUC18 pET-26SJ construct was totally inhibited in higher BLIP concentration.

IV.8. *IN-VIVO* β -LACTAMASE INHIBITION BY PEPTIDES

Peptides derived from BLIP have been shown to inhibit β -lactamase. These small molecules are very effective to use in medical applications with their easy uptake. For this purpose, the inhibition potential of peptides was investigated.

Investigation of *in-vivo* β -lactamase inhibition by peptides was performed by incubating actively growing cell culture with peptides and then measuring viable cell.

IV.8.1. Analysis of Growth Profiles

In order to investigate the *in-vivo* β -lactamase inhibition by different peptides, *E. coli* K12 (pUC18) cells were used since M9 medium was able to support their growth. According to the procedure explained in Section III.2.15, the cells were grown in M9 minimal medium which composed of only salts and minerals, in addition to the basic carbon and nitrogen sources for growth. Therefore uptake of the peptides was expected to be triggered under these conditions. After binding, the cells were expected to lose their resistance to ampicillin and die.

The growth profile of *E. coli* K12 cells harboring plasmid pUC18 was determined by absence of peptides which was shown in Figure IV.10.

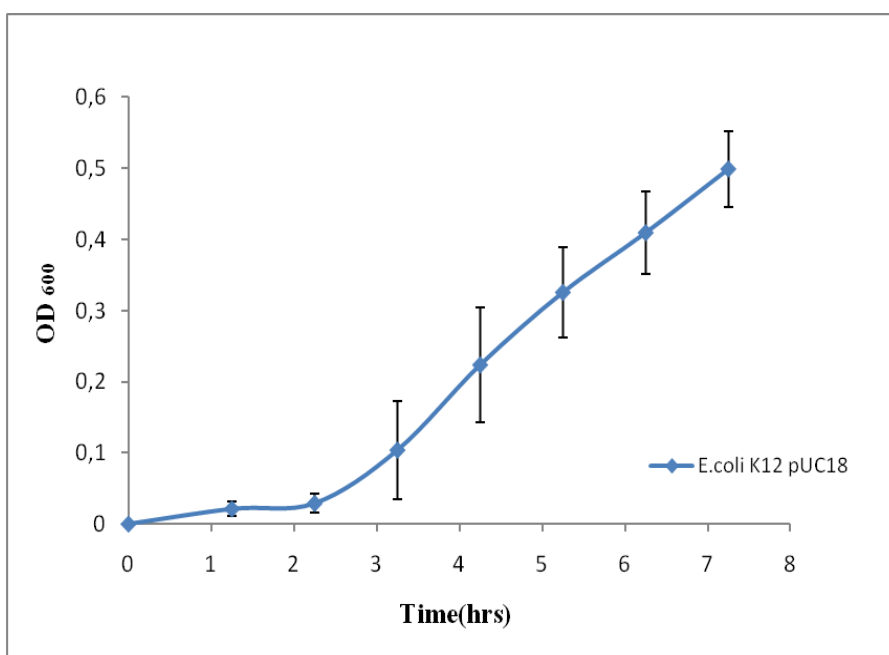


Figure IV.10. Growth profile of *E. coli* K12 pUC18 cells in M9 Medium

β -lactamase inhibition was tested with 100 μ M concentrations of the three different peptides containing the residues A45-Y53 of BLIP which is known to be most effective for binding. B45-53 is a peptide containing residues A45-Y53 of BLIP without an insert. B45-53-fluor was labelled with fluoresceine to

track cells in fluorescence microscopy applications. Biotin is an essential vitamin for many microorganisms and they prefer to uptake from cell culture to synthesis.

Table IV.9. Peptides used in *in-vivo* β -lactamase Inhibition

Peptides	
B45-53	NH-HA AGD YYA Y-CONH ₂
B45-53- fluor	5(6)-Fluoresceine-NH-LL IIL HAA GDY YAY- CONH ₂
B45-53-biotin	Biotin-NH-HA AGD YYA Y-CONH ₂

The cells were incubated with 100 μ M B45-53 peptide and their growth profile monitored every hour at OD₆₀₀ nm. The cells, which were not incubated with B45-53, were used as a control set.

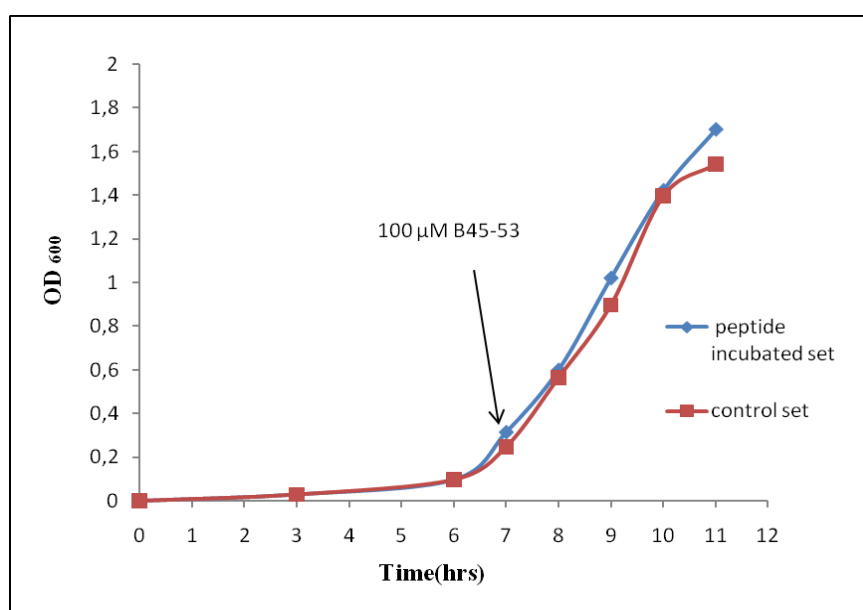


Figure IV.11. Growth profile of *E. coli* K12 pUC18 cells: in the presence of B45-53 peptide control set (■), incubated set with B45-53 peptide (◆).

According to the results given in Figure IV.11, B45-53 peptide had no effect on the growth profile of *E. coli* K12 cells harboring plasmid pUC18. Similarity of both of the growth profiles may be due to low concentration of peptide. On the other hand, the peptides could not have been taken up and/or broken down outside the periplasm by the cells to use as protein sources.

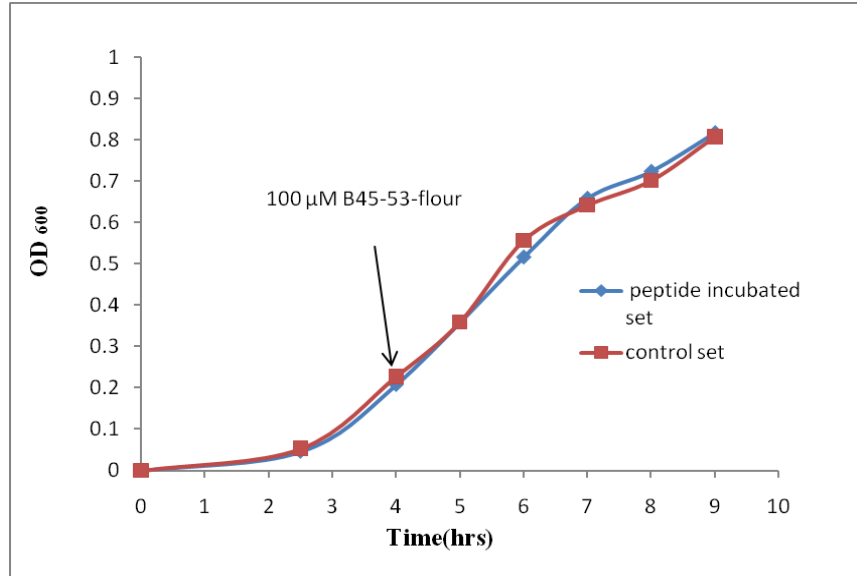


Figure IV.12. Growth profile of *E. coli* K12 (pUC18) cells: in the presence of B45-53- flour peptide control set (■), incubated set with B45-53- biotin peptide (◆)

In-vivo β -lactamase inhibition was also performed with B45-53-fluor containing fluorescein. In cellular biology, the isothiocyanate derivative of fluorescein is often used to label and track cells in fluorescence microscopy applications. As Figure IV.12 indicates, no differences between the control and data set was seen. The cells could have used up the fluorescein labeled peptide as a protein source.

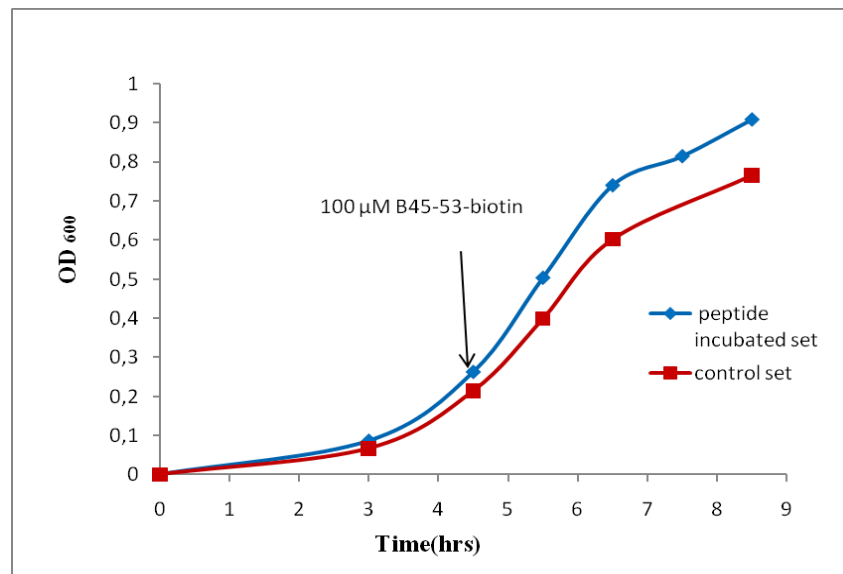


Figure IV.13. Growth profile of *E. coli* K12 (pUC18) cells in the presence of B45-53- biotin peptide: control set (■), incubated set with B45-53- biotin peptide (◆)

The biotinylated B45-53-biotin peptide was used to investigate the uptake and inhibition effect of biotinylated peptides. Biotin is an essential vitamin for many microorganisms and they prefer to taken up from cell culture for synthesis. The main reason in using biotinylated peptide was to force the cells to take the peptide up using the biotin uptake mechanism. The results in Figure IV.13 showed that incubation with B45-53-biotin caused only a slight difference between two set of experiment. The cells may break down biotinylated peptide and use only biotin.

In order to consider the effect of only biotin on the growth profile of *E.coli* K12 (pUC18) cells, they were grown in a medium containing 100 μ M biotin. The results were shown in Figure IV.14. The growth profiles of the control and data set was similar.

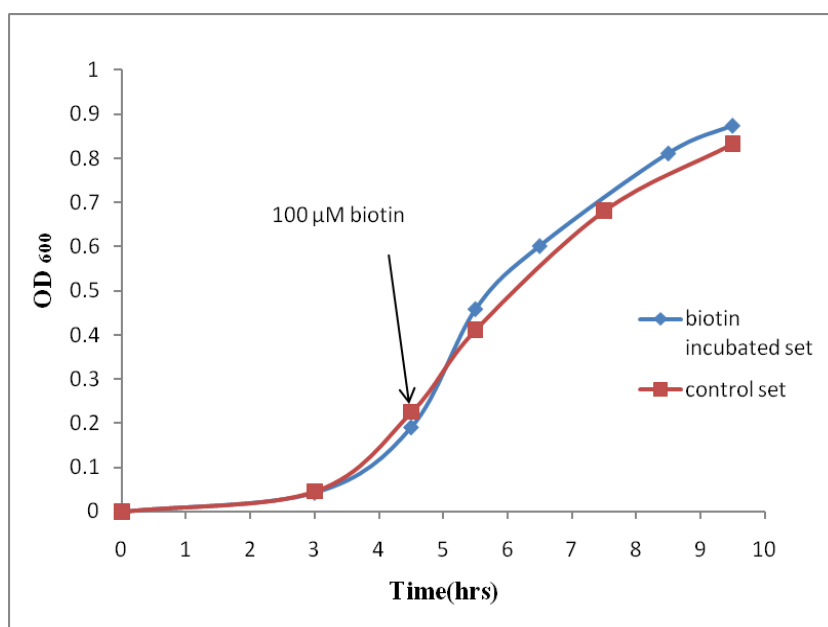


Figure IV.14. Growth profile of *E. coli* K12 (pUC18) cells in the presence of biotin: control set (■), incubated set with biotin (◆)

No significant inhibition in growth has been observed when cells were treated with inhibitory peptides. In these experiments, it could not be distinguished if the peptides bound to β -lactamase at all.

IV.8.2. Viable Cell Count

Measurement of growth by monitoring the optical density of growing cultures in the presence of peptides did not give sufficient and clear result. In order to differentiate between dead cells and live cells, viable cell counts were performed. As a result of this, it could be possible to see whether peptide addition reduces β -lactamase resistance and causes cell death.

E. coli K12 pUC18 cells were grown in both LB and M9 medium in order to investigate the inhibition potential of B45-53 peptide. After 100 μ M peptide addition, the cells were grown for 4 hours and plated to investigate viable cells every hour.

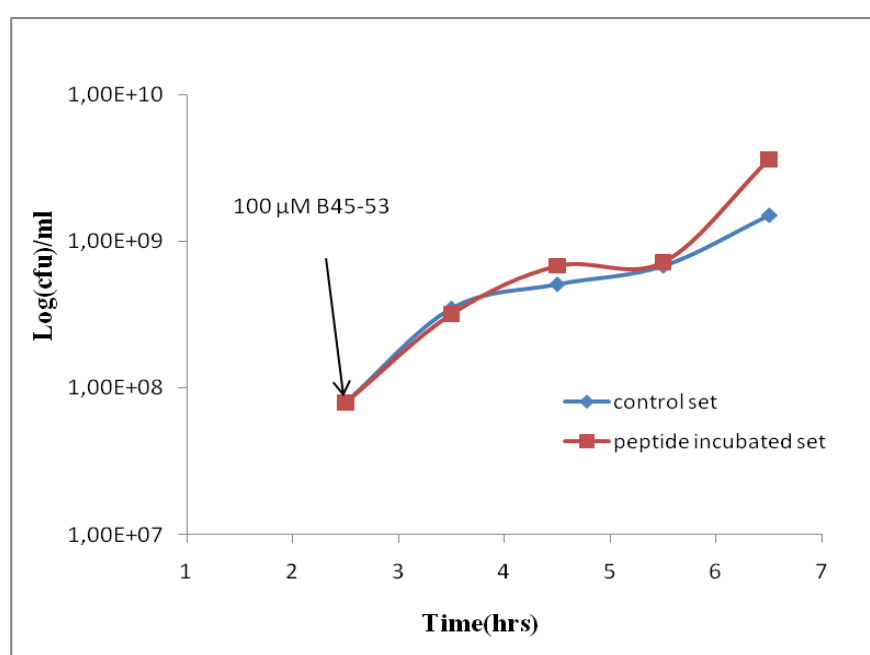


Figure IV.15. Cell viability profiles of *E. coli* K12 (pUC18) cells incubated with 100 μ M B45-53 peptide in LB medium: control set(◆), incubated set with B45-53 peptide (■)

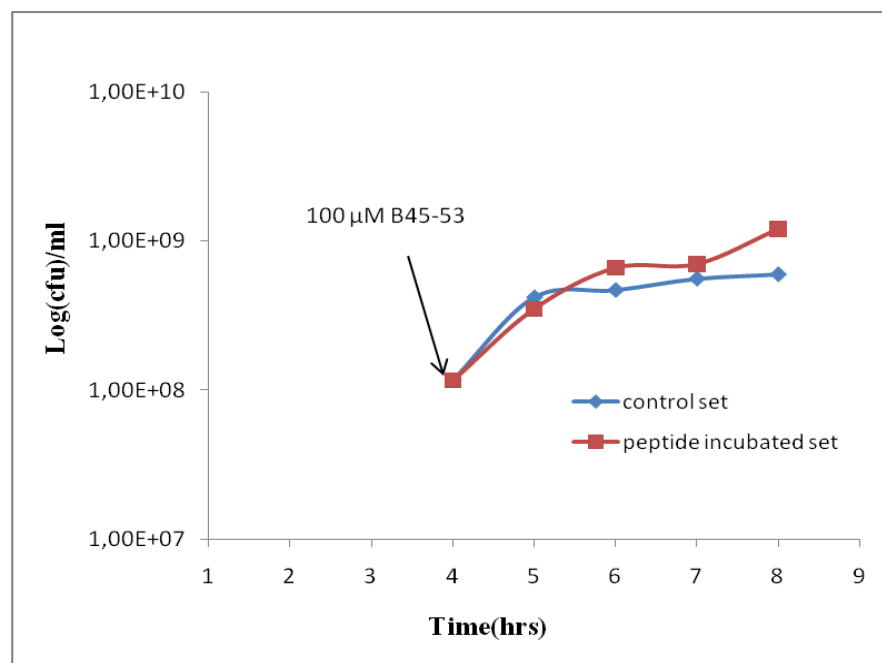


Figure IV.16. Cell viability profiles of *E. coli* K12 (pUC18) cells vector incubated with 100 μ M B45-53 peptide in M9 medium: control set (◆), incubated set with B45-53 peptide (■)

As Figure IV.15 and IV.16 indicate, cell viability profiles of the cells incubated with peptide did not have a significant change after peptide addition in both LB and M9 medium. A slight difference was observed between viable cells of the incubated set with peptide and control set after 2 hours addition of peptide. The result of viable cell counts and growth profiles were similar to each other. *In-vivo* inhibition potential of peptides was not observed.

IV.9. *IN-VITRO* β -LACTAMASE INHIBITION BY PEPTIDES

The studies on β -lactamase inhibition by peptides showed that peptides could not inhibit cell growth. Is this because the peptides could not be taken up or could not be effective in binding and inhibiting β -lactamase found in the periplasm of the cells. To differentiate these possibilities, the inhibition potential of peptides under *in-vitro* conditions was investigated.

In order to investigate *in-vitro* inhibitor effect of peptides, enzyme activity measurements were performed with periplasmic protein extract containing β -lactamase from uninduced *E. coli* BL21(DE3) (pUC18 and pET-26SJ) cells. Two

different peptides, B45-53 and B45-53-biotin, were used at concentrations of 50, 75, 100, 150, and 200 μM . According to the procedure explained in Section III.2.16, the reaction mixture was incubated at 25°C and 30 rpm for one hour in the presence of phosphate buffer.

Table IV.10. *in-vitro* β -lactamase inhibition by the B45-53 peptide.

	Activity(U/L)
control	125
50 μM peptide	124
100 μM peptide	129
150 μM peptide	121
200 μM peptide	121

Table IV.11. *in-vitro* β -lactamase inhibition by the B45-53-biotin peptide.

	Activity(U/L)
control	115
50 μM peptide	121
75 μM pepide	128
100 μM peptide	118
150 μM peptide	112

As Table IV.10 and Table IV.11 indicate, increasing concentration of B45-53 and B45-53-biotin had not a considerable effect on β -lactamase activity. The peptides and β -lactamase could not interact *in-vitro* conditions under these assay conditions.

IV.10. *IN-VITRO* COMMERCIAL β -LACTAMASE INHIBITION

Commercial β -lactamase was used to investigate inhibition effect of BLIP and peptides. The amount of β -lactamase protein in a periplasmic protein extracts is not known. Therefore, activity measurements with commercial β -lactamase are important for kinetic calculations.

During incubation of the reaction mixture, commercial β -lactamase could lose its activity because of the effect of incubation conditions such as temperature rather than inhibition effect of BLIP and the peptides tested. Thus, the activity of β -lactamase was measured before and after incubation to determine whether the activity changed during incubation. Phosphate buffer- enzyme mixture was incubated at 30 rpm and 25 °C for one hour.

Table IV.12. Decrease in activity (%) of commercial β -lactamase during incubation

non-incubated TEM-1 β -lactamase activity (U/L)	38
incubated TEM-1 β -lactamase activity (U/L)	20
Decrease in activity (%)	47

The activity of the enzyme decreased 47 per cent during a one hour incubation as shown in Table IV.12. As a result of this, it was clear that phosphate buffer and/or temperature could be cause a considerable decrease in enzyme activity.

Activity measurements were performed with different β -lactamase concentrations; 0.002, 0.003 and 0.005 μ M. The enzyme was not incubated in these experiments.

Table IV.13. Activities of different β -lactamase concentrations.

Concentration of TEM-1 β -lactamase in reaction mixture (μ M)	0.002	0.003	0.005	0.01
Activity (U/L)	35	38	44	53

As expected, the activity measurements of different β -lactamase concentrations showed that the activity increased with an increasing amount of enzyme as expected. On the other hand, the activity values were too low compared to osmotic shock extract. As Table IV.8 indicates, the activity of the enzyme in 100 μ L of periplasmic extract of *E. coli* BL21 (DE3) harboring pUC18 plasmid was 1186 U/L. The activity of 100 μ L of commercial enzyme was only 53 U/L which was lower than 95 percent than osmotic shock extract.

In order to investigate the inhibition effect of B45-53 peptide and BLIP on commercial β -lactamase, the enzyme was incubated with B45-53 and BLIP at 30 rpm and 25 °C for one hour in the presence of phosphate buffer. The activity results and concentrations of enzyme, BLIP and the peptide are given in Table IV.14. The inhibition effect was seen in slope of lines in Figure IV.17. The initial slope of the absorbance measurements is used to calculate the β -lactamase activity using the Equation III.1.

Table IV.14. Commercial β -lactamase inhibition by BLIP and B45-53 peptide

Concentration of TEM-1 β -lactamase in reaction mixture(μ M)	Concentration of B45-53 peptide in reaction mixture(μ M)	Protein Concentration of BLIP in reaction mixture(μ g/ μ L)	Activity (U/L)
0.003	-	-	22
0.003	0.2	-	37
0.01	-	-	25
0.01	0.4	-	27
0.01	-	0.0078	5

As Table IV.14 indicates, the enzyme was not inhibited by 0.2 and 0.4 μ M concentrations of peptide compared with the activity results of absence of the peptide. However, 0.0078 μ g/ μ L (7.8 μ g in total reaction medium) concentration of BLIP caused 80 per cent inhibition of 0.01 μ M of the enzyme. The results were also similar with *in-vitro* inhibition measurements of the enzyme in periplasmic extracts. Table IV.5 showed that the inhibition of β -lactamase in the periplasmic extract with 7.8 μ g BLIP was 69 per cent.

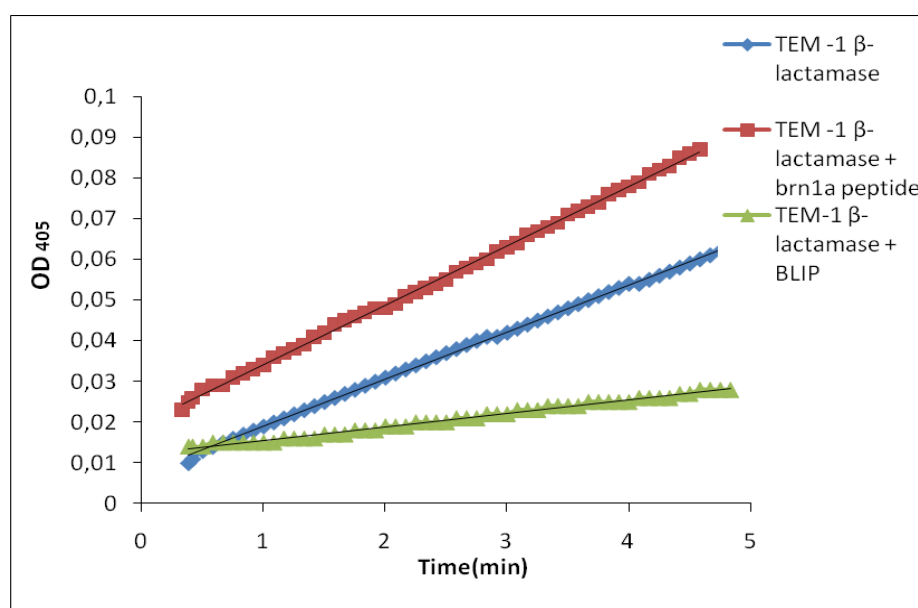


Figure IV.17. Centa hydrolysis by TEM-1 β -lactamase (◆), TEM-1 β -lactamase - B45-53 peptide mixture(■) and TEM-1 β -lactamase - BLIP mixture (▲)

CHAPTER V

CONCLUDING REMARKS and RECOMMENDATIONS

V.1. CONCLUDING REMARKS

In this study, *in-vitro* and *in-vivo* methods have been used to investigate β -lactamase inhibition by BLIP and BLIP derived peptides. Inhibition has been investigated by examining the growth characteristics of recombinant cells, colony forming units, SDS-PAGE and Native-PAGE analysis.

In-vivo binding experiments have been performed with *E. coli* BL21(DE3) cells harboring both pUC18 and pET-26SJ vectors for simultaneous expression of R-TEM-1 β -lactamase and BLIP. In the presence of BLIP, the activity of β -lactamase in the periplasm decreased to 96 and 95 per cent respectively after 1.5 and 3 hours incubation period.

On the other hand, the change in β -lactamase activity per viable cell was investigated and a data range was obtained. Induction of BLIP production led to a 35 fold decrease in β -lactamase activity per viable cell. The results suggested that BLIP efficiently inhibits R-TEM-1 β -lactamase in the periplasm of the cells.

The presence of BLIP and BLIP- β -lactamase complexes in the periplasmic fraction were verified by SDS-PAGE and Native-PAGE analysis, respectively. In order to verify the presence of β -lactamase protein in Native-PAGE gels, an activity measurement was performed with gel bands.

On the other hand, *in-vivo* enzyme inhibition by BLIP derived peptides was performed with *E. coli* K12 cells harboring pUC18 plasmid, but inhibition potential of peptides was not observed in both LB and M9 medium. *In-vitro* activity measurements were performed with both β -lactamase in the periplasm as well as commercial β -lactamase. While BLIP inhibited the enzymes respectively 69 and 80 per cent, inhibition property of peptides was not observed. The results suggested that BLIP efficiently inhibits R-TEM-1 β -lactamase in *in-vitro* conditions, but BLIP derived peptides do not.

V.2. RECOMMENDATIONS

The periplasmic extract contains different proteins, metabolites and enzymes. The presence of these molecules may prevent the BLIP- β -lactamase interaction. As a result of this, the pure β -lactamase should be used to investigate *in-vitro* inhibition potential of BLIP derived peptides.

Different BLIP based peptides should be investigated to find R-TEM-1 β -lactamase inhibitors with high inhibition potential. Mutations can be used on these new peptides to elucidate the contribution of these residues to β -lactamase binding. The kinetic parameters for *in-vitro* BLIP based peptides - β -lactamase interaction also should be applied to characterize their kinetics *in-vitro*.

After the kinetic parameters for *in-vitro* BLIP based peptides - β -lactamase interaction is established, these can be the initial step for *in-vivo* inhibition of β -lactamase. The penetration of the peptides into the periplasm should be investigated by fluorescence microscope.

The presence of BLIP in cellular fractions should also be confirmed by Western blot analysis.

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APPENDIX A

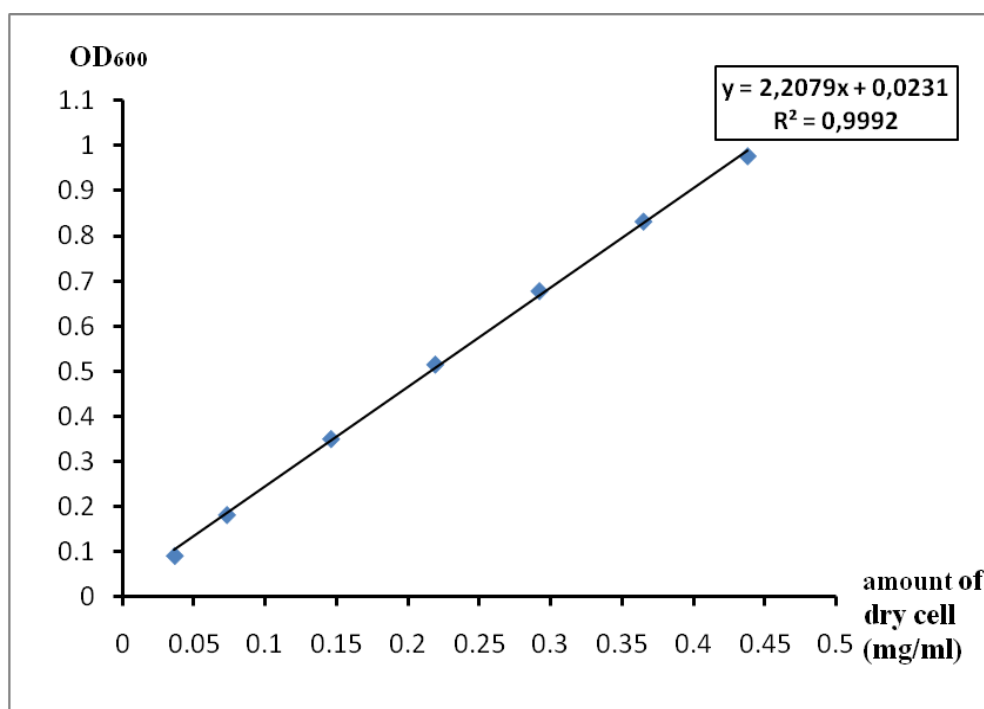


Figure A.1. Correlation factor of dry weights to optical densities

APPENDIX B

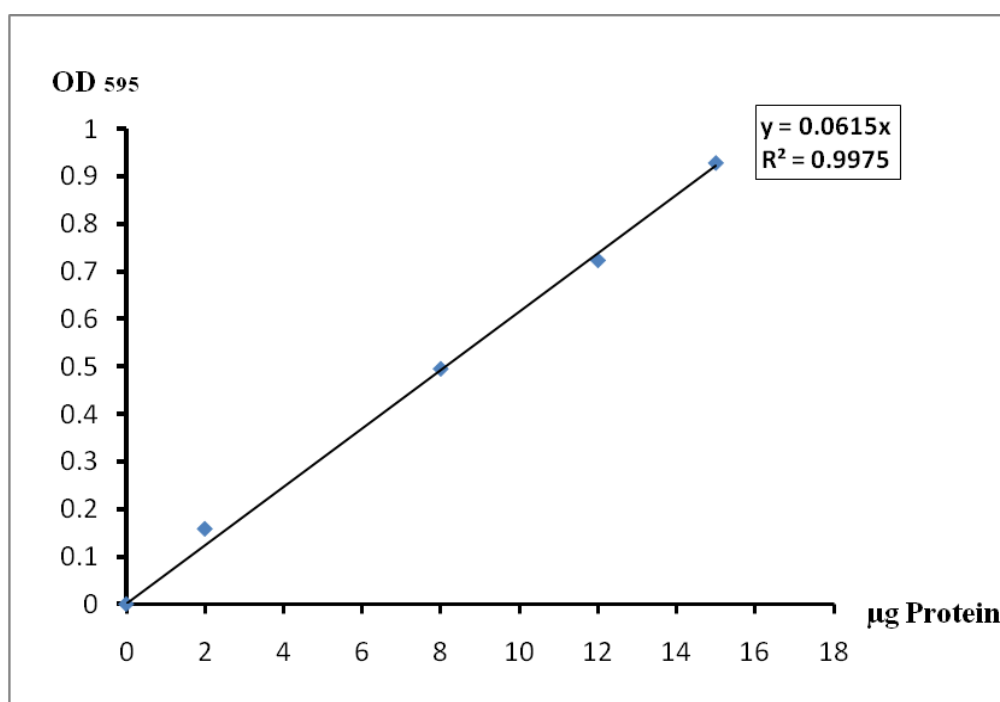


Figure B.1. BSA calibration curve

APPENDIX C

Table C.1. Datas of uninduced experiments

	Uninduced Experiments					
	Experiment 2		Experiment 3		Experiment 4	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26
Activity(U/L)	47.49	45.79	27.80	18.02	143.75	37.10
Harvestion OD	0.44	0.43	0.49	0.43	0.52	0.5220
CFU(10 ⁶)	62	30	83	23	135	29
Activity/OD	107.02	107.29	56.83	41.72	276.76	71.08
Activity/CFU	0.77	1.54	0.33	0.78	1.06	1.28

Table C.2. Datas of induced experiments

	Induced Experiments			
	Experiment 1		Experiment 2	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26
Activity(U/L)	4.35	63.74	2.41	27.47
Induction OD	0.51	0.48	0.46	0.49
Harvestion OD	1.72	1.70	1.46	1.62
CFU(10 ⁶)	73	37	62	16
Activity/OD	2.53	37.52	1.65	17.01
Activity/CFU	0.06	1.72	0.04	1.72

APPENDIX D

Table D.1. Datas of induced experiments in medium containing high concentration of antibiotic.

	Experiment1		Experiment2		Experiment3	
	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26	<i>E. coli</i> BL21(DE3) pUC18 pET-26SJ	<i>E. coli</i> BL21(DE3) pUC18 pET-26
Activity(U/L)	79.82	145.74	48.24	125.20	5.12	76.86
Induction OD	0.55	0.45	0.53	0.44	0.61	0.42
Harvesting OD	1.62	1.64	1.07	1.13	1.64	1.80
CFU(10 ⁶)	540	770	16	137	53	116
Activity/OD	49.40	88.93	45.00	110.80	3.12	42.70
Activity/CFU	0.15	0.19	3.02	0.91	0.10	0.66

AUTOBIOGRAPHY

I graduated from Chemical Engineering Department of Marmara University in 2009. I started master in science at Marmara University Bioengineering Department in 2009.

Projects

- Research of thermal inactivation of *Bacillus licheniformis* BA17 alkaline proteases in the presence of metal ions and research of inactivation kinetics of proteases in organic solvents.”, TUBITAK Project (2209)
- “Investigation of Penetration of Inhibitor Peptide Into Cells and Effects of Intracellular Enzyme with Experimental Methods.”, TUBITAK Project (1001)

Selected Posters

- Gizem Buldum, Burcu Bulak, Berna Sarıyar Akbulut (2009): “Research of thermal inactivation of *Bacillus licheniformis* BA17 alkaline proteases in the presence of metal ions and research of inactivation kinetics of proteases in organic solvents”, XXI. National Biochemistry Congress, Istanbul.
- Gizem Buldum, Kadriye Simay Yalaz, Özkırımlı Ölmez, Berna Sarıyar Akbulut(2011): “Investigation of *in vivo* inhibition potential of β -lactamase and BLIP”, 36th FEBS Congress, Italy.