TEM-1 β-LACTAMASE ACTIVITY IN THE PRESENCE OF BLIP IN CELLULAR ENVIRONMENTS

by

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To my family...

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ABSTRACT

TEM-1 β-LACTAMASE ACTIVITY IN THE PRESENCE OF BLIP IN CELLULAR ENVIRONMENTS

β-lactams are an important class of antibiotics used in the treatment of various infections. These antibiotics are the most widely utilized by virtue of their high efficacy, low cost and safety profile. This widespread, heavy and sometimes irresponsible usage exerts considerable selection for resistance in bacteria. The most prevalent bacterial resistance mechanism to the β-lactam antibiotics is the production of β-lactamases. β-lactamases hydrolyze the amide bond of the four-membered β-lactam ring of the antibiotic rendering it inactive. Therefore, β-lactamase inhibitors emerge as an important research field to restore the therapeutic value of β-lactam antibiotics. *Streptomyces clavuligerus* produces a proteinaceous β-lactamase inhibitor (BLIP), which is a 165 amino acid protein. It inhibits several class A β-lactamases such as TEM-1 and SHV-1 β-lactamase with different affinities.

In this study, *in-vitro* and *in-vivo* methods have been used to investigate β-lactamase inhibiton by BLIP. Periplasmic expression of BLIP has been achieved using two expression systems with different leader sequences, the native BLIP leader sequence and the *pelB* leader sequence. pUC18 vector was used for the expression of R-TEM-1 β-lactamase in *E. coli* BL21(DE3) cells. *In-vivo* binding experiments have been performed with cells expressing both BLIP and R-TEM-1 β-lactamase. Inhibition has been investigated by examining the growth characteristics of recombinant cells, colony forming units, SDS-PAGE analysis and measuring β-lactamase activity. In the presence of BLIP with the native leader sequence total β-lactamase activity dropped from 16450 U/L to 984 U/L. The reduction in β-lactamase activity was 40.9 per cent in the presence of BLIP with the native leader sequence while it was 62.4 per cent in the presence of BLIP with *pelB* leader sequence. Increasing the amount of BLIP to 531 μg and 325 μg, respectively resulted in 98 per cent decline in the activity.

ÖZET

HÜCRE ORTAMINDA BLIP VARLIĞINDA TEM-1 β-LAKTAMAZ AKTİVİTESİ

β-laktam antibiyotikleri çeşitli enfeksiyonların tedavisinde kullanılan önemli bir antibiyotik türüdür. β-laktam antibiyotikleri yüksek etkinlik, düşük maliyet ve güvenlik profili gibi avantajlarından dolayı en yaygın kullanılan antibiyotiklerdir. Bu antibiyotiklerin yaygın, aşırı ve bazen sorumsuz kullanımı bakterilerin direnç geliştirmesine sebep olmaktadır. Bakterilerde β-laktam antibiyotiklerine karşı görülen en yaygın direnç türü β-laktamaz enzimlerinin üretimidir. β-laktamazlar antibiyotiklerin β-laktam halkasındaki amid bağını hidroliz ederek antibiyotiği etkisiz hale getirirler. Bu nedenle β-laktamaz inhibitörleri, β-laktam antibiyotiklerinin tedavi edici etkisini geri kazandırmak amacıyla önemli bir araştırma konusu haline gelmektedir. *Streptomyces clavuligerus,* 165 aminoasitten oluşan protein yapılı bir β-laktamaz inhibitör proteini (BLIP) üretmektedir. Bu inhibitör proteini TEM-1 ve SHV-1 gibi birçok A sınıfı β-laktamaz'ı değişik afinitelerle inhibe etmektedir.

Bu çalışmada, *in-vitro* ve *in-vivo* metotlar kullanılarak β-laktamaz'ın BLIP tarafından inhibisyonu incelenmiştir. BLIP'in periplazmada üretilmesi için farklı sinyal dizilerine sahip iki üretim sistemi kullanılmıştır: orijinal sinyal dizisini içeren BLIP ve *pelB* sinyal dizisi içeren BLIP. pUC18 vektörü *E. coli* BL21(DE3) hücrelerinde R-TEM-1 β-laktamaz üretimi için kullanılmıştır. *In-vivo* bağlanma deneyleri BLIP ve R-TEM-1 β-laktamazı birlikte üreten hücrelerle gerçekleştirilmiştir. İnhibisyon, rekombinant hücrelerin büyüme özellikleri, koloni oluşturan hücreler, SDS-PAGE analizi ve *in-vitro* β-laktamaz aktivitesi çalışmaları ile incelenmiştir. Orijinal sinyal dizisine sahip BLIP'in varlığında, toplam β-laktamaz aktivitesi 16450 U/L değerinden 984 U/L değerine düşmüştür. β-laktamaz aktivitesindeki düşüş orijinal sinyal dizisi içeren BLIP varlığında yüzde 40.9 iken *pelB* sinyal dizisine sahip BLIP varlığında 62.4'tür. BLIP miktarı sırasıyla 531 μg ve 325 μg'a arttırıldığında aktivitedeki düşüş yüzde 98 değerine ulaşmıştır.

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LIST OF SYMBOLS / ABBREVATIONS

K_i	Inhibition constant
µg	Microgram
µL	Microliter
µM	Micromolar
APS	Ammonium persulfate
BLIP	β-lactamase inhibitor protein
bp	Base pair
CFU	Colony forming unit
EDTA	Ethylenediaminetetraacetic acid
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
L	Liter
LB	Luria-bertani broth
M	Molar
mg	Miligram
mL	Mililiter
mM	Milimolar
MW	Molecular weight
nm	Nanometer
nM	Nanomolar
OD	Optical density
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N,N-tetramethylethylendiamine
U	Unit

1. INTRODUCTION

Medical science witnessed one of the most significant developments in its history with the advent of numerous classes of antibiotics in 1930s. Among these, β-lactam antibiotics have been widely used due to their comparatively high effectiveness, minimal side effects, low cost and ease of delivery (Gretes *et al.*, 2009; Wilke *et al.*, 2005; Doran *et al.*, 1990). They account approximately 50% of world antibiotic consumption (Essack, 2001; Poole, 2004).

β-lactam antibiotics target transpeptidase enzymes which are responsible for the synthesis of the bacterial cell wall and kill bacteria (Wang *et al*., 2007; Wilke *et al.*, 2005; Kang *et al.*, 2000). The irresponsible and extensive use of these antibiotics has contributed to the emergence of widespread antibiotic resistant bacteria (Maiti *et al.*, 2006; Majududdin *et al.*, 2002). The bacterial resistance against antibiotics is a growing concern in the treatment of infectious diseases (Schroeder *et al.*, 2002; Palumbi *et al.*, 2001).

The most common mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamase enzymes. These enzymes degrade the β-lactam antibiotics by hydrolyzing the amide bond of the four-membered β-lactam ring thus rendering the antibiotic ineffective. The TEM-1 β-lactamase is the most widely distributed, accounting for 70–80 pre cent of plasmid-mediated β-lactamases (Mroczkowska and Barlow, 2008; Maiti *et al.*, 2006). An approach that has been utilized to cope with bacterial resistance to β-lactam antibiotics is the use of small molecule β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Petrosino *et al.*, 1999; Rudgers and Palzkill, 2001). β-lactamases often exhibit high affinity to these inhibitors, are irreversibly bound and finally are inactivated by them (Handal and Olsen, 2000). These inhibitors do not have antimicrobial activity themselves; however, they are used in combination with various β-lactam antibiotics. They bind to β-lactamases and prevent the enzyme from hydrolyzing the antibiotic. This in turn repairs the therapeutic value of the antimicrobial agent. Nevertheless, variants of β-lactamases have evolved that are no longer sensitive to these inhibitors while still maintaining the ability to hydrolyze β-lactam antibiotics (Rudgers and Palzkill, 2001).

The inhibitor clavulanic acid was initially purified from the bacterium *Streptomyces clavuligerus*, which also produces a protein inhibitor of β-lactamases called β-lactamase inhibitory protein, BLIP (Doran *et al.*, 1990). BLIP is a 165 amino acid protein composed of two domains. It binds TEM-1 β-lactamase with wide range affinities (K_i = 0.1–0.6 nM) (Yuan *et al.*, 2009, Gretes *et al.*, 2009). BLIP also inhibits β-lactamases from both gram-positive and gram-negative bacteria (Strynadka *et al.*, 1994; Petrosino *et al.*, 1999; Rudgers and Palzkill, 1999). Therefore, small peptides mimicking the binding and inhibition activity of BLIP would aid the development of novel β-lactamase inhibitors and antibiotics (Rudgers and Palzkill, 2001).

In previous studies, it has been 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 is to investigate *in-vivo* and *in-vitro* binding of BLIP to R-TEM-1 β-lactamase. pET-26b(+) vector carrying the BLIP gene with either the native or the *pelB* leader sequence was used for periplasmic BLIP expression in *E. coli* BL21(DE3) cells. These cells were also transformed with pUC18 plasmid vector for simultaneous expression of BLIP and β-lactamase for *in-vivo* inhibition studies. The growth characteristics, colony forming units and protein analysis of the cells harboring both two plasmid vectors and plasmid vectors individually were studied.

2. BIOLOGICAL BACKGROUND

2.1. β-lactam Antibiotics and β-lactamase Mediated Resistance

Antibiotics are important therapeutic agents used in the treatment of a variety of infections caused by Gram-negative and Gram-positive bacteria. β-lactams are the most important group of antibiotics. Penicillins, monobactams, carbapenems and cephalosporins (Figure 2.1) are all considered β-lactam antibiotics, which of all are characterized by a four-membered β-lactam ring (Essack, 2001; Poole, 2004).

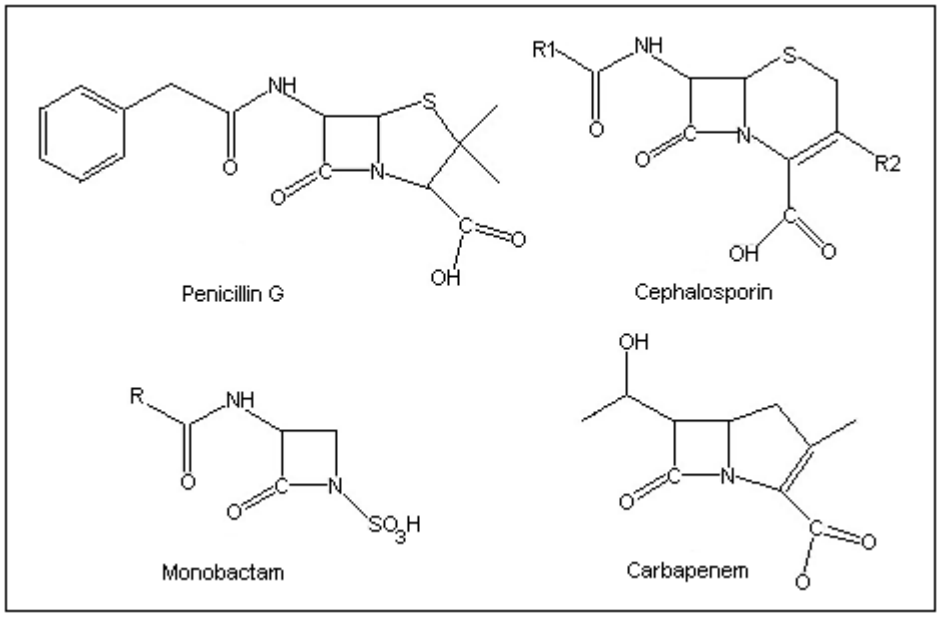

Figure 2.1. Chemical structures of β-lactam antibiotics (Handal and Olsen, 2000)

β-lactam antibiotics were discovered by Fleming 80 years ago (Fleming, 1929) and since then β-lactam antibiotics are the most widely utilized of all different antimicrobial agents that they account for approximately 50% of world antibiotic consumption (Livermore, 1998; Essack, 2001; Poole, 2004). β-lactam antibiotics are of great importance owing to their comparatively high effectiveness, minimal side effects, low cost and ease of delivery (Wilke *et al.*, 2005; Doran *et al.*, 1990).

β-lactam antibiotics interfere in a special way with the biosynthesis of the cell wall (peptidoglycan layer). The cell wall is an essential element in maintaining the shape and rigidity of the cell (Handal and Olsen, 2000). This cell wall is comprised of a basic repeating unit of an alternating disaccharide, *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM). The individual peptidoglycan units are produced in the cytoplasm while their final cross-linking takes place on the external surface of the cytoplasmic membrane and the reaction is catalyzed by the cell wall transpeptidases which are bound to the membrane. The cell wall transpeptidases, known as the penicillin-binding proteins (PBPs), are responsible for synthesis and remodeling of the peptidoglycan. They are serine active-site enzymes and perform their catalytic cycle according to an acylation/deacylation mechanism. β-lactam antibiotics acylate the active-site serine residue of PBPs forming rather stable covalent non-catalytic acyl-enzymes. This results in formation of non-functional peptidoglycan layer and, eventually, cell death. The β-lactam antibiotics act as steric analogs and are recognized as natural substrates by the PBPs (Wilke *et al.*, 2005; Handal and Olsen, 2000; Tipper and Strominger, 1965).

The irresponsible and heavily use of β-lactam antibiotics have caused the bacteria to develop sophisticated resistance mechanisms against them (Maiti *et al.*, 2006). The emergence of antibiotic resistant bacteria is a serious problem to the antibiotic therapy and β-lactams, the most efficient antibiotics against pathogenic bacteria, are threatened by this problem (Doran *et al.*, 1990; Rudgers *et al.*, 2001).

There are three types of primary resistance mechanisms utilized by bacteria to protect themselves. The most common bacterial resistance to β-lactam antibiotics is the production of β-lactamases. β-lactamases are hydrolytic enzymes that efficiently catalyze the irreversibly hydrolysis of the amide bond of the β-lactam ring resulting in biologically inactive products. Another mechanism is the modifications in PBPs. PBPs are the targets of β-lactam antibiotics and any mutation may lead to less susceptible PBPs preventing them to acylate or inactivate by antibiotics. Alteration of the permeability or efflux action, which prevents the access of β-lactam antibiotics to the PBPs, is the third mechanism (Essack, 2001; Maiti *et al.*, 2006).

2.2. β-lactamases and TEM-1 β-lactamase

The β-lactamases are hydrolytic enzymes degrading β-lactam antibiotics by hydrolysis of the amide bond of the four-membered β-lactam ring (Figure 2.2), thus conferring resistance to their host organism (Wilke *et al.*, 2005). There are four classes of β-lactamases according to Ambler classification which is based on primary sequence homology. Class A, C and D β-lactamases are serine active site enzymes while class B β-lactamases require zinc ions for catalytic activity (Yuan *et al.*, 2009; Wilke *et al.*, 2005; Majiduddin *et al.*, 2002). The number of different β-lactamases which was reported as around 200 in 2003 (Page *et al.*, 2003) have increased to 530 in three years period (Babic *et al.*, 2006).

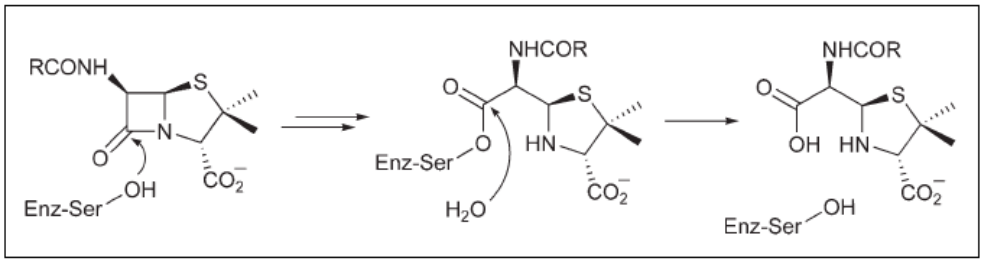

Figure 2.2. The hydrolysis of acyl-enzyme complex catalysed by a serine β-lactamase (Kershaw *et al.*, 2005)

The spread of β-lactamase genes have been aided by their integration with mobile genetic elements such as plasmids or transposons, which is also known as plasmid-mediated or plasmid-borne resistance. The expression of β-lactamases in the cell can be either constitutive or inducible. Once expressed, β-lactamases are secreted into the periplasmic space in gram-negative bacteria while they are either bound to the cytoplasmic membrane or excreted into the medium in gram-positive bacteria (Wilke *et al.*, 2005; Rudgers *et al.*, 2001; Rudgers and Palzkill, 1999).

TEM-1 is the most common plasmid-mediated β-lactamase encountered in gram-negative bacteria and has significantly contributed to resistance against β-lactam antibiotics. The TEM-1 β-lactamase confers the bacteria resistance to penicillins (Yuan *et al.*, 2009; Mroczkowska and Barlow, 2008; Majiduddin *et al.*, 2002; Williams, 1999).

The TEM-1 β-lactamase was first described in 1965 by Datta and Kontomichalou in an ampicillin-resistant strain of *E. coli* isolated from the blood culture of a young Greek girl named Temoniera (Maiti *et al.*, 2006; Jacoby, 2006). It has been the most prevalent plasmid-mediated β-lactamase ever since.

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). The amino acid sequence of TEM-1 β-lactamase is given in Figure 2.3.

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

Figure 2.3. The sequence of TEM-1 β-lactamase (Reynolds *et al.*, 2006)

Plasmid mediated TEM-1 β-lactamase was produced utilizing pUC18 vector. The pUC18 vector possesses the 861 bp long $bla_{\text{TEM-1}}$ gene which confers resistance to ampicillin (Figure 2.4). TEM-1 is naturally found in the periplasm of gram-negative bacteria such as *E. coli* (Lim *et al.*, 2001).

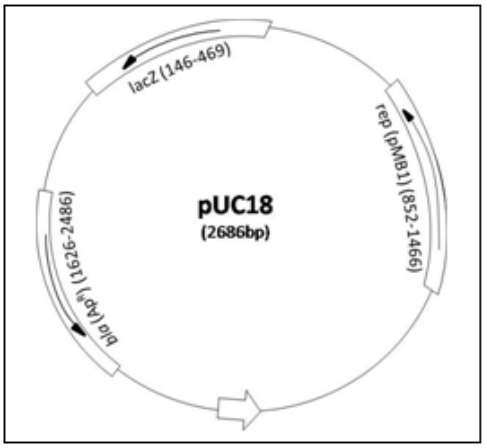

Figure 2.4. The pUC18 vector map

With the aim of overcoming resistance to β-lactam antibiotics mediated by β-lactamases, new β-lactam antibiotics were introduced to the market in 1980s, which are called third generation cephalosporins. However, with each new class that has been used to treat patients, new β-lactamases have emerged that caused resistance to the new class of drug. Presumably, the selective pressure of the use and overuse of new antibiotics in the treatment of infections has selected for new variants of β-lactamases. Thus the newer third generation cephalosporins were challenged by an unexpected set of mutational events shortly after their introduction into clinic use (Maiti *et al.*, 2006).

One response to the continuing problem of β-lactam resistance in pathogenic bacteria is to develop entirely new drugs, exhibiting great affinity for PBPs and are unable to be cleaved, or poorly cleaved by β-lactamases. However, these new drugs could not be efficient due to the emergence of new TEM mutants resistant to the agents. Another strategy that has been employed to combat antimicrobial resistance is the administration of β-lactamase inhibitors. The use of an inhibitor along with an existing β-lactam antibiotic is an effective means to treat various β-lactamase producing bacterial pathogens (Petrosino *et al.*, 1999).

2.3. Significance of β-lactamase Inhibitors

The use of β-lactamase inhibitors in combination with penicillins and cephalosporins started in the late 1980s. This strategy has been effective for three decades. There are three clinically important β-lactamase inhibitors: clavulanic acid, sulbactam and tazobactam (Figure 2.5). β-lactamases often exhibit a high affinity for these compounds, become irreversibly bound, and are thereby inactivated (Handal and Olsen, 2000).

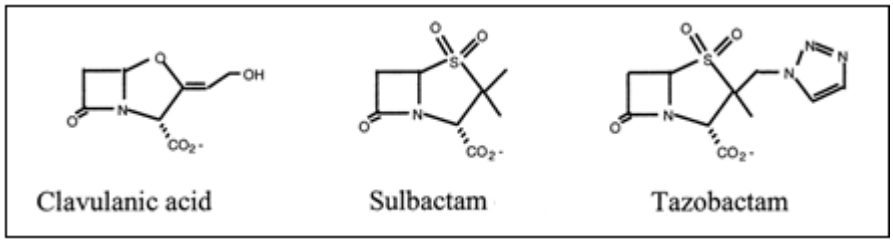

Figure 2.5. Chemical structures of β-lactamase inhibitors in clinical use

In recent years, however, β-lactam/ β-lactamase inhibitor combination resistant bacteria have evolved. The resistance is due to mutations in TEM-1 that do not react efficiently with the small molecule inhibitors but retain the ability to hydrolyze β-lactam antibiotics (Huang et al., 2003).

A protein β-lactamase inhibitor (BLIP) was described in the culture supernatants of *Streptomyces clavuligerus*. It is important in terms of being the first protein-based inhibitor (Doran *et al.,* 1990).

2.4. β-lactamase Inhibitory Protein (BLIP)

The *bli* gene was isolated from the genomic DNA of *Streptomyces clavuligerus* and the mature BLIP was first characterized by Doran *et al.* in 1990. BLIP is a 165 amino acid protein (Figure 2.6) with the molecular weight of 17.523 kDa, which calculated from the gene sequence. The *bli* gene also includes an N-terminal 36-amino-acid native leader sequence of *S. clavuligerus* presumably important for translocation of the protein (Doran *et al.,* 1990). The crystal structure of BLIP was determined by Strynadka *et al.* in 1994.

BLIP	MRTVGIGAGVRRLGRAVVMAAAVGGLVLGSAGASNAAGVMTGAKFTQIQF	50
BLIP	GMTRQQVLDIAGAENCETGGSFGDSIHCRGHAAGDYYAYATFGFTSAAAD	100
BLIP	AKVDSKSQEKLLAPSAPTLTLAKFNQVTVGMTRAQVLATVGQGSCTTWSE	150
BLIP	YYPAYPSTAGVTLSLSCFDVDGYSSTGFYRGSAHLWFTDGVLQGKRQWDLV	201

Figure 2.6. DNA sequence of region encompassing *S.clavuligerus* BLIP gene. The native leader sequence of *S.clavuligerus* is indicated by underlying

BLIP inhibits several class A β-lactamases such as TEM-1 and SHV-1 with a wide range of affinities, from nanomolar to picomolar range (Yuan *et al.*, 2009; Gretes *et al.*, 2009; Zhang and Palzkil, 1999; Strynadka *et al.*, 1994). Strynadka *et al.*(1994) found 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. *In-vitro* binding of BLIP to TEM-1 β-

lactamase was also studied by Albeck and Schreiber (1999) and the related kinetic parameters were determined. The binding affinity was calculated as 0.4 nM which was very similar to that of Strynadka *et al.*(1994) indicating that the activity of the recombinant protein is identical to that of the secreted protein.

Another *in-vitro* β-lactamase inhibition study was conducted with 6xHis-tagged BLIP (Petrosino *et al.*, 1999). Wild type 6xHis-tagged BLIP was found to inhibit TEM-1 β-lactamase with a K_i of 0.11 nM. This result also showed that N-terminal His-tag has no considerable effect on BLIP binding. The results of Rudgers and Palzkill (1999) were favorably comparable to that of Petrosino *et al.* The inhibiton of wild type β-lactamase in the presence of 1 nM BLIP resulted in a K_i of 0.22 nM, which is in good agreement with the previous study. This study also showed that BLIP inhibited several mutant β-lactamases with affinities varying from 0.42 pM to 0.49 nM (Rudgers and Palzkill, 1999). Although several studies indicate that BLIP is a potent inhibitor of several class A β-lactamases, it does not inhibit class B, C and D β-lactamases (Strynadka *et al.*, 1994).

2.5. *In-vitro* β-lactamase – BLIP Interaction

Chromogenic substrates have been widely used in kinetic studies. CENTA (Figure 2.4) is a chromogenic cephalosporin structurally resembling nitrocefin, cephaloridine and cephalotin and can be used for the kinetic characterization of β-lactamase. This chromogenic substrate displays a change in color from light yellow (λ maximum 340nm) to chrome yellow (λ maximum 405nm) concomitant with hydrolysis of the β-lactam ring (Jones *et al.*, 1982).

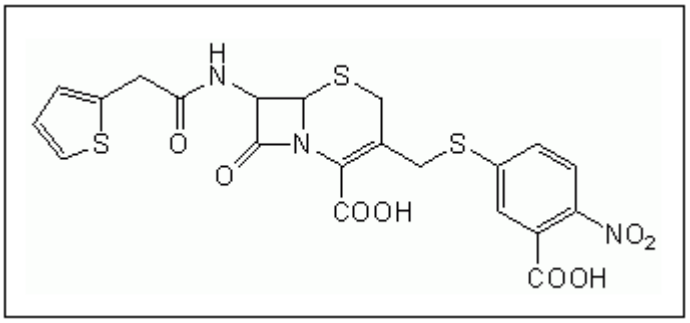

Figure 2.7. The structure of CENTA

CENTA can easily be used not only for the rapid identification of active fractions during β-lactamase purification or for the detection of β-lactamase in bacterial crude extracts or in chromatographic fractions during enzyme purification but also in high-throughput screening tests for the selection of new β-lactamase inactivators (Bebrone *et al.,* 2001). The kinetic parameters resulting from the interactions between TEM-1 β-lactamase and CENTA were given in Table 2.4 (Bebrone *et al.*, 2001).

Table 2.1. The kinetic parameters for TEM-1 β-lactamase and CENTA

Enzyme	CENTA (mM)	Enzyme (nM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m($\mu M^{-1}s^{-1}$)
TEM-1	0.025	11.5	110	70	1.6

2.6. Recent Studies on Inhibition of β-lactamase by BLIP and Peptides

The emergence of antibiotic resistant bacteria brings about the need for novel classes of antibiotics and inhibitors. In this respect many studies focusing on β-lactam resistance in both gram-negative and gram-positive bacteria were conducted.

A strategy that has been employed to combat antibiotic resistance is to use β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Rudgers and Palzkill, 1999). These small molecules do not have antimicrobial activity, however, they are used with various β-lactam antibiotics to bind β-lactamase and prevent the hydrolysis of the antibiotic thus repairing the therapeutic value of the antibiotic. Unfortunately, clinical isolates that are no longer sensitive to these inhibitors were reported (Rudgers *et al.,* 2001; Thai *et al.,* 2001).

TEM-1 β-lactamase is strongly inhibited by BLIP. The determinations of crystal structures of TEM-1 – BLIP complex, BLIP only and TEM-1 individually (Strynadka et al., 1996; Strynadka et al., 1994; Strynadka et al., 1992) enabled to study different aspects of protein-protein interactions between TEM-1 β-lactamase and its novel inhibitor BLIP. These studies provided substantial information about the important residues of each protein (Albeck and Schreiber, 1999).

The identification of the residues of BLIP responsible for binding and inhibition will make the design of tighter and smaller inhibitors for β-lactamase possible (Petrosino *et al.,* 1999). These residues were utilized to propose small peptides imitating the binding and inhibition activity of BLIP. It was shown that TEM-1 β-lactamase was inhibited by peptides derived from BLIP with different *Ki* values changing from 446 μM to 0.01 nM (Rudgers and Palzkill, 2001; Rudgers *et al.,* 2001; Huang *et al.,* 2000).

Huang et al. (2003) found and optimized a linear 6-mer peptide with the sequence Arg-Arg-Gly-His-Tyr-Tyr using phage display method. This peptide which is 50% identical with a type II β-turn sequence of BLIP was found to inhibit TEM-1 β-lactamase with a Ki of 136 μM. This peptide was also shown to inhibit different classes of β-lactamases (Huang *et al.,* 2003).

Another study reports the inhibition potential of peptides based on the 46 to 51 region of BLIP. The cyclic peptide with cysteine residues at both ends inhibits TEM-1 with a *Ki* of 603 μM. This cyclic peptide was then reduced to see the effect of the disulfide bond and *Ki* was found to be 488 μM. Another peptide having residues 41 to 50 of BLIP was constructed. This peptide bound to TEM-1 β-lactamase with a *Ki* of 359 μM. (Rudgers *et al.,* 2001).

Like phage display method, the two-hybrid system is used for the high-throughput screening of protein interactions. A study using yeast two-hybrid system was conducted by Sun *et al.* (2005). Two peptides were shown to inhibit TEM-1 β-lactamase *in-vivo* while *in-vitro* studies indicated that only one of these peptides having nucleotide sequence of TTCACTATCCACTGCAGTGTCACTGCTGCAGGTGACTACTACTGTGTTCATGGC GCTAATGGCACCTCTTTCTAG inhibited TEM-1 β-lactamase (Sun *et al.,* 2005).

The *in-vitro* and *in-vivo* binding of BLIP to β-lactamase will be an important starting point for the design and development of new peptide based drugs that could inhibit β-lactamase. It is the common target for antibiotic resistance in both gram-negative and gram-positive bacteria.

3. MATERIALS

3.1. Bacterial Strains and Plasmids

In this study, pET-26b(+) vector was used for periplasmic expression of BLIP and pET-28a(+) vector was used for cytoplasmic expression of BLIP. These vectors are highly efficient for expression under the control of the strong T7 promoter (Novagen, 2006). The experiments were performed with the *E. coli* BL21(DE3) strain since this strain can be used with protein expression vectors that are under the control of the T7 promoter, such as pET vectors.

The expression of BLIP was achieved using two different expression systems in pET-26b(+) vector with different leader sequences: the first one with the *pelB* leader sequence of the pET-26b(+) and the second one with the native BLIP leader sequence.

E. coli XL1, *E. coli* TB1 cells and pUC18 plasmid vector were from our laboratory stock. *E. coli* BL21(DE3) cells were obtained from TÜBİTAK. pET-26b(+) plasmid vector was purchased from Novagen. pET-26b(+) vector carrying the BLIP gene with native leader sequence was a generous gift from Susan Jensen (University of Alberta) whereas pET-26b(+) vector carrying the BLIP gene with *pelB* leader sequence and pET-28a(+) vector carrying the BLIP gene used for cytoplasmic expression were from a previous study by Ezgi Akkaya.

3.2. Chemicals and Enzymes

All the chemicals and the solutions used in this study were purchased from APPLICHEM (Germany), MERCK (Germany), MOLEKULA (Germany) or SIGMA (USA). Restriction endonucleases were from New England Biolabs (USA), Promega (USA) or Fermentas (USA). DNA ladders and protein molecular weight markers were purchased from Fermentas (USA).

3.3. Laboratory Equipments

Laboratory equipments used in this study and functions are shown in Table 3.1.

Table 3.1. List of laboratory equipments

Purpose	Equipment
Absorbance Measurement	DU 640 Spectrophotometer (Beckman, USA) Specord 200 (Analytikjena, UK)
Agarose Gel Electrophoresis	Mini-Sub Cell GT (Biorad, USA)
Centrifugation	1-15 centrifuge (SIGMA, Germany) 2-16PK centrifuge (SIGMA, Germany)
Deepfreezer	Ultra Low Temperature Freezer U410 Premium (New Brunswick Scientific, USA)
Incubation	FN500 Incubator (Nüve, Turkey)
Orbital Shaker	ZHWY-211B Shaker Incubator (ZHICHENG) Innova 4340 (New Brunswick Scientific, USA)
Sterilization	Autoclave (ALP, Japan)
pH Measurement	pH meter (SCHOTT, Germany)
Pipetting	1-10, 10-100, 100-1000 µl pipettes (Thermo Electron Corporation, CANADA)
Polyacrylamide Gel Electrophoresis	Mini-PROTEAN® 3 Cell (Biorad, USA)
Power Supply	Power EC250-90 (Thermo Electron Corporation, CANADA)
Refrigerating	Refrigerator (Frigidaire, USA)
Sonification	Branson Sonifier 450 (VWR Scientific, USA)
Sterile Environment	LaminAir HBB 2460 (Holten, Denmark) MN 120 (Nüve, Turkey)
UV Monitor	UV Lamb (Biolab, USA)
Vortexing	Reax Top Vortex (Heidolph, Germany)
Water Purification Systems	MILLI-Q UF Plus (MILLIPORE, USA)
Weighing	Balance XB 220A (Precisa, Switzerland)

3.4. Growth Media

Luria Broth (LB) medium and LB-agar medium were used as growth media for Escherichia coli throughout the study.

Table 3.2. LB medium

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Add dH_2O up to 1 liter	

Table 3.3. LB-agar medium

Chemical	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Add dH_2O up to 1 liter	

3.5. Buffers and Solutions

3.5.1. Agarose Gel Electrophoresis Buffers and Solutions

The buffers and solutions used for preparing agarose gel and running agarose gel electrophoresis are shown below.

Table 3.4. 0.5M EDTA stock solution (pH 8.0)

Chemical	Amount
Na_2EDTA	18.61 g
Adjust pH to 8.0 with NaOH and add dH_2O up to 100 ml	

Table 3.5. 5X TBE buffer

Chemical	Amount
Tris base	27 g
Boric acid	13.75 g
0.5M EDTA stock solution (pH 8.0)	10 ml
Add dH_2O up to 500 ml	

Table 3.6. Agarose gel electrophoresis running buffer (0.5X TBE)

Chemical	Amount
5X TBE buffer	50 ml
Add dH_2O up to 500 ml	

Table 3.7. Ethidium bromide

Chemical	Amount
Ethidium bromide	10 mg/ml

3.5.2. Polyacrylamide Gel Electrophoresis Buffers and Solutions

The buffers and solutions used for preparing sodium dodecyl sulfate (SDS) polyacrylamide gel and running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are shown below.

Table 3.8. Electrophoresis seperating gel buffer (1.5M Tris, pH 8.8)

Chemical	Amount
Tris	18.15 g
Adjust pH to 8.8 with HCl and add dH_2O up to 100 ml	

Table 3.9. Electrophoresis stacking gel buffer (0.5M Tris, pH 6.8)

Chemical	Amount
Tris	3 g
Adjust pH to 6.8 with HCl and add dH_2O up to 100 ml	

Table 3.10. Acrylamide-bisacrylamide solution (30:0.8)

Chemical	Amount
Acrylamide	29.2 g
Bisacrylamide	0.8 g
Add dH_2O up to 100 ml	

Table 3.11. 10% SDS

Chemical	Amount
SDS	10 g
Add dH_2O up to 100 ml	

Table 3.12. Electrophoresis 12% seperating gel (% w/v, pH 8.8)

Chemical	Amount
Acrylamide bisacrylamide solution (30:0.8)	4 ml
Electrophoresis separating gel buffer	2.5 ml
dH_2O	3.5 ml
10% SDS	100 µl
10% APS	50 µl
TEMED	5 µl

Table 3.13. 10% APS

Chemical	Amount
APS	0.5 g
Add dH_2O up to 5 ml	

Table 3.14. Electrophoresis 5% stacking gel (% w/v, pH 6.8)

Chemical	Amount
Acrylamide bisacrylamide solution (30:0.8)	0.67 ml
Electrophoresis stacking gel buffer	1 ml
dH_2O	2.5 ml
10% SDS	42 µl
10% APS	30 µl
TEMED	5 µl

Table 3.15. 2X Sample buffer

Chemical	Amount
Electrophoresis separating gel buffer	2.5 ml
10% SDS	4 ml
Glycerol	2 ml
2-Mercaptoethanol	1 ml
Bromophenol blue	0.02 g
Add dH_2O up to 10 ml, store at -20°C	

Table 3.16. 10X Electrophoresis running buffer

Chemical	Amount
Tris	30.3 g
Glycine	144 g
SDS	10 g
Add dH_2O up to 1 liter	

Table 3.17. Gel fixing solution

Chemical	Amount
Ethanol	50%
Phosphoric acid	2%

Table 3.18. Gel washing solution (Solution 2)

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

Table 3.19. Gel staining solution

Chemical	Amount
Methanol	34%
Ammonium sulfate	17%

Table 3.19. Gel staining solution (continued)

Phosphoric acid	2%
Coomasie Brilliant Blue – G250	0.066%

3.5.3. Enzyme Activity Measurement Buffers and Solutions

3.5.3.1. Preparation of Substrate Solution: 25 mg CENTA was dissolved in 10 ml 50 mM K^+PO_4 buffer and aliquoted and stored at -20°C. The concentration of the stock solution was 4.7 mM.

Table 3.20. K^+PO_4 buffer (1M, pH 7.0)

Chemical	Amount
1M K_2HPO_4	450 ml
1M KH_2PO_4	550 ml

3.5.4. Other Stock Solutions

The stock solutions of antibiotics (ampicillin and kanamycin) and IPTG are prepared as shown in Table 3.21.

Table 3.21. Other stock solutions

Chemical	Amount
Ampicillin	100 mg/ml
Kanamycin	50 mg/ml
1M IPTG	23.8302 g/10 ml

3.6. Kits

Genopure Plasmid Midi Kit and High Pure Plasmid Isolation Kit were purchased from Roche.

4. METHODS

In all experiments, sterile equipment was used. Glassware, tips, tubes, solutions and media used in the study were sterilized in an autoclave at 1.02 atm and 121°C for 15 minutes prior to use and kept in containers or bottles sealed with autoclave tape. Antibiotics and IPTG were filter sterilized using a membrane with a pore size of 45μm. Experimental work required sterile environment was carried out under laminar flow.

4.1. Preparation of Bacterial Stocks

A single colony from a master plate was inoculated with a sterile tip into fresh LB medium supplemented with appropriate antibiotic and grown overnight at 37°C and 180 rpm. 300μl grown cells were added to sterile centrifuge tubes containing sterile 30 per cent glycerol and stored at -80°C.

4.2. Preparation of Preculture

10 ml sterile LB medium was supplemented with appropriate antibiotic, if necessary and inoculated with 50 μl frozen stock culture. Preculture was incubated at 37°C and 180 rpm overnight, approximately 12-16 hours.

4.3. Culture Conditions

Cells were grown in orbital shakers at 37°C and 180 rpm. Culture volume was adjusted to one fifth of the flask volume to provide efficient aeration. LB medium was used as media in all experiments. Media were supplemented with 50 μg/ml kanamycin for the selection of pET-26b(+) harboring cells and 100 μg/ml ampicillin for the selection of pUC18 harboring cells. The expression of the recombinant protein was easily and efficiently achieved by inducing the promoter with IPTG (isopropyl β-D-1-thiogalactopyranoside). IPTG was added to the culture to give a final concentration of 0.5 mM or 1 mM. The induction was performed when OD_{600} reached the value of 0.5 per ml of culture.

4.4. Quantitation of Growth

Growth of *E. coli* cultures was determined by measuring optical densities of the cells at 600 nm using a spectrophotometer. LB medium was used as the blank and it was also used to dilute the samples to keep the spectroscopic readings within reliable limits which are between 0.2 and 0.6. Preculture of *E. coli* cells were inoculated into fresh LB medium at a 1:100 dilution. 0.1-1 ml samples were taken at the desired time intervals to monitor the cell growth.

4.5. Viable Cell Count

Bacterial samples were plated to determine the number of viable cells. For a ten fold dilution, 500 µl of the bacterial sample taken at the desired time interval was transferred into a glass tube containing 4.5 ml of fresh liquid medium and vortexed to homogenize. For another ten-fold dilution, 500 µl from this mixture was taken and placed in a new glass tube containing 4.5 ml fresh liquid medium. This process continued until the desired dilution was obtained. Finally, 1 ml of the appropriate dilution was deposited in a sterile Petri dish, in triplicate, and agar containing appropriate antibiotic were poured into Petri dishes. The sample and the liquid agar were 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.

4.6. Preparation of Plasmid pET-26b(+) – BLIP gene with Native Leader Sequence

The plasmid DNA was obtained as lyophilized powder with an amount of 1 µg. It was dissolved in 50 µl sterile distilled water to give a final concentration of 20 ng/µl. pET-26b(+) vector possessing the BLIP gene was constructed in such a manner that it contained the native leader sequence of *Streptomyces clavuligerus*, which transports BLIP to the periplasm of the cell.

4.7. Plasmid DNA Isolation

Roche Genopure Plasmid Midi Kit and Roche High Pure Plasmid Isolation Kit were used for plasmid DNA isolation operations. Isolation processes were performed according to the instructions of the manufacturer.

4.7.1. Midi-Prep Plasmid DNA Isolation

30 ml *E. coli* cells from an overnight grown culture were centrifuged at 5,000 x *g* and 4°C for 10 minutes. The supernatant was discarded and the cell pellet was resuspended gently in 4 ml Suspension Buffer + RNase by pipetting. 4 ml Lysis Buffer was added to the suspension and the new suspension was mixed by inverting the tube 6 to 8 times. The suspension was incubated at +15 to +25°C for 2-3 minutes. 4 ml chilled Neutralization Buffer was added to the suspension. The suspension was mixed by inverting the tube 6 to 8 times and incubated on ice for 5 minutes. The cell lysate was cleared by centrifugation at 12,000 x *g* and 4°C for 30 minutes. After centrifugation, the supernatant was filtered through a filter paper wetted by Equilibration Buffer to remove the white precipitate. The sealing ring was mounted to the column to fix the column into the collection tube. One column was inserted into one collection tube; the column was equilibrated with 2.5 ml Equilibration Buffer and allowed to empty by gravity flow. The flow-through was discarded. The cleared lysate was loaded onto the pre-equilibrated column and allowed to empty by gravity flow. The column was washed with 5 ml Wash Buffer, allowed to empty by gravity flow and the flow-through was discarded. This step was carried out one more time to make sure that all present contaminants were removed. The column and the collection tube were centrifuged together at 15,000 x *g* and 4°C for 10 minutes to remove the Wash Buffer left in the column. The column was inserted into a new collection tube and 5 ml prewarmed (50°C) Elution Buffer was added to the column and allowed to empty by gravity flow. The collected flow-through was treated with 3.6 ml isopropanol to precipitate the plasmid DNA and centrifuged immediately at 15,000 x *g* and 4°C for 30 minutes. The supernatant was discarded and the precipitated plasmid DNA was resuspended with 3 ml chilled 70 per cent ethanol. Resuspension was followed by centrifugation at 15,000 x *g* and 4°C for 10 minutes. Ethanol was removed and the plasmid

DNA pellet was allowed to dry for 10 minutes under the laminar flow. The plasmid DNA pellet was redissolved in 100 µl sterile distilled water and stored at -80°C for further use.

4.7.2. Mini-Prep Plasmid DNA Isolation

1.5 ml overnight grown *E. coli* cells were centrifuged at 6,000 x *g* for 30 seconds. This step was repeated three times using the same centrifuge tube. The supernatant was discarded and the cell pellet was resuspended with 250 µl Suspension Buffer + RNase. 250 µl Lysis Buffer was added to the suspension from previous step and the new one was mixed by inverting the tube 3 to 6 times. The suspension was incubated at +15 to +25°C for 5 minutes. After adding 350 µl chilled Binding Buffer, the tube was inverted 3-6 times and incubated on ice for 5 minutes. In this step, the solution became cloudy and a white precipitate formed. The suspension was centrifuged at 13,000 x *g* for 10 minutes. Afterwards, one high pure filter tube was inserted into one collection tube and all the supernatant from the previous step was transferred into the upper reservoir of the filter tube. The collection tube and the filter tube were centrifuged together at 13,000 x *g* for 1 minute. The flow-through in the collection tube was discarded and the collection tube was re-inserted into the filter tube. 700 µl Wash Buffer II was added to the upper reservoir of the filter tube and the tubes were centrifuged together at 13,000 x *g* for 1 minute. The flow-through was discarded and the filter tube was centrifuged for additional 1 minute to remove the remaining Wash Buffer in the filter. The flow-through and the collection tube were discarded and the filter tube was inserted into a clean, sterile 1.5 ml centrifuge tube. To elute the DNA, 100 µl Elution Buffer was added to the upper reservoir of the filter tube and the tubes were centrifuged at 13,000 x *g* for 1 minute. The flow-through contained the DNA and stored at -80°C.

4.8. Plasmid Transformation into *E. coli* Cells

$CaCl_2$ protocol was used to transform *E. coli* BL21(DE3) cells with either pUC18 plasmid or pET-26b(+) plasmid or both. Preculture of *E. coli* BL21(DE3) cells, grown overnight in LB medium, was used to inoculate fresh LB medium culture at a 1:100 dilution. Cells were grown at 37°C and 180 rpm to facilitate aeration. When optical density of the culture reached the value of 0.6 at 600 nm, 30 ml of the culture was transferred into

a clean, sterile centrifuge tube and allowed to incubate on ice for 10 minutes. After incubation, the cells were centrifuged at 3,000 rpm and 4°C for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 1.2 ml 0.1 M chilled $CaCl_2$. The suspension was incubated on ice for 10 minutes and then centrifuged at 3,000 rpm and 4°C for 10 minutes. The supernatant was discarded and the pellet was allowed to air-dry under laminar flow for 5 minutes. The pellet was resuspended in 1.2 ml 0.1 M chilled $CaCl_2$. 200 µl of suspended cells was transferred into a sterile centrifuge tube and 10 µl plasmid DNA was added to the suspension while other 200 µl of suspended cells without plasmid was used as the control. Both cell suspensions were incubated on ice for 30 minutes. Following a heat shock at 42°C for 45 seconds, the cells were incubated on ice for 2 more minutes. 800 µl of LB medium was added to the cells and the cells were incubated at 37°C for 1 hour. 100 µl of cell suspensions was homogenized and then plated on selective plates containing appropriate antibiotic. The plates were incubated overnight at 37 °C.

4.9. Agarose Gel Electrophoresis

Agarose gel electrophoresis method was used in order to separate DNA molecules according to their size. Agarose powder was dissolved in 0.5X TBE Buffer to obtain a final concentration of 0.7 per cent (w/v) by heating. When the agarose powder was dissolved completely, ethidium bromide was added to the solution to a final concentration of 0.5 µg/ml. Ethidium bromide was utilized to visualize DNA under UV light. The solution was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel cooled and solidified, the comb was removed and the gel was placed into electrophoresis tank filled with 0.5X TBE Buffer. The DNA samples were mixed with loading dye, loaded into the wells and run at 110 V for approximately 75 minutes. The DNA fragments were visualized under UV light.

4.10. Extraction of Periplasmic Proteins

The osmotic shock protocol was described by Nossal *et. al.,* in 1966, to extract the periplasmic proteins of *E. coli* cells. 100 ml sterile LB medium was inoculated with a preculture at a 1:100 dilution and placed in the orbital shaker at 37°C and 180 rpm. The

cells were grown until OD_{600} reached the value of 0.5 units per ml of culture and then the cells were induced with 0.5 mM IPTG. After 3 hours after induction, the cells were harvested by centrifugation at 7,000 rpm and 4°C for 10 minutes. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 2 ml osmotic shock solution containing 20 per cent (w/v) sucrose, 30 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA). The suspension was incubated at room temperature for 20 minutes. Followed by centrifugation at 9,000 rpm and 4°C for 20 minutes, supernatant was discarded and the cell pellet was resuspended in 4 ml 5 mM ice-cold $MgCl_2$ and gently incubated on ice for 20 minutes. The cells were removed from the periplasmic extract by centrifugation at 9,000 rpm and 4°C for 20 minutes. The supernatant contained periplasmic proteins and was stored at -20°C for further use.

4.11. Extraction of Cytoplasmic Proteins

The protocol for the extraction of cytoplasmic proteins of *E. coli* cells was described by Qiagen, the manufacturer of affinity chromatography method used in this study. Grown cells were used to inoculate 100 ml sterile LB medium at a 1:100 dilution and were allowed to grow at 37°C and 180 rpm. The cells were grown until OD_{600} reached the value of 0.5 and then the cells were induced with 0.5 mM IPTG for 2 or 3 hours. The cells were harvested by centrifugation at 7,000 rpm and 4°C for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 4 ml lysis buffer. The cells were sonicated on ice for 5 times for 10 s with 10 s cooling period between each burst. The lysate was centrifuged at 9,000 rpm and 4°C for 30 minutes. The supernatant was transferred into a clean tube and stored at -20°C.

4.12. Determination of Protein Concentration

Bradford *et. al.,* 1976, described a fast and accurate protocol, known as Bradford Protein Assay, to determine the protein concentration of any cell extracts. Bovine Serum Albumin (BSA) was used as the standard for the preparation of the calibration curve. Bradford samples with different BSA concentrations were prepared and the absorbances were measured at 595 nm. A standard curve of absorbance versus micrograms of protein

was plotted using linear regression analysis. This plot was used to determine the protein concentration of cell extracts. The calibration curve obtained is given in Appendix B.

4.13. SDS-PAGE Analysis of Periplasmic Proteins

The purpose of SDS-PAGE is to separate proteins according to only their sizes. For this purpose, osmotic shock 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 per cent SDS polyacrylamide gel and allowed to run at 110 V for approximately 75 minutes. When the electrophoresis was completed, the gels were gently removed from glass plates and placed in fixing solution overnight to fix the proteins into the gel. On the following day, the gels were washed with Solution 2 for an hour. Afterwards, the gels were stained with Coomasie G-250 at least overnight and distilled water was used as the destain. The gels were dried for ease of keeping.

4.14. *In-vitro* Enzyme Activity Measurement

β-lactamase activity was measured in order to determine any *in-vitro* β-lactamase-BLIP interaction. For this purpose, periplasmic protein extract from *E. coli* BL21(DE3) cells harboring pUC18 plasmid were used as the source of β-lactamase and periplasmic protein extracts from *E. coli* BL21(DE3) cells harboring pET-26b(+) plasmid carrying the BLIP gene was used as the source of BLIP. All activity measurements were performed in a total reaction volume of 1 ml. CENTA (Calbiochem) was used as the substrate and in all experiments, 470 μM CENTA was mixed with pre-determined amount of periplasmic protein extract. The hydrolysis of CENTA was observed by monitoring the change in the absorbance at 405 nm. Periplasmic protein extract from cells not harboring pUC18 plasmid was used as control.

One Unit (U) of β-lactamase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at 25°C and pH 7.0 (Equation 4.1):

$$U = \frac{\frac{dA}{dt} \times V_t \times 10^6}{\varepsilon_\lambda \times V_S \times d} \quad (4.1)$$

V_t = Total reaction volume (ml)

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

ε_λ = Extinction coefficient of the substrate (6400 M^{-1} cm^{-1} for CENTA)

V_S = Volume of enzyme (ml)

d = Light path (1 cm)

4.15. Ni-NTA Affinity Chromatograhy

1 ml of the 50 per cent Ni-NTA slurry was added to 4 ml cleared lysate and mixed gently by shaking at 200 rpm at 4°C for 60 min. The lysate–Ni-NTA mixture was loaded into a column with the bottom outlet capped. The bottom cap was removed the column flow-through was collected for SDS-PAGE analysis. The column was washed twice with 4 ml wash buffer containing 20 mM inmidazole; wash fractions were collected. The column was washed twice with 4 ml wash buffer containing 70 mM inmidazole to remove any contaminant proteins. The protein was eluted four times with 0.5 ml elution buffer containing 250 mM imidazole. The eluate fractions were pooled and analyzed on SDS-PAGE.

5. RESULTS AND DISCUSSION

5.1. Expression Systems Used in This Study

pET-26b(+) vector possessing the BLIP gene with the native leader sequence was a generous gift of Susan Jensen (University of Alberta). This construct was called pET-26_{SJ} throughout the thesis. pET-26b(+) vector containing the BLIP gene with *pelB* leader sequence at its N-terminus and pET-28a(+) vector carrying the BLIP gene used for cytoplasmic expression of BLIP were from a previous study by Ayşe Ezgi Akkaya and these constructs were called pET-26_{EA} and pET-28_{EA}, respectively throughout the thesis. pUC18 plasmid was used for the expression of RTEM-1 β-lactamase.

For simultaneous expression of BLIP and β-lactamase and investigate their binding properties in the periplasm of the cell, in addition to pUC18, pET-26_{SJ} or pET-26_{EA} were transformed into *E. coli* BL21(DE3) cells.

5.1.1. Transformation of pET vectors into *E. coli* cells

E. coli XL1 cells were used as host cells for initial cloning of plasmid DNA since this strain has been proven to have high transformation efficiency and good plasmid yield (Tu *et al.,* 2005). pET-26_{SJ} construct was transformed into *E. coli* XL1 cells by $CaCl_2$ method as described in Section 4.8. The recombinant cells transformed with pET-26_{SJ} construct were screened on LB agar plates containing 50μg/ml kanamycin. The cells were grown in liquid LB medium and the recombinant plasmids were isolated according to the protocol described in Section 4.6.1. Isolated plasmids were double digested by *NdeI* and *BamHI* restriction enzymes in order to verify the presence of the BLIP gene in the plasmid. Following digestion, DNA fragments were analyzed on 0.7 per cent agarose gel (Figure 5.1). The digestion of the recombinant plasmid produced two separate DNA fragments with sizes of 5270 bp and 750 bp containing the BLIP gene. After the presence of BLIP containing fragment in pET-26_{SJ} construct was verified, the vector was transformed into *E.coli* BL21(DE3) cells to be used in expression studies.

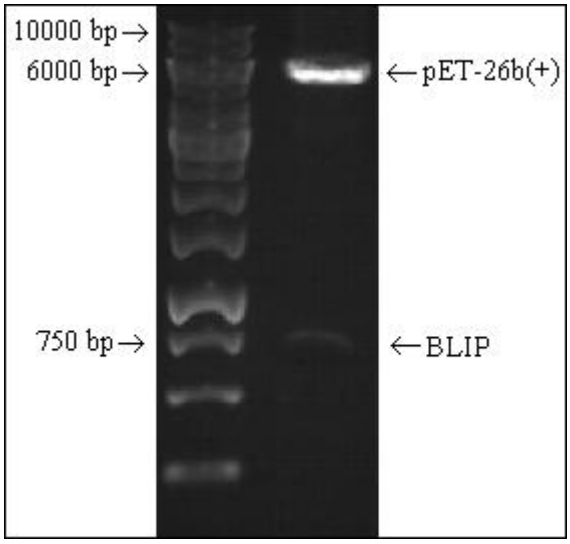

Figure 5.1. Electrophoretic analysis of the double digest of the recombinant plasmid pET-26_{SJ}. Lane 1: Marker, Lane 2: Double digested recombinant plasmid

pET-28_{EA} construct was transformed into *E. coli* BL21(DE3) cells as described in Section 4.8. The cells transformed with pET-28a(+) vector were screened on LB agar plates containing 50µg/ml kanamycin. In order to verify the presence of BLIP gene, the plasmids were isolated according to the manufacturer's protocol described in Section 4.7.1. Isolated plasmids were double digested by *NcoI* and *HindIII* restriction enzymes to verify the presence of the BLIP gene (Figure 5.2).

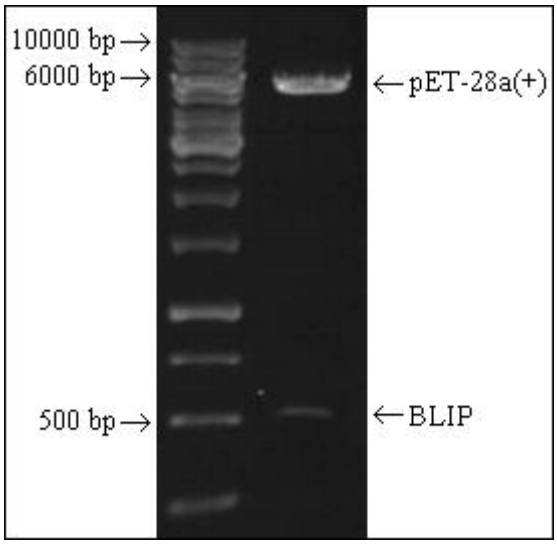

Figure 5.2. Electrophoretic analysis of the double digest of the recombinant plasmid pET-28_{EA}. Lane 1: Marker, Lane 2: Double digested recombinant plasmid

As Figure 5.2 indicates, double digestion of the recombinant plasmid pET-28_{EA} created two DNA fragments of 5246 and 507 bp in length confirming the presence of the BLIP gene. Isolated recombinant plasmids were then transformed into *E. coli* BL21(DE3) cells for expression studies.

5.1.2. Transformation of pUC18 Vector into *E. coli* Cells

In addition to its function as a cloning vector, pUC18 carries the *bla* gene and encodes the protein RTEM-1 β-lactamase. In this study, pUC18 vector was used for β-lactamase expression. To investigate the interaction between β-lactamase and BLIP, pUC18 vector was transformed in both *E. coli* BL21(DE3) cells harboring pET-26_{EA} construct and *E. coli* BL21(DE3) cells harboring pET-26_{SJ} construct. The cells harboring both vectors were selected on LB agar plates, supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin.

5.2. The Effect of Expression of BLIP on Growth Characteristics

5.2.1. Analysis of *E. coli* Growth Curves

In an effort to compare the growth profiles of wild type *E. coli* BL21(DE3) cells and *E. coli* BL21(DE3) cells harboring pUC18 vector, growth curve analysis was performed according to the protocol described in Section 4.4. The optical densities of the samples were monitored at 600 nm and growth profiles of the cells were determined (Figure 5.3). The growth curve for *E. coli* BL21(DE3) cells harboring pUC18 vector was performed in triplicate and the error bars represent the range of values obtained from triplicate samples.

Both wild type cells and cells with pUC18 plasmid entered the exponential phase immediately after inoculation into the culture. During exponential phase, they showed a similar growth pattern with similar maximum specific growth rates, which are 0.579 h^{-1} for wild type *E. coli* BL21(DE3) cells and 0.587 h^{-1} for *E. coli* BL21(DE3) cells harboring pUC18 plasmid. After 13 hours, both cells entered the stationary phase with a maximum OD_{600} value of 3.3 nm per ml of culture.

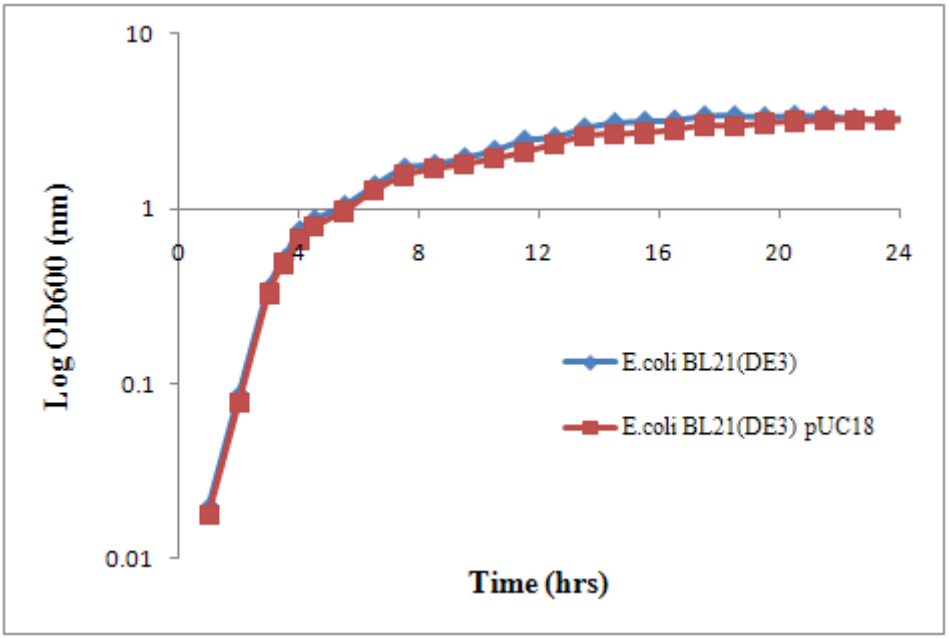

Figure 5.3. Growth profiles of *E. coli* BL21(DE3) cells (◆) and *E. coli* BL21(DE3) cells harboring pUC18 plasmid (■).

In order to investigate the effect of β-lactamase and/or BLIP expression on the cells and to determine whether the presence of BLIP would have an inhibitory effect on β-lactamase, wild type *E. coli* BL21(DE3) cells, *E. coli* BL21(DE3) cells harboring only pUC18 vector, *E. coli* BL21(DE3) cells harboring only pET-26_{SJ} construct and *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct were grown. If coexpression of BLIP and β-lactamase allows these two proteins to interact, β-lactamase was expected to be inhibited by BLIP. This would results in cell death in the presence of ampicillin. β-lactamase production from pUC18 vector is constitutive. BLIP production is under the control of T7 promoter, therefore the cells were induced with 0.5 mM IPTG when OD_{600} of the cell cultures reached 0.5. The optical densities of the samples were monitored at 600 nm and growth profiles of the cells were determined (Figure 5.4). The growth curves for the cells harboring both pUC18 vector and pET-26_{SJ} construct and the cells harboring only pET-26_{SJ} construct were performed in duplicate and the error bars represent the range of values obtained from duplicate experiments.

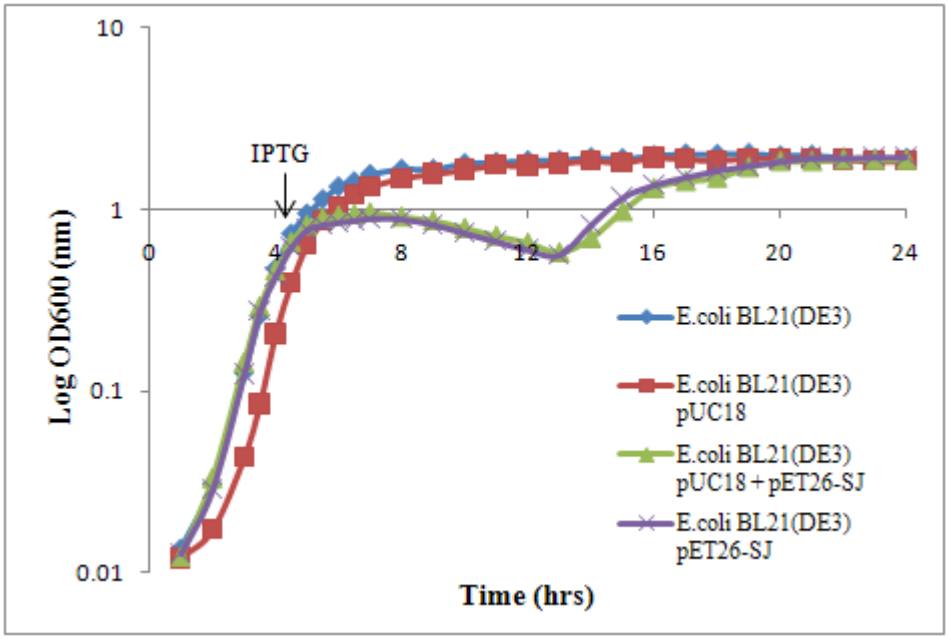

Figure 5.4. Growth profiles of wild type *E. coli* BL21(DE3) cells (◆), *E. coli* BL21(DE3) cells harboring pUC18 vector (■), *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct (▲), *E. coli* BL21(DE3) cells harboring only pET-26_{SJ} construct (✕)

Based on Figure 5.4, all cultures entered exponential growth phase 1 hour following inoculation. During exponential phase, wild type *E. coli* BL21(DE3) cells (◆) and *E. coli* BL21(DE3) cells harboring pUC18 vector (■) showed a similar growth patterns. Growth rates of these cells were calculated as 0.520 h^{-1} for wild type *E. coli* BL21(DE3) cells and 0.522 h^{-1} for *E. coli* BL21(DE3) cells harboring pUC18 vector. The slight difference between the two may result from the metabolic burden caused by the β-lactamase expression. Although the induced cells had two exponential phases during their 24 hour growth profile, growth rates until the time of induction for the cells harboring only pET-26_{SJ} construct (✕) and cells harboring both pUC18 vector and pET-26_{SJ} construct (▲) were calculated to be 0.514 h^{-1} and 0.521 h^{-1}, respectively. From these results, it can be concluded that β-lactamase expression has no significant effect on cell growth.

The cells harboring only pET-26_{SJ} construct (✕) and the cells harboring both vectors (▲) grew until OD_{600} was reached 0.90 and 0.96, respectively and then OD_{600} of the cultures started to decrease. At the end of the 13^{th} hour, OD_{600} of the cells was measured as

0.57 and 0.58, respectively. After 13 hours, the cells began to grow again and at the end of 20 hours, they reached the stationary phase.

E. coli BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct (▲) and cells harboring only pET-26_{SJ} construct (✕) showed almost the same growth pattern. This suggests that induction caused a similar metabolic burden on the cells. On the basis of these results, it can not be claimed that the decrease in the growth of the cells is either inhibition of β-lactamase by BLIP or metabolic burden from BLIP expression.

5.2.2. Viable Cell Count

In an effort to elucidate the reason for the decline in growth upon induction and to determine whether this was due to cell death, the number of colony forming units was determined. *E. coli* BL21(DE3) cells harboring only pUC18 vector, uninduced *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct, induced *E. coli* BL21(DE3) cells harboring only pET-26_{SJ} construct, induced *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct were grown until OD_{600} reached the value of 0.5 and the cells harboring recombinant plasmids were induced with 0.5 mM IPTG for three hours. Samples were taken at specific time intervals and plated according to the protocol described in Section 4.5. Colonies on the plate were counted and plotted against time (Figure 5.5). In Figure 5.5, time "0" indicates inoculation of the cells into the culture. The analysis was performed in duplicate for induced cells harboring both pUC18 vector and pET-26_{SJ} construct and the error bars were used.

The number of viable cells from the culture that was induced and harboring both plasmids (✕) was 1.5 fold higher than that of the culture that was uninduced and harboring both plasmids (■) at the time of induction, although OD_{600} values of the cultures were approximately the same (0.5850 and 0.5947, respectively). One hour after induction, the CFU of the induced cells decreased by 99.93 per cent. This suggested that induction affected the cells immediately. However, the CFU of the uninduced control (■) showed a 2.5 fold increase in the same time interval.

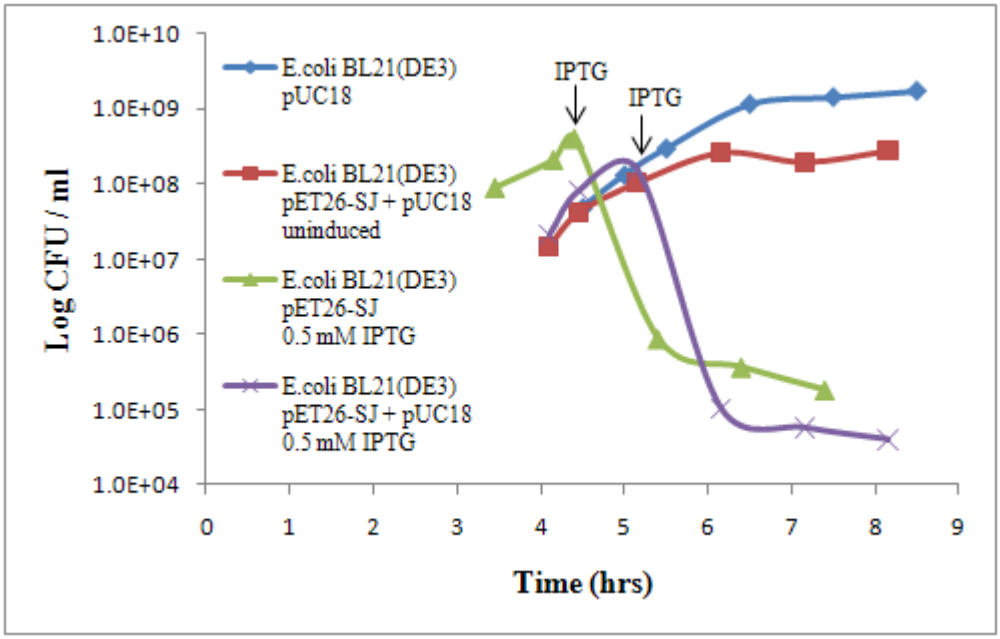

Figure 5.5. Cell viability profiles of *E. coli* BL21(DE3) cells harboring only pUC18 vector (◆), uninduced *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct (■), induced *E. coli* BL21(DE3) cells harboring only pET-26_{SJ} construct (▲), induced *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct (✕)

E. coli BL21(DE3) cells harboring pET-26_{SJ} construct (▲) had a 99.78 per cent decrease in the CFU. Since the decrease was very similar to that of cells harboring both pUC18 vector and pET-26_{SJ} construct (✕), it may be argued that the decrease in the CFU of the cells was mainly due to the metabolic burden caused by BLIP production.

The analysis showed that one hour after induction, *E.coli* BL21(DE3) cells harboring only pUC18 vector (◆) had the highest cell viability ($117x10^7$ CFU/ml). The second highest cell viability ($26x10^7$ CFU/ml) was observed in uninduced cells harboring both pUC18 vector and pET-26_{SJ} construct (■). Although these cells were expected to show similar cell viability, the CFU of the cells harboring two plasmids was 4.5 fold lower than the cells harboring only pUC18 vector. This result suggested that even the cells were not induced; plasmid maintenance imposed a metabolic burden on the host cells resulting in reduced cell viability.

5.3. *In-vitro* β-lactamase Inhibition by BLIP

Before its inhibition, β-lactamase activity in *E. coli* BL21(DE3) cells was investigated. The cells harboring pUC18 plasmid were used to determine β-lactamase expression and its progress with respect to time. Four separate cultures were grown, harvested at OD_{600} values indicated and β-lactamase activity from these cultures was measured (Figure 5.6). *In-vitro* R-TEM-1 β-lactamase activity was calculated as described in Section 4.12 and the results are summarized in Table 5.1.

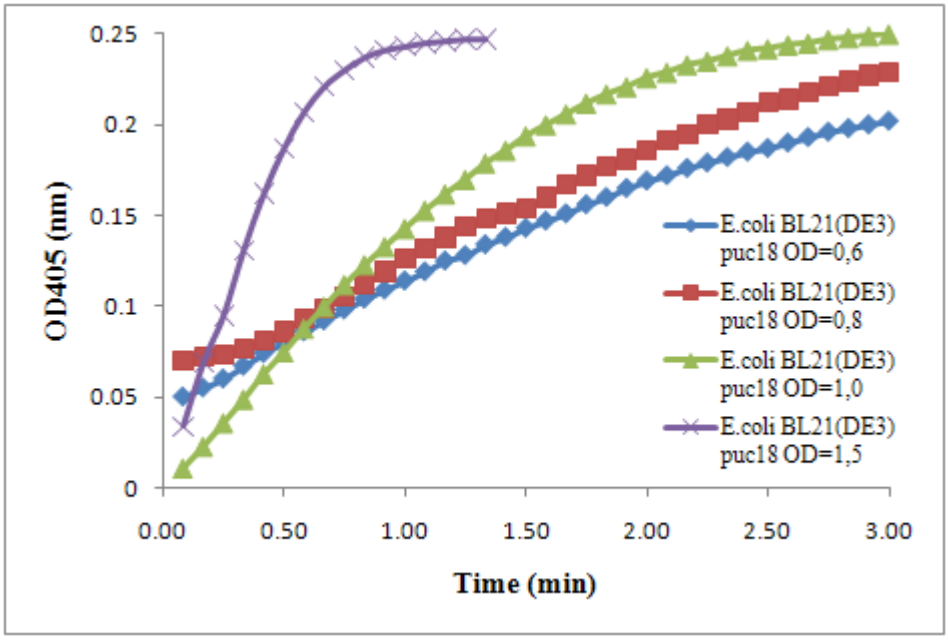

Figure 5.6. *In-vitro* β-lactamase activity of periplasmic extracts of *E. coli* BL21(DE3) cells harboring pUC18 plasmid with different OD_{600} values

In Table 5.1, optical densities of the cell cultures were converted to dry cell weight by using the equation g DCW = 0.6083 x OD_{600} (Appendix A). Activity of each sample was divided by its own biomass value. As the results indicate, β-lactamase activity increases with increasing OD_{600} values with cells at OD_{600}= 1.498 having the highest yield (12452 U) per g DCW. It can be concluded that the amount of β-lactamase produced within the cell is directly proportional to the time that the cells are grown.

Table 5.1. β-lactamase activity results for *E. coli* BL21(DE3) cells from different cell cultures with different cell culture densities

Expression system	OD_{600} of cell culture (nm)	Biomass (g DCW/L)	Activity (U/L)	β-lactamase activity (U/g DCW)
E. coli BL21(DE3) pUC18	0.596	0.363	1,844	5,080
	0.799	0.486	2,031	4,179
	1.024	0.623	4,406	7,072
	1.498	0.911	11,344	12,452

5.3.1. Binding of BLIP to β-lactamase in Periplasmic Protein Extracts

In an effort to investigate *in-vitro* inhibitory potential of BLIP, β-lactamase and BLIP each expressed in different cell cultures were used. The periplasmic protein extract containing β-lactamase was incubated with the periplasmic protein extract containing BLIP for two hours. After treatment, the β-lactamase activity in the periplasmic protein extract was measured. Variations in the activity were interpreted as the inhibition of β-lactamase by BLIP.

E. coli BL21(DE3) cells harboring pET-26_{SJ} construct and *E. coli* BL21(DE3) cells harboring pET-26_{EA} construct were tested to determine whether leader sequence has an effect on the binding of BLIP to β-lactamase or not. For this purpose, cells harboring pET-26_{SJ} construct and cells harboring pET-26_{EA} construct were grown in LB medium until OD_{600} reached 0.5 and both cultures were induced with 0.5 mM IPTG for 3 hours. *E. coli* TB1 cells harboring pUC18 vector were used to express TEM-1 β-lactamase in the periplasm.

Using periplasmic protein extracts has one drawback because the exact amount of desired protein is not known. Besides, the amount of total protein and the desired protein may vary in each periplasmic extract. For this reason, the amount of periplasmic protein extract to be added to the incubation mixture should be determined. Initial experiments were carried out with 100 µl periplasmic protein extract containing β-lactamase from *E. coli* TB1 cells and the results are given in Figure 5.7.

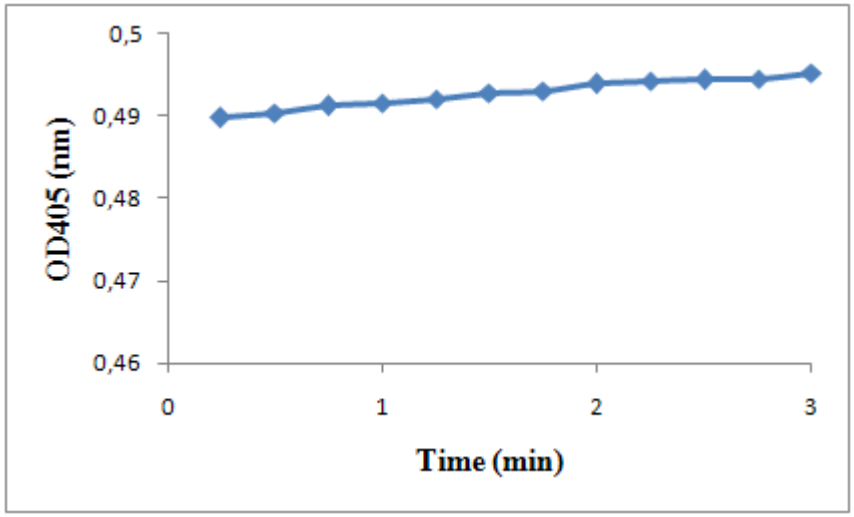

Figure 5.7. CENTA product formation using 100 µl periplasmic protein extract

As Figure 5.7 indicates, the reaction is complete within a minute. This could be due to the high amount of β-lactamase in the periplasmic protein extract. Therefore the amount of periplasmic protein extract was reduced to 1 µl (0.54 µg), 3 µl (1.63 µg), 5 µl (2.72 µg), 10 µl (5.43 µg) and 15 µl (8.15 µg). The activity results of different amounts of periplasmic protein extracts containing β-lactamase are shown in Figure 5.8.

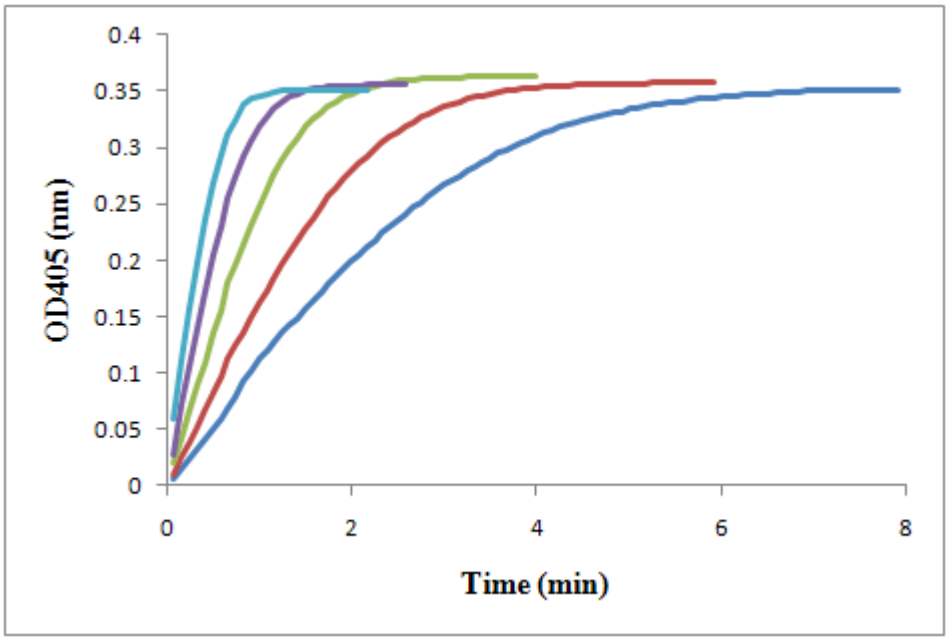

Figure 5.8. CENTA hydrolysis by 1 µl (blue), 3 µl (red), 5 µl (green), 10 µl (purple) and 15 µl (cyan) periplasmic protein extract

From Figure 5.8, it was concluded that 15 µl (8.15 µg, cyan) periplasmic protein extract containing β-lactamase was sufficient to measure the reaction rate. However, as already stated, the amount of β-lactamase in the periplasmic protein extract may vary. The amount of protein in this sample was taken as the standard in further experiments.

15 µl periplasmic protein extract containing β-lactamase was mixed with equal amount of BLIP containing periplasmic protein extracts (8.15 µg) from BL21(DE3) cells harboring pET-26_{SJ} construct or BL21(DE3) cells harboring pET-26_{EA} construct induced with 0.5 mM IPTG for three hours. The mixtures were incubated at 25°C and 180 rpm for two hours. The activity results for equal amounts of BLIP and β-lactamase are shown in Table 5.2.

The same experiments were repeated with a higher BLIP concentration as well. As expected, there is a higher decrease in the activity with higher amounts of BLIP. The results related to β-lactamase activity in the presence of higher BLIP concentration are also shown in Table 5.2.

Table 5.2. β-lactamase activity in the presence of BLIP containing periplasmic extracts

Expression system	**Total periplasmic protein in reaction mixture (µg)**		**β-lactamase activity (U/L)**	**Inhibition (%)**
	β-lactamase	**BLIP**		
E. coli TB1 pUC18	8.15	-	5750	0
E. coli TB1 pUC18 and *E. coli* BL21(DE3) pET-26_{SJ}	8.15	8.15	3400	40.9
	8.15	531	130	97.7
E. coli TB1 pUC18 and *E. coli* BL21(DE3) pET-26_{EA}	8.15	8.15	2160	62.4
	8.15	325	90	98.4

If BLIP binds and inhibits β-lactamase in the mixture after 2 hour incubation, the β-lactamase activity should be lower than 5750 U/L obtained in *E. coli* TB1 pUC18 periplasmic extract. According to the results summarized in Table 5.2, incubation of equal amounts of periplasmic extract containing BLIP with periplasmic extract containing β-lactamase resulted in 40.9 per cent decrease in the activity for pET-26_{SJ} construct while pET-26_{EA} construct showed 62.4 per cent decrease in the activity compared to *E. coli* TB1 pUC18. This result suggests that BLIP with *pelB* leader sequence inhibits β-lactamase 20 per cent more efficiently than BLIP with native leader sequence at low concentrations of BLIP.

The activity was further reduced by higher concentrations of BLIP. When higher amounts of periplasmic protein extracts containing BLIP, volumes (500 µl) kept same for each, were tested from pET-26_{SJ} and pET-26_{EA} constructs, they showed a very similar reduction in the activity, 97.7 per cent and 98.4 per cent, respectively. It can be concluded that at higher concentrations of BLIP, β-lactamase is strongly inhibited by BLIP in the prepared periplasmic fractions and leader sequence does not play a significant role in the binding properties of BLIP.

Since *E. coli* BL21(DE3) cells were used for BLIP production, β-lactamase from *E. coli* BL21(DE3) cells was also tested for inhibition studies. When the periplasmic protein extract containing BLIP and the periplasmic protein extract containing β-lactamase were incubated, precipitation occurred and the tests could not be completed.

5.3.2. Investigation of BLIP – β-lactamase Binding in the Periplasm

Binding of BLIP to β-lactamase in the periplasm of the cells was investigated. *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct were grown in LB medium until OD_{600} reached 0.5 and induced with 0.5 mM IPTG. The cells harboring only pUC18 vector were used as the control group. The β-lactamase activity in the periplasmic protein extracts was determined and the results are shown in Figure 5.9.

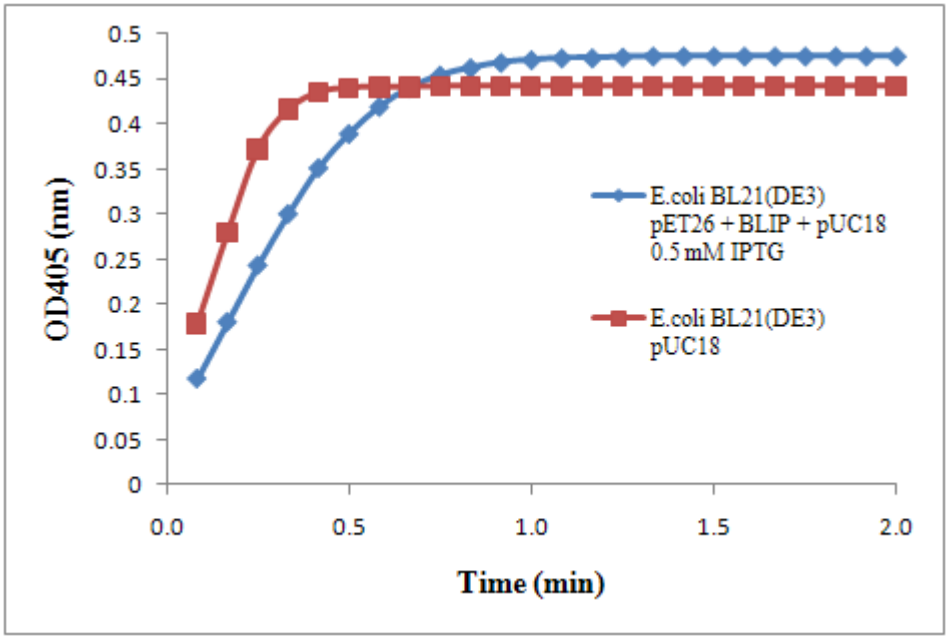

Figure 5.9. CENTA hydrolysis by periplasmic protein extracts from *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct (◆) and *E. coli* BL21(DE3) cells harboring only pUC18 vector (■)

The initial slope of absorbance measurements is used to calculate β-lactamase activity using Equation 4.1. In Figure 5.9, the cells harboring both plasmids had a lower initial speed compared to that of cells harboring only pUC18 vector. β-lactamase activity in the periplasmic extracts was calculated by using Equation 4.1 and the results are summarized in Table 5.3.

Table 5.3. Activity results for β-lactamase inhibition in the periplasm of the cells

Expression system	**Proteins expressed**	**Total periplasmic protein in reaction mixture (µg)**	**β-lactamase activity (U/L)**
E. coli BL21(DE3) pUC18	β-lactamase	10	16450
E. coli BL21(DE3) pUC18 + pET-26_{SJ}, induced	β-lactamase + BLIP	117	984

As Table 5.3 indicates, 16450 U/L of activity was found in the cells harboring only pUC18 vector. However, in the presence of BLIP, the activity was reduced by 94 per cent to 984 U/L. The decrease in activity suggests that BLIP binds and inhibits β-lactamase in the prepared periplasmic fraction when they are co-expressed.

In an effort to compare β-lactamase activity in induced and uninduced cells, *E. coli* BL21(DE3) cells harboring both pUC18 vector and pET-26_{SJ} construct were grown to OD_{600} values of 0.5 and one of the cultures was induced with 0.5 mM IPTG for 7.5 hours while the other was not induced. β-lactamase activity of the periplasmic protein extracts was measured at the end of 7.5 hours. The results are summarized in Table 5.4.

Table 5.4. β-lactamase activity in uninduced and induced *E. coli* BL21(DE3) cells harboring both pET-26_{SJ} construct and pUC18 vector

Expression system	**Proteins expressed**	**Total periplasmic protein in reaction mixture (μg)**	**β-lactamase activity (U/L)**
E. coli BL21(DE3) pUC18 + pET-26_{SJ}, uninduced	β-lactamase	250	306.3
E. coli BL21(DE3) pUC18 + pET-26_{SJ}, induced	β-lactamase + BLIP	250	24

β-lactamase production is constitutive while BLIP production from pET-26_{SJ} construct is inducible. When BLIP was not expressed, the cells showed an activity of 306.3 U/L. However, expression of BLIP resulted in a 13 fold reduction in the activity, 24 U/L, suggesting that β-lactamase is inhibited by BLIP when they are coexpressed.

Comparison of induced cells from Table 5.3 and Table 5.4 showed that the cells induced for 7.5 hours showed a lower activity (24 U/L) than the cells induced for 3 hours (984 U/L). It can be concluded that β-lactamase is further inhibited by BLIP when they are coexpressed for longer periods.

5.4. Electrophoretic Analysis of Protein Extracts

Protein analysis was performed on periplasmic and cytoplasmic extracts of cells harboring vectors. Protein concentrations were determined by Bradford Assay and the samples were loaded to 12 per cent SDS-polyacrylamide gel with the same amount of protein. The proteins were analyzed after Coomasie G-250 staining.

5.4.1. Periplasmic extracts containing β-lactamase

In this study, β-lactamase expression from both *E. coli* TB1 cells and *E. coli* BL21(DE3) cells was examined. Periplasmic protein extract of each cell was analyzed by SDS-PAGE to confirm the expression of β-lactamase in these cells. In an effort to investigate expression and the effect of time on β-lactamase production in *E. coli* TB1 cells, four separate cell cultures were grown and harvested at four OD_{600} values: 0.4, 0.7, 1.0 and 1.3. The periplasmic protein extracts were analyzed by SDS-PAGE (Figure 5.10).

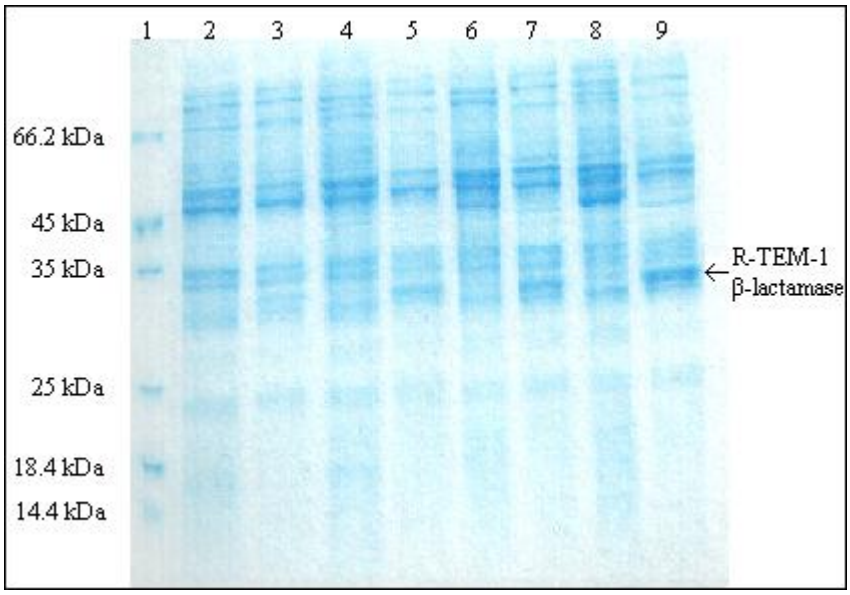

Figure 5.10. Analysis of periplasmic protein extracts. Lane 1: Molecular weight marker; *E. coli* TB1 cells without and with pUC18 plasmid Lane 2-3: at OD_{600}= 0.4; Lane 4-5: at OD_{600}= 0.7; Lane 6-7: at OD_{600}= 1.0; Lane 8-9: at OD_{600}= 1.3

In lanes 3, 5, 7 and 9, the thickness of the band between 25 kDa and 35 kDa from the extract of *E. coli* TB1 cells harboring pUC18 plasmid suggested that β-lactamase is expressed in these cells and maximum β-lactamase expression occurs at OD_{600} = 1.3. This information can be useful when a large quantity of β-lactamase production is desirable.

When β-lactamase expression from *E. coli* BL21(DE3) cells were examined, a band corresponding to R-TEM-1 β-lactamase was observed in the periplasmic space of *E. coli* BL21(DE3) cells harboring the pUC18 vector (Fig. 5.11, lane 3) compared to wild type *E. coli* BL21(DE3) cells (Fig. 5.11, lane 2).

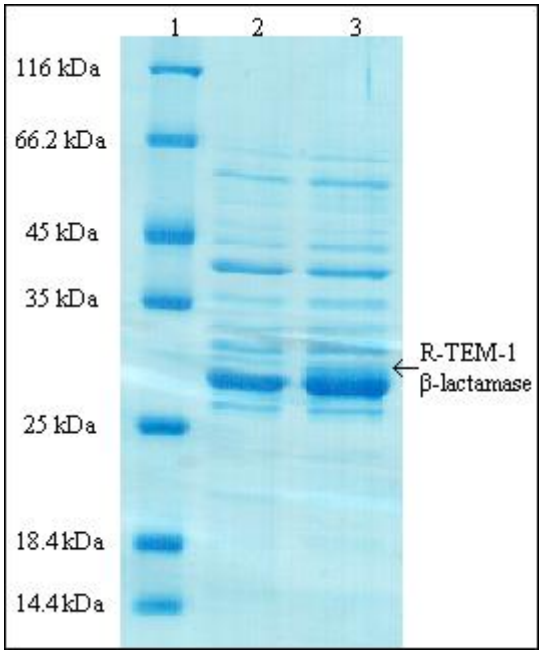

Figure 5.11. Analysis of β-lactamase on SDS-PAGE. Lane 1: Molecular weight marker, Lane 2: *E. coli* BL21(DE3) cells, Lane 3: *E. coli* BL21(DE3) cells harboring pUC18 vector

5.4.2. Periplasmic extracts containing β-lactamase and/or BLIP

Possible leaky expression of BLIP was tested in the pET-26_{EA} construct. *E. coli* BL21(DE3) cells harboring pET-26b(+) plasmid and *E. coli* BL21(DE3) cells harboring pET-26_{EA} construct were grown and none of the cultures were induced with IPTG. The cells were harvested two hours after OD_{600} reached 0.5 and the samples were analyzed by SDS-PAGE (Figure 5.12). It can be said that leaky expression did not occur in pET-26_{EA}

construct since there was not any band differences between lanes 2 and 3.

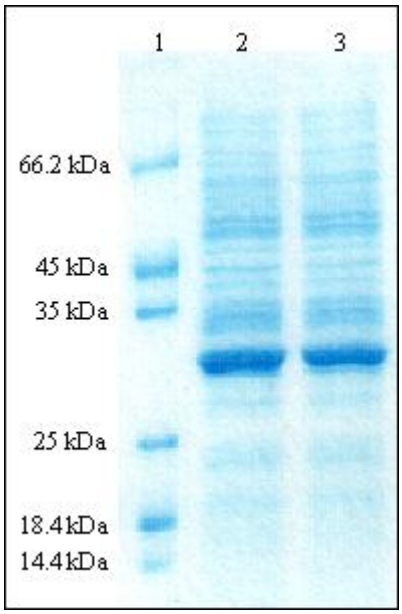

Figure 5.12. Analysis of leaky expreesion of BLIP by SDS-PAGE. Lane 1: molecular weight marker; *E. coli* BL21(DE3) cells harboring Lane 2: uninduced pET-26b(+) plasmid, Lane 3: uninduced pET-26_{EA} construct

After BLIP expression was confirmed to be under the control of the T7 promoter and there was no leaky expression, two cultures of *E. coli* BL21(DE3) cells harboring pET-26_{EA} construct were grown and one of the cultures was induced with 1 mM IPTG for 2 hours. Uninduced *E. coli* BL21(DE3) cells harboring pET-26_{EA} construct was used as control. The samples were analyzed by SDS-PAGE (Figure 5.13).

When the cells harboring pET-26_{EA} construct were induced, (Figure 5.13, lane 4) the two thick bands in lanes 2 and 3 disappeared and a new band appeared at about 40 kDa. Induction results in the expression of a different set of proteins and the band at 40 kDa may be a BLIP dimer. However, BLIP expression cannot be confirmed by these SDS-PAGE results.

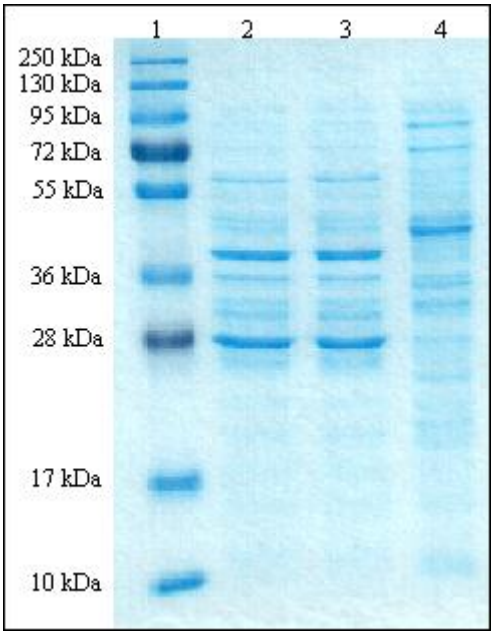

Figure 5.13. Analysis of BLIP by SDS-PAGE. Lane 1: molecular weight marker; *E. coli* BL21(DE3) cells harboring Lane 2: uninduced pET-26b(+) plasmid, Lane 3: uninduced pET-26_{EA} construct, Lane 4: pET-26_{EA} construct induced with 1 mM IPTG

In an effort to investigate simultaneous expression of β-lactamase and BLIP by SDS-PAGE analysis, the cells harboring both pET26_{SJ} construct and pUC18 vector and the cells harboring only pET-26_{SJ} construct were induced with 0.5 mM IPTG when OD_{600} reached 0.5 for three hours. At the same time, the cells harboring both pET-26_{SJ} construct and pUC18 vector and the cells harboring only pUC18 vector were grown to be used as controls. The results are given in Figure 5.14.

The sample in Lane 3 of Figure 5.14 was prepared from the cells harboring both plasmids but not induced with IPTG, while the sample in Lane 5 was from the cells harboring only pUC18 vector. R-TEM-1 β-lactamase band is present in Lanes 3 and 5. However, when the cells harboring both plasmids were induced with IPTG (Lane 1), the R-TEM-1 β-lactamase band could not be observed. Furthermore, no difference was detected between Lane 1 and Lane 2 at the BLIP band. This result suggested that BLIP is either not produced within the cell or it is produced in such small quantities that it is not visible on SDS-PAGE. Another reason for not observing BLIP may be the formation of inclusion bodies which are the insoluble aggregates of inactive proteins. Since the activity results

suggested β-lactamase inhibition in the presence of induction, BLIP is expected to be present in the periplasmic extract. Further purification of these fractions containing BLIP or Western blotting will enable the observation of BLIP.

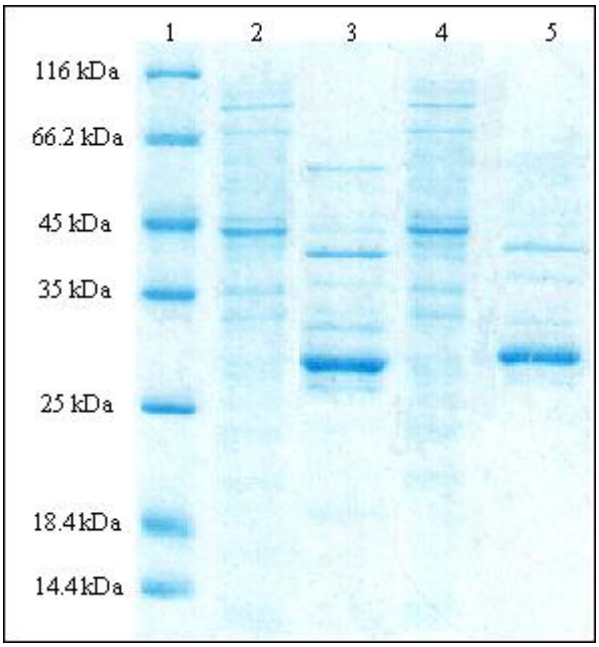

Figure 5.14. Electrophoretic analysis of periplasmic protein extracts from E.coli BL21(DE3) cells. Lane 1: Molecular weight marker; Lane 2: The cells harboring both plasmids, induced with 0.5 mM IPTG; Lane 3: The cells harboring both plasmids, uninduced control; Lane 4: the cells harboring only recombinant plasmid, induced with 0.5 mM IPTG; Lane 5: the cells harboring only pUC18 plasmid

5.4.3. Electrophoretic analysis of cytoplasmic extracts

Expression studies were also performed with pET-28_{EA} construct. To investigate the effect of different IPTG concentrations, the cells were induced with 0.5 mM and 1 mM IPTG for 2 hours. Since there is a high risk for inclusion body formation in the cytoplasm and BLIP in pET-28_{EA} construct is expressed in the cytoplasm, the cultures were grown at 30°C after induction. The samples were analyzed on SDS-PAGE (Figure 5.15). No band was observed corresponding to the size of BLIP between lane 2 and lanes 3&4.

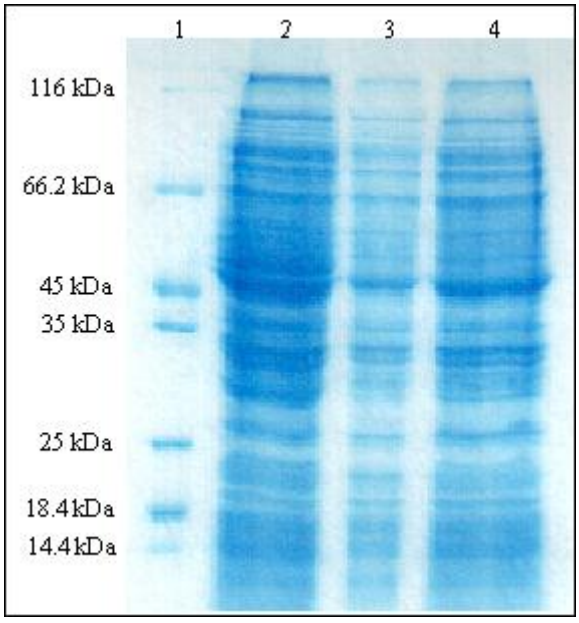

Figure 5.15. BLIP on SDS-PAGE. Lane 1: Molecular weight marker; *E. coli* BL21(DE3) cells Lane 2: uninduced; Lane 3: induced, 0.5 mM IPTG, Lane 4: induced, 1 mM IPTG

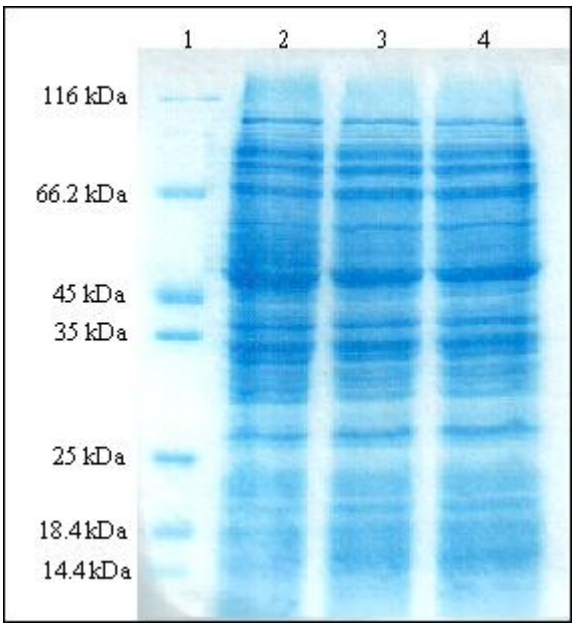

Figure 5.16. BLIP on SDS-PAGE. Lane 1: Molecular weight marker, *E. coli* BL21(DE3) cells Lane 2: uninduced ; Lane 3: induced, 0.5 mM IPTG, Lane 4: induced, 1 mM IPTG

E. coli BL21(DE3) cells harboring pET-28_{EA} construct were grown at 30°C before and after induction (Figure 5.16). Comparison of Figure 5.15 and Figure 5.16 showed that growing cells at 30°C before induction did not have an effect on the proteins expressed.

In an effort to confirm BLIP expression by the construct that contains the BLIP gene, cells harboring pET-28$_{EA}$ construct and pET-28a(+) plasmid were induced with 0.5 mM IPTG for 2 hours. Uninduced cells were used as the control and the samples were analyzed on SDS-PAGE (Figure 5.17).

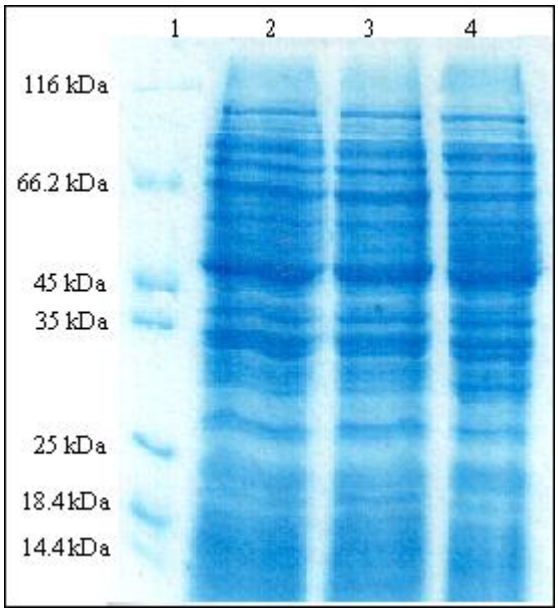

Figure 5.17. SDS-PAGE analysis of cells containing the recombinant pET-28$_{EA}$ construct and the nonrecombinant pET-28a(+) plasmid . Lane 1: molecular weight marker; *E. coli* BL21(DE3) cells harboring Lane 2: pET-28a(+) plasmid induced with 0.5 mM IPTG, Lane 3: pET-28$_{EA}$ construct induced with 0.5 mM IPTG, Lane 4: uninduced pET-28$_{EA}$ construct

Comparison of the different lanes in Figure 5.17 shows that the band between 18.4 and 25 kDa is thicker in Lane 3. On the other hand, this band is present in Lanes 2 and Lane 4 as well, showing that it is a protein expressed by both pET-28a(+) plasmid and pET-28$_{EA}$ construct and it was expressed in larger amounts when the cells were induced. No other difference was observed in the cytoplasmic extracts of the cells harboring the recombinant (contains BLIP) pET-28$_{EA}$ construct and the non-recombinant pET-28a(+) plasmid. The presence of BLIP could not be verified by SDS-PAGE analysis.

To observe if there is any possible leaky expression of BLIP, *E. coli* BL21(DE3) cells harboring pET-28a(+) plasmid and cells harboring the pET-28$_{EA}$ construct were grown with no induction. The control group, cells harboring pET-28$_{EA}$ construct, was

induced with 1 mM IPTG for 3 hours. All cultures were grown at 30°C before and after induction. The results are shown in Figure 5.18.

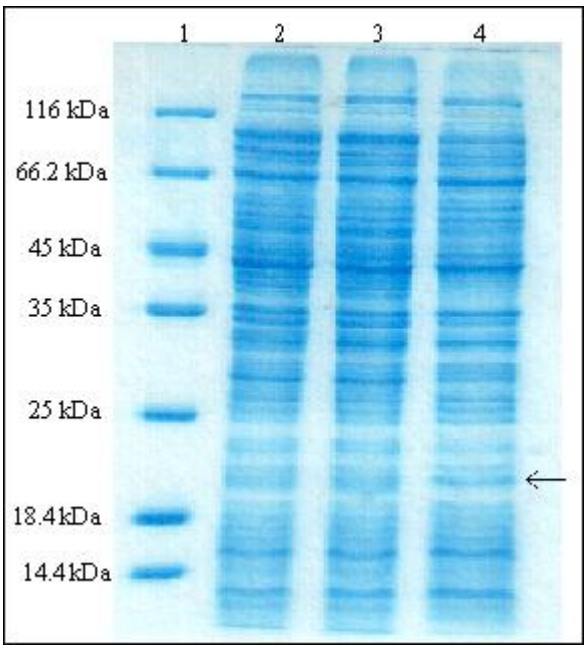

Figure 5.18. Analysis of BLIP by SDS-PAGE. Lane 1: molecular weight marker; *E. coli* BL21(DE3) cells harboring Lane 2: uninduced pET-28a(+) plasmid, Lane 3: uninduced pET-28_{EA} construct, Lane 4: pET-28_{EA} construct induced with 1 mM IPTG

From Figure 5.18, it can be said that leaky BLIP expression did not occur since no difference could be observed between the cytoplasmic extract of cells with the uninduced pET-28a(+) plasmid and that with the uninduced pET-28_{EA} construct. A sharp band, indicated with an arrow, was observed in the sample in lane 4. It cannot be argued that it was BLIP since the sharp band was also present in uninduced cells (lane 3).

In order to observe if BLIP is expressed at longer induction times, *E. coli* BL21(DE3) cells harboring pET-28_{EA} construct were induced with 1 mM IPTG for 3 hours and 5 hours and the uninduced cells were used as the control. The samples were diluted analyzed on SDS-PAGE (Figure 5.19). BLIP was not observed in the prepared samples.

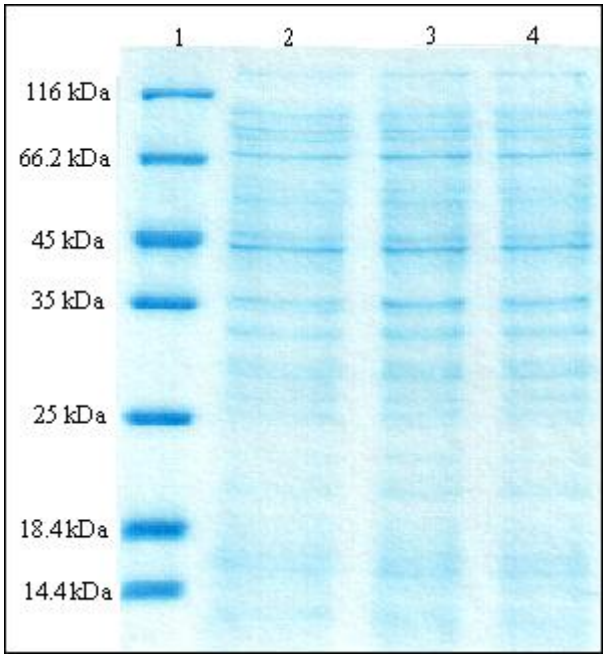

Figure 5.19. Analysis of BLIP by SDS-PAGE. Lane 1: molecular weight marker; *E. coli* BL21(DE3) cells harboring pET-28_{EA} construct Lane 2: uninduced, Lane 3: induced for 3 hours, Lane 4: induced for 5 hours

5.4.4. Affinity chromatography of extracts containing BLIP

The 6xHis affinity tag placed at the C- or N- terminus of the protein of interest facilitates purification of recombinant proteins by Ni-NTA affinity chromatography. It allows purification of proteins from less than 1 per cent of the total protein preparation to more than 95 per cent homogeneity in just one step (Qiagen, 2003). Since BLIP has a C-terminal 6xHis-tag in pET-26_{EA} plasmid, it can be purified by affinity chromatography.

E. coli BL21(DE3) cells and *E. coli* BL21(DE3) cells harboring pET-26_{EA} plasmid were grown and the latter one was induced with 1 mM IPTG when OD_{600} reached 0.5. The cells were harvested 2 hours after induction. The samples were prepared for affinity chromatography as described in Section 4.15. They were analyzed on SDS-PAGE and the results are given in Figure 5.20.

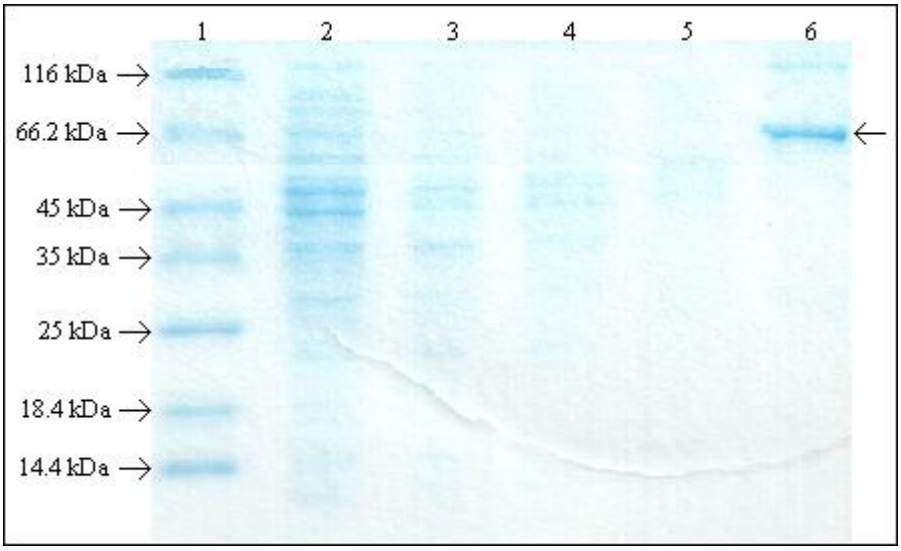

Figure 5.20. Electrophoretic analysis of affinity samples. Lane 1: molecular weight marker, Lane 2: Wild type *E. coli* BL21(DE3), *E. coli* BL21(DE3) cells harboring pET-26_{EA} plasmid Lane 3: flow-through, Lane 4-5: first and second washes, Lane 6: eluate

In affinity chromatography, the protein of interest is expected to be present in eluate fraction in large amounts. However, in the figure, the sharp band in the eluate corresponds to 66 kDa in size. Therefore, it cannot be BLIP since BLIP is an 18 kDa protein. In this respect, BLIP was not observed in the extract prepared from *E. coli* BL21(DE3) cells harboring pET-26_{EA} plasmid on SDS-PAGE after Ni-NTA affinity chromatography.

6xHis affinity chromatography method was also employed to purify BLIP from cytoplasmic extract of *E. coli* BL21(DE3) cells harboring pET-28_{EA} construct. The cells were grown until OD_{600} reached 0.5 and induced with 0.5 mM IPTG for 2 hours. Cytoplasmic extract from wild type *E. coli* BL21(DE3) cells were used as the control. SDS-PAGE analysis of the samples is shown in Figure 5.21.

When samples from the various stages of affinity chromatography were examined by SDS-PAGE analysis, very little protein was captured by the resin so that after first wash with 20 mM imidazole, almost no protein was present in the following fractions. In the elution step, ~ 0.1 mg/ml protein was present in the eluate and BLIP was not observed in this fraction. This may be due to low expression of BLIP under these conditions.

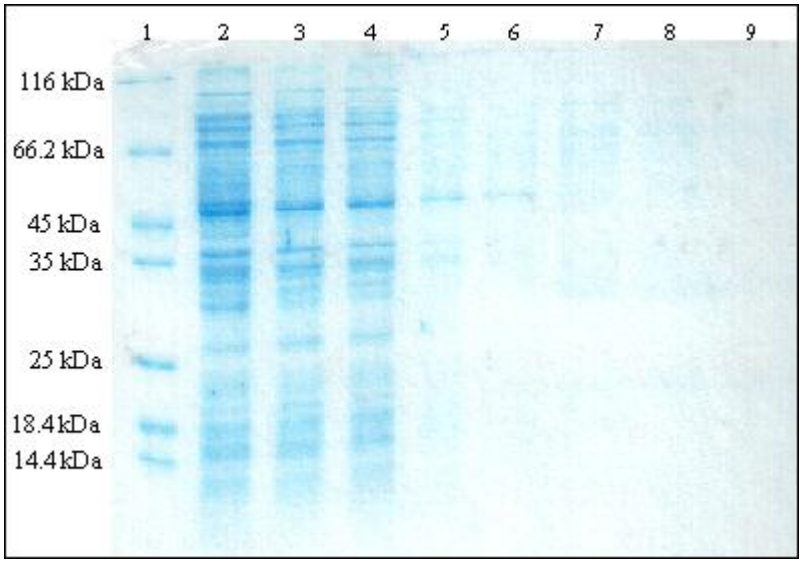

Figure 5.21. Analysis of BLIP containing affinity samples by SDS-PAGE. Lane 1: molecular weight marker; Lane 2: cytoplasmic extract of wild type *E. coli* BL21(DE3) cells; cytoplasmic extract of *E. coli* BL21(DE3) cells harboring pET-28_{EA} construct Lane 3: before column, Lane 4: flow-through, Lane 5-6: first and second washes with 20 mM imidazole, Lane 7-8: first and second washes with 70 mM imidazole, Lane 9: eluate

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The aim of this study was to investigate *in-vivo* and *in-vitro* binding of BLIP to R-TEM-1 β-lactamase using experimental procedures. Accordingly, two different expression systems with different leader sequences were used for periplasmic expression of BLIP: the first one with the *pelB* leader sequence and the second one with the native BLIP leader sequence. Cytoplasmic expression of BLIP by pET-28_{EA} construct was analysed. pUC18 vector was used for the expression of R-TEM-1 β-lactamase.

The pET-26 vectors and the pUC18 vector were transformed into *E. coli* BL21(DE3) cells to express BLIP and R-TEM-1 β-lactamase simultaneously to examine *in-vivo* binding properties of BLIP to β-lactamase. *In-vitro* studies were conducted by measuring β-lactamase activity in the periplasmic extracts of the cells. It was achieved by incubating the periplasmic protein extract of the cells harboring recombinant plasmids and the periplasmic protein extract of the cells harboring pUC18 vector.

Growth characteristics of the cells were examined to determine if any inhibition occurs on the cells. The induction of recombinant cells with IPTG resulted in a substantial retardation of cell growth, which is mainly due to metabolic burden. Since all cells harboring recombinant plasmids were affected in a similar manner, it cannot be argued that the decrease in the growth rate is related to inhibition of β-lactamase by BLIP. The number of colony forming units was determined to examine the effect of expression of BLIP. It was observed that one hour after induction, CFU of the induced cells harboring recombinant plasmids (pET-26_{SJ} + pUC18 or pET-26_{SJ} alone) decreased by 99.93 per cent, which suggested that induction affected the cells immediately.

BLIP could not be observed by electrophoretic analysis in periplasmic or cytoplasmic protein extracts. It may be due to the fact that either BLIP is not expressed within the cells or it is expressed in little amounts. To verify the expression and presence of BLIP in the cells, *in-vitro* β-lactamase activity was measured. 94 per cent decrease in β-

lactamase activity suggested that BLIP efficiently inhibits R-TEM-1 β-lactamase in the periplasm of the cells. After incubation of periplasmic protein extract containing BLIP with periplasmic protein extract containing β-lactamase, the activity decreased by 40.9 per cent by BLIP with native leader sequence (from pet26EA construct) and 62.4 per cent by BLIP with *pelB* leader sequence (from pET-26_{SJ} construct). These results suggest that *pelB* BLIP inhibits R-TEM-1 β-lactamase more efficiently than native BLIP with the native leader sequence. Higher concentrations of BLIP gave approximately a 98 per cent decrease in the activity for both leader sequences, which indicated that leader sequence does not play a significant role in the binding properties of BLIP at higher concentrations.

6.2. Recommendations

Further experiments should be carried out to observe BLIP by electrophoresis. To this end, different parameters affecting the expression of BLIP could be optimized. Western blot analysis should be performed to confirm the presence of BLIP in cellular fractions.

BLIP with *pelB* leader sequence has been constructed such that it contains an N-terminal His-tag, which allows for easy and efficient purification of BLIP. Once BLIP is purified via affinity chromatography, the kinetic parameters such as K_m and K_i for *in-vitro* BLIP – β-lactamase inhibition should be determined.

After the kinetic parameters for *in-vitro* BLIP – β-lactamase interaction are established, the same procedure should be applied to new BLIP based peptides to characterize their kinetics *in-vitro*. This is the initial step for *in-vivo* inhibition of β-lactamase.

APPENDIX A: DRY CELL WEIGHT CORRELATION FACTOR

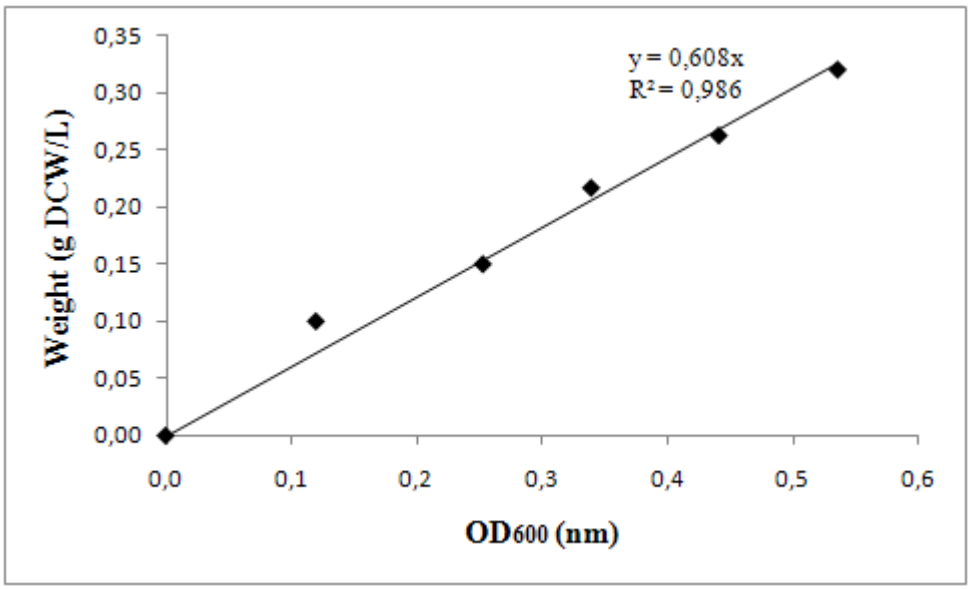

Figure A.1. Correlation factor of dry weights to optical densities

APPENDIX B: BSA CALIBRATION CURVE

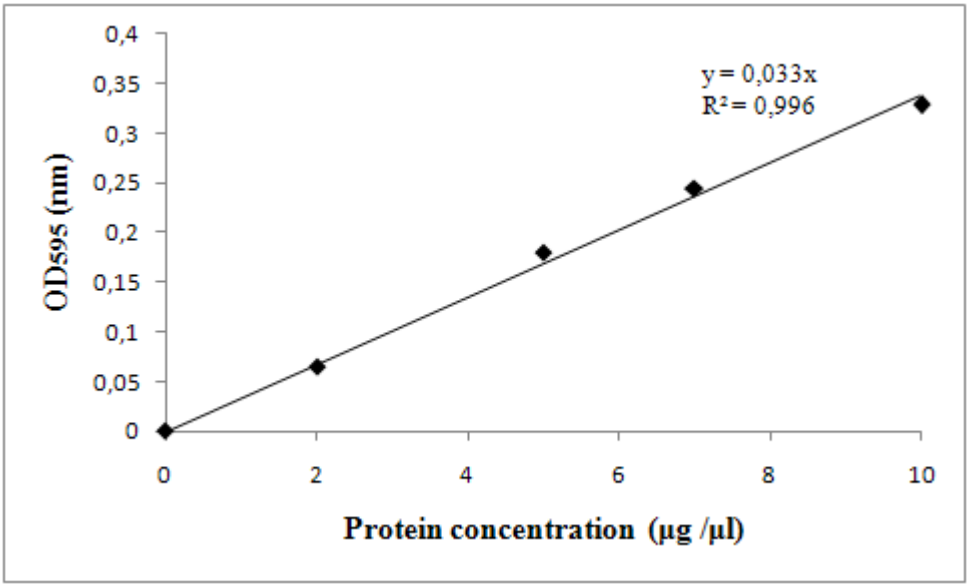

Figure B.1. BSA calibration curve

REFERENCES

Albeck, S. and G. Schreiber, 1999, "Biophysical Characterization of the Interaction of the β-Lactamase TEM-1 with Its Protein Inhibitor BLIP", *Biochemistry,* Vol. 38, pp. 11-21.

Babic, M., A. M. Hujer and R. A. Bonomo, 2006, "What's new in antibiotic resistance? Focus on beta-lactamases", *Drug Resistance Updates,* Vol. 9, pp. 142-156.

Bebrone, C., C. Moali, F. Mahy, S. Rival, J. D. Docquier, G. M. Rossolini, J. Fastrez, R. F. Pratt, J. M. Frère and M. Galleni, 2001, "CENTA as a Chromogenic Substrate for Studying β-Lactamases", *Antimicrobial Agents and Chemotherapy,* Vol. 45, pp. 1868-1871.

Bradford, M., 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding", *Analytical Biochemistry,* Vol. 72, pp. 248-254.

Datta, N. and P. Kontomichalou, 1965, "Penicillinase Synthesis Controlled by Infectious R factors in *Enterobacteriaceae*", *Nature (London),* Vol. 208, pp. 239-241.

Doran, J. L., B. K. Leskiw, S. Aippersbachand and S. E. Jensen, 1990, "Isolation and Characterization of a β-Lactamase-Inhibitory Protein from *Streptomyces clavuligerus* and Cloning and Analysis of the Corresponding Gene", *Journal of Bacteriology,* Vol. 172, pp. 4909-4918.

Essack, S. Y., 2001, "The Development of β-lactam Antibiotics in Response to the Evolution of β-lactamases", *Pharnaceutical Research Review Article,* Vol. 18, No. 10, October 2001.

Fleming, A., 1929, "On the antibacterial action of cultures of a Penicillium, with special reference to their use in the isolation of *B. influenza*", *British]ournal of Experimental Pathology*, Vol. 10, pp. 226-236.

Gretes, M., Lim, D. C., Jensen, S. E., Kang, S. G., Lee, K. J. and Strynadka, N. C. J., 2009, "Insights into Positive and Negative Requirements for Protein–Protein Interactions by Crystallographic Analysis of the β-Lactamase Inhibitory Proteins BLIP, BLIP-I, and BLP", *Journal of Molecular Biology*.

Handal, T. and Olsen, I., 2000, "Antimicrobial Resistance with Focus on Oral Beta-lactamases", *Europian Journal of Oral Sciences*, Vol. 108, pp. 163-174.

Huang, W., Z. Zhang and T. Palzkill, 2000, "Design of Potent β-Lactamase Inhibitors by Phage Display of β-Lactamase Inhibitory Protein", *The Journal of Biological Chemistry*, Vol. 275, No. 20, pp. 14964-14968.

Huang, W., Z. Beharry, Z. Zhang and T. Pazkill, 2003, "A broad spectrum peptide inhibitor of β-lactamase identified using phage display and peptide arrays", *Protein Engineering*, Vol. 16, pp. 853-860.

Jacoby, G. A., 2006, "β-Lactamase Nomenclature", *Antimicrobial Agents and Chemotherapy*, Vol. 50, No. 4, pp. 1123-1129.

Jones, R. N., H. W. Wilson, J. William, J. R. Novick, A. L. Barry and C. Thornsberry, 1982, "In vitro Evaluation of CENTA, a New Beta-Lactamase-Susceptible Chromogenic Cephalosporin Reagent", *Journal of Clinical Microbiology*, Vol. 15, pp. 954-958.

Kang, S. G., H. U. Park, H. S. Lee, H. T. Kim and K. J. Lee, 2000, "New β-Lactamase Inhibitory Protein (BLIP-I) from *Streptomyces exfoliatus* SMF19 and Its Roles on the Morphological Differentiation", *The Journal of Biological Chemistry*, Vol. 275, No. 22, pp. 16851-16856.

Kershaw, N. J., Caines, M. E. C., Sleeman, M. C., Schofield, C. J., 2005, "The enzymology of clavam and carbapenem biosynthesis", *The Royal Society of Chemistry*, pp. 4251-4263.

Lim, D., H. U. Park, L. De Castro, S. G. Kang, H. S. Lee, S. Jensen, K. J. Lee and N. C. J. Strynadka, 2001, "Crystal structure and kinetic analysis of β-lactamase inhibitor protein-II in complex with TEM-1 β-lactamase", *Nature Structural Biology*, Vol. 8, No. 10, pp. 848-852.

Livermore, D. M., 1998, "β-Lactamase-Mediated Resistance and Opportunities for Its Control", *Journal of Antimicrobial Chemotherapy,* Vol. 41, pp. 25-41.

Maiti, S. N., Babu, R. P. K., Shan, R., 2006, "Overcoming Bacterial Resistance: Role of *β*-Lactamase Inhibitors", *Top Heterocyclic Chemistry,* Vol. 2, pp. 207-246.

Majiduddin, F. K., I. C. Materon, and T. G. Palzkill, 2002, "Molecular analysis of beta-lactamase structure and function", *International Journal of Medical Microbiology*, Vol. 292, pp. 127-137.

Mroczkowska, J. E. and Barlow, M., 2008, "Fitness Trade-Offs in bla_{TEM} Evolution", *Antimicrobial Agents and Chemotherapy*, Vol. 52, No. 7, pp. 2340-2345.

Nossal, N. G. and L. A. Heppel, 1966, "The Release of Enzymes by Osmotic Shock from *Escherichia coli* in Exponential Phase", *The Journal of Biological Chemistry,* Vol. 241, pp. 3055-3062.

Novagen, 2006, "pET System Manual, 11th Edition", http://www.emdchemicals.com/life-science-research/novagen-technical bulletins/c_IMOb.s1OXkUAAAEj2xsYzMkq

Page, M. I., P. S. Hinchliffe, J. M. Wood, L. P. Harding and A. P. Laws, 2003, "Novel mechanism of inhibiting β-Lactamases by sulfonylation using β-Sultams", *Bioorganic & Medicinal Chemistry Letters*, Vol. 13, pp. 4489-4492.

Palumbi, S. R., 2001, "Humans as the World's Greatest Evolutionary Force", *Science,* Vol. 293, pp. 1786-1790.

Petrosino, J., G. W. Rudgers, H. Gilbert and T. Palzkill, 1999, "Contributions of Aspartate 49 and Phenylalanine 142 Residues of a Tight Binding Inhibitory Protein of β-Lactamases", *Journal of Biological Chemistry,* Vol. 274, pp. 2394-2400.

Poole, K., 2004, "Resistance to β-lactam Antibiotics", *Cellular and Molecular Life Sciences,* Vol. 61, pp. 2200-2223.

Reynolds, K. A., J. M. Thomson, K. D. Corbett, C. R. Bethel, J. M. Berger, J. F. Kirsch, R. A. Bonomo and T. M. Handel, 2006, "Structural and Computational Characterization of the SHV-1 β-lactamase- β-lactamase Inhibitor Protein Interface", *The Journal of Biological Chemistry,* Vol. 281, pp. 26745-26753.

Rudgers, G. W. and T. Palzkill, 1999, "Identification of Residues in β-Lactamase Critical for Binding β-Lactamase Inhibitory Protein", *The Journal of Biological Chemistry,* Vol. 274, No. 11, pp. 6963-6971.

Rudgers, G. W., W. Huang and T. Palzkill, 2001, "Binding Properties of a Peptide Derived from β-lactamase Inhibitory Protein", *Antimicrobial Agents and Chemotherapy,* Vol. 45, pp. 3279-3286.

Rudgers, G. W. and T. Palzkill, 2001, "Protein minimization by random fragmentation and selection", *Protein Engineering,* Vol. 14, No. 7, pp. 487-492.

Savard, P. Y. and S. M. Gagne, 2006, "Backbone Dynamics of TEM-1 Determined by NMR: Evidence for a Highly Ordered Protein", *Biochemistry,* Vol. 45, pp. 11414-11424.

Schroeder, W. A., T. R. Locke and S. E. Jensen, 2002, "Resistance to β-Lactamase Inhibitor Protein Does Not Parallel Resistance to Clavulanic Acid in TEM β-Lactamase Mutants", *Antimicrobial Agents and Chemotherapy*, Vol. 46, No. 11, pp. 3568-3573.

Strynadka, N. C. J., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh and M. N. G. James, 1992, "Molecular structure of the acyl-enzyme intermediate in β lactam hydrolysis at 1.7 Å resolutions", *Nature*, Vol. 359, pp. 700-705.

Strynadka, N. C. J., S. E. Jensen, K. Johns, H. Blanchard, M. Page, A. Matagne, J. M. Frere and M.N.G. James, 1994, "Structural and Kinetic Characterization of a β-lactamase-inhibitor protein", *Nature*, Vol. 368, pp. 657-660.

Strynadka, N. C., S. E. Jensen, P. M. Alzari and M. N. G. James, 1996, "A potent new mode of β-lactamase inhibition revealed by the 1.7 Å X-ray crystallographic structure of the TEM-1-BLIP complex", *Nature Structural Biology*, Vol. 3, pp. 290-297.

Sun, W., Y. Hu, J. Gong, C. Zhu and B. Zhu, 2005, "Identification of β-lactamase Inhibitory Peptide Using Yeast Two-Hybrid System", *Biochemistry (Moscow)*, Vol. 70, No. 7, pp. 753-760.

Thai, W., Ashish S. Paradkar and Susan E. Jensen, 2001, "Construction and Analysis of β-lactamase Inhibitory Protein (BLIP) Non-producer Mutants of *Streptomyces clavuligerus*", *Microbiology*, Vol. 147, pp. 325-335.

Tipper, D. J. and J. L. Strominger, 1965, "Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine", *Proceedings of the National Academy of Sciences*, Vol. 54, pp. 1133-1141.

Tu, Z., G. He, K. X. Li, M. J. Chen, J. Chang, L. Chen, Q. Yao, D. P. Liu, H. Ye, J. Shi, X. Wu, 2005, "An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains", *Electronic Journal of Biotechnology*, Vol.8, No.1, pp. 114-120.

Wang, J., Zhang, Z., Palzkill, T. and Chow, D. C., 2007, "Thermodynamic Investigation of the Role of Contact Residues of β-Lactamase-inhibitory Protein for Binding to TEM-1 β-Lactamase", *The Journal of Biological Chemistry*, Vol. 282, No. 24, pp. 17676-17684.

Wilke, M. S., A. L. Lovering and N. C. J. Strynadka, 2005, "β-Lactam antibiotic resistance: a current structural perspective" *Current Opinion in Microbiology*, Vol. 8, pp. 525-533.

Williams, J. D., 1999, "β-Lactamases and β-lactamase inhibitors", *International Journal of Antimicrobial Agents*, Vol. 12, pp. 3-7.

Yuan, J., Huang, W., Chow, D. C. and Palzkill, T., 2009, "Fine Mapping of the Sequence Requirements for Binding of β-lactamase Inhibitory Protein (BLIP) to TEM-1 β-lactamase Using a Genetic Screen for BLIP function", *Journal of Molecular Biology.*