Chapter 4

Catalytic selection of b-lactamase with mRNA display library

Abstract: A novel catalytic selection approach for improvement of enzymatic function is demonstrated. An immobilized mechanism-based inhibitor, whose inhibition stimulated by the same mechanism of enzyme as to normal substrate, is able to enrich the active mutants of enzyme quickly and efficiently. Biotinylated penicillanic acid sulfone, a β-lactamase mechanism-based inhibitor, is employed to select active mutants of β-lactamase from an mRNA-lactamase fusion library. This strategy can be applied on the functional evolution of other enzymes, such as aldolase.

4.1 Introduction

1. Functional evolution of enzymes

The enzymes present in modern biochemistry catalyze a great variety of chemical reaction precisely and efficiently as a result of billions of years of natural evolution. A clear understanding of their structures, mechanisms and functions is important to the advancement of medicine, synthetic chemistry, material science, as well as many other fields. Despite tremendous efforts, many aspects of enzymes remain a mystery. One example is the minimum size necessary to keep their active site residues in their proper positions for catalysis. Indeed, the smallest enzymes known tend to be ~100 residues, for example, acylphosphatase[1], a 98-residue-long enzyme. It is unclear if short catalytic proteins existed in the early stages of evolution and then were replaced by larger enzymes due to the demands of high efficiency and precision. If the existence of short catalytic proteins that mimic the function of natural enzymes can be demonstrated, it will lend insight into the origins of life. Despite the failure of many de novo efforts to produce such short protein catalysts with biologically interesting properties[2,3], the advancement of combinatorial approaches including enzyme evolution provides an attractive alternative.

Enzyme evolution has been used widely to improve key properties of many enzymes, such as substrate specificity and thermo stability[4,5]. Generally, enzyme evolution starts with the creation of a library of mutated genes. Mutants with enhanced properties are identified from the background of neutral or harmful mutants, and subjected to further cycles to accumulate beneficial mutations. Currently, screening is the primary method to identify positive mutants because it is easy to implement and apparently suitable to any enzymes, as long as the size of libraries is kept manageable.

However, to manipulate even a small size of library is not trivial. Even though the advance of robotic systems in recent years already exceedingly facilitates the screening process, the size of libraries that can be handled by screening is still its major limitation. *In vivo* selection, by contrast, has apparent advantages over screening for searching larger libraries. Nevertheless, the *in vivo* selection suffers from its difficulty to be used in an enzyme that is not critical to the survival of the host organism. In addition, organisms are notoriously adept at evading imposed selective pressure by unexpected mechanisms. In stead, *in vitro* selection can be employed on any molecules that are capable of being copied. The stringency of selection pressure is also controllable *in vitro*. Thus, an *in vitro* catalytic selection strategy based on mRNA display library is reported herein.

2. Catalytic selection with mechanism based inhibitors

Affinity selection has been extensively utilized to recognize the epitopes for antibodies, detect new receptors with natural ligands, and select DNA-binding proteins in either *in vivo* or *in vitro* libraries. A variation of affinity selection, where the transition state analogs of chemical reactions are used as antigens, can generate catalytic antibodies[6]. This technique is based on the fact that the antibodies specific for the transition state analog of chemical reactions should have catalytic power. However, catalytic antibodies are usually much less efficient than natural occurring enzymes. In many cases, antibodies bound to the transition state analog tightly fail to accelerate the chemical reaction. Recently, reactive molecules during immune response have been used as antigens to generate catalytic antibodies[7-9]. This approach has proven to be superior over the conventional catalytic antibody selection. Nevertheless, the immune system has

several limitations: 1) the scaffold for recombination is limited to the immunoglobin family, 2) the selection stringency cannot be controlled, and 3) the optimization of selected antibodies is difficult. Thus, the concept of "reactive immune response" is applied on mRNA-display library to select active enzymes from a library instead.

In this new strategy, the immobilized mechanism-based inhibitor, which is defined as an unreactive compound that inhibits an enzyme upon being activated, is employed as target to search the mRNA display library. A mechanism-based inhibitor, which shares structural similarity with the substrate or product of a specific enzyme, is converted to an intermediate product that is generally very reactive to target enzyme. While in the active site, this active intermediate quickly forms a covalent bond with target enzyme, thus inactivating the enzyme. The elegant inhibitory mechanism of mechanism-based inhibitor differs from that of normal competitive or non-competitive inhibitors in that a chemical reaction via the enzyme's normal catalytic pathway must take place to activate the mechanism-based inhibitor. This mechanism provides a powerful method for catalytic selection. When the entire mRNA display library of a specific enzyme is incubated with the immobilized mechanism-based inhibitor, only mutants maintaining the normal functions are able to form covalent bond with inhibitor and bind to solid support. All other inactive individuals are removed by one simple washing step. After identifying functional mutants from the library, error prone PCR can be used to add diversity to the library prior to the next cycle of selection.

3. A model system – b-lactamase

RTEM-1 β-lactamase, a 30 kD enzyme, has been chosen as a model system because of its unique features, including the simplicity of its structure, extensive understanding of its mechanism, and the availability of its mechanism-based inhibitors.

β-lactamases are a wide family of proteins encoded by either chromosome or plasmid. These enzymes are normally responsible for the bacterial resistance towards β-lactam antibiotics because they can hydrolyze β-lactam ring efficiently. RTEM-1 β-lactamase is a plasmid-encoded enzyme that consists of only 263 amino acid residues in a single polypeptide chain[10]. It has attained a kinetic optimum in that its catalytic activity towards penicillin is diffusion-limited.

a. Mechanism of RTEM-1 b-lactamase

Extensive investigations on the mechanism of RTEM-1 β-lactamase indicate that a serine residue in position 70 plays an essential role in catalytic reaction[11] (Figure 4.1). Catalysis is initiated by nucleophilic attack of Ser70 on the β-lactam ring to form a tetrahedral acyl intermediate. Then, the product is released by the nucleophilic attacking of a water molecule, which is activated by Glu166. The E166A mutant of β-lactamase is hydrolysis-deficient[12,13].

b. Penicillanic acid sulfone (PAS) – a mechanism-based inhibitor of b-lactamase

Penicillanic acid sulfone is a potent mechanism-based inhibitor of β-lactamase[11,14-17] (Figure 4.2). Inhibition starts from nucleophilic attack of Ser70 on the β-lactam ring to form an acyl-enzyme intermediate. However, the pentacyclic ring of penicillanic acid sulfone is labile because the strong electron-withdrawing sulfone group

can stabilized the cleaved product. Thus, the acyl-enzyme intermediate can follow three possible pathways[18]. The first is normal deacylation, which regenerates the enzyme. The

Figure 4.1 Two-step mechanism of RTEM-1 β-lactamase.

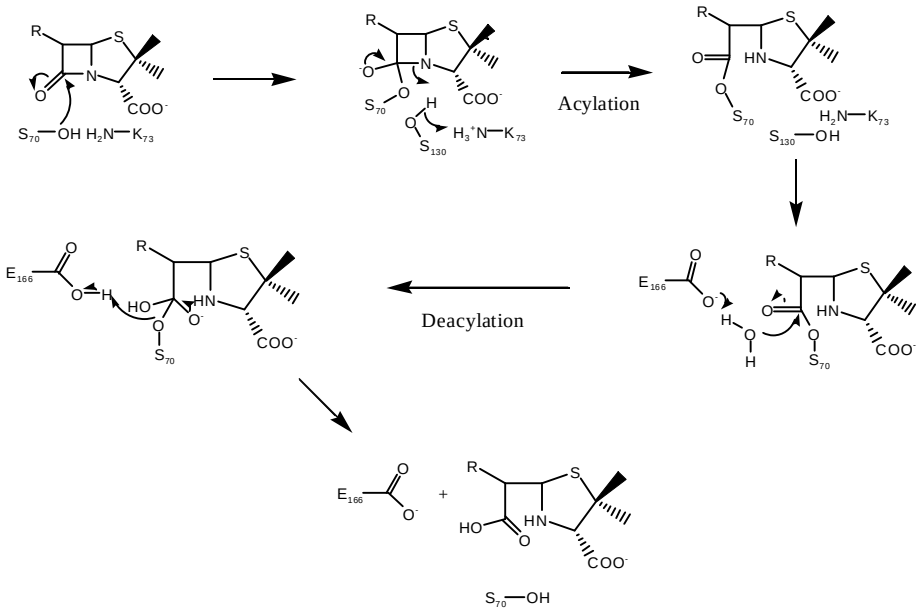
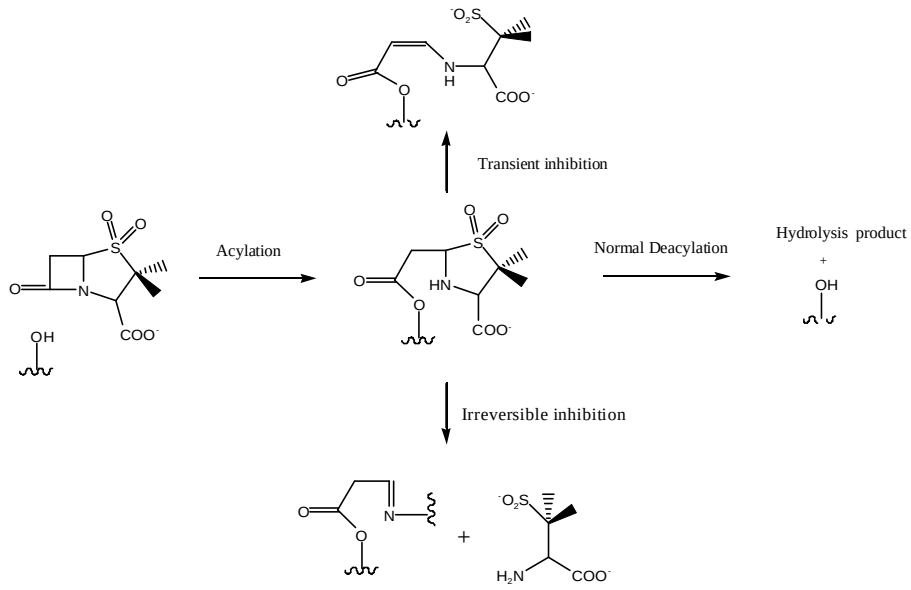


Figure 4.2 Inhibitory mechanism of penam sulfone.



second pathway is transient inhibition where the acyl-enzyme intermediate is tautomerized to another much more stable enamine intermediate. The enamine intermediate can be slowly released from the active site to accomplish the turnover of β-lactamase. As the last possibility, the inhibitor forms an intermolecular crosslink with the ε-amine of a lysine proximal to the active site, presumably either Lys73 or Lys234[19], accompanied by the concomitant release of penicillamine sulfuric acid. Thus the activity of β-lactamase is totally lost due to this irreversible inhibition. It was reported that partition of acyl-enzyme intermediate in these three pathways depends on the nature of 6-substituent on the β-lactam, as well as the pH of reaction condition. Generally, in lower pH, the irreversible inhibition is more efficient[20]. Electron-withdraw substituents on the 6-position of β-lactam also facilitate the formation of irreversible crosslink product.

4. Methodology

The catalytic selection involves three steps: 1) the generation of fusion library, 2) the preparation of immobilized inhibitor, and 3) the selection scheme.

a. Generation of mRNA-protein fusion library

The diversity of the β-lactamase fusion library is introduced by error-prone PCR. The mutation rate in each base of the DNA template is controlled as 1.0%[21]. Thus, the resulting protein library has a high percentage of multiple mutations. The probability P of having k mutations in a sequence of length n can be calculated using equation below (Figure 4.3 A).

$P(k,n,e) = (n!/[(n-k)!k!])e^{k}(1-e)^{n-k}$, where ε is the error rate per position.

In a library containing 10^{13} different sequences, the fraction of coverage of N-sites mutants, defined as the number of N-site mutants in library divided by the total possible recombinants of N-site mutants, can also be calculated (Figure 4.3 B). The high mutation rate can guarantee the exploration of multiple mutations with compensatory, additive or co-operative effects[22]. Evolution under low mutation rate, however, can only survey additive mutants in the library.

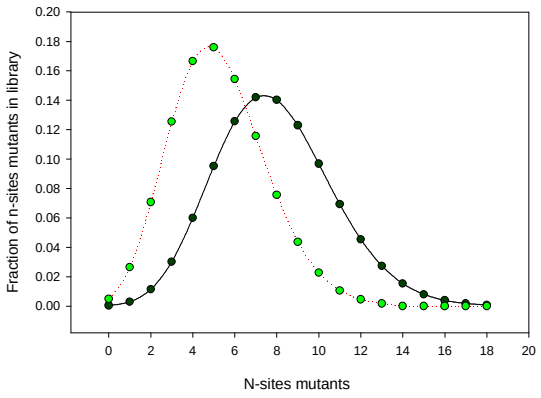
b. Preparation of immobilized inhibitor

Biotinylated penicillanic sulfone is used as the immobilized mechanism-based inhibitor of β-lactamase (Figure 4.4). The biotin moiety allows the inhibitor to be immobilized on the streptavidin agarose gel under harsh conditions. The long polyether chain provides space to avoid potential interference of the solid support on the catalytic reactions. In addition, the existence of a disulfide bond makes it feasible to liberate the immobilized enzymes from the streptavidin agarose under mild reductive conditions.

c. Scheme for selection of b-lactamase turnover

Following the generation of β-lactamase fusion library, the entire library is incubated with the biotinylated penicillanic sulfone in the presence of ampicillin. Both active mutants that can regenerate themselves and mutants with the properties of penicillin binding proteins have the capability to form covalent link with inhibitor. Thus these two types of mutants can not be distinguished if only biotinylated penicillanic sulfone is used. The presence of excess ampicillin, the substrate of β-lactamase, can prohibit the mutants similar to penicillin binding proteins from binding with inhibitors[23]. Only mutants that are able to turnover can be immobilized and subjected for further cycles of selection.

Figure 4.3 [A]. Distribution of N-site mutants in β-lactamase library. The size of DNA template is 789 bp and mutation rate is 1.0% base. The black points represent the number of mutants in DNA template and the green points represent the number of mutants in respective protein. [B]. Log(fraction of coverage) to N-site mutants. The fraction of coverage is defined as the number of N-site mutants in a library containing 10^{13} different sequences divided by the total possible recombinants of N-sites mutants when the mutation rate is 1% for β-lactamase DNA template. For 1- 2- 3-site mutants, the number of copies that have the same sequence is show on the top of respective columns.



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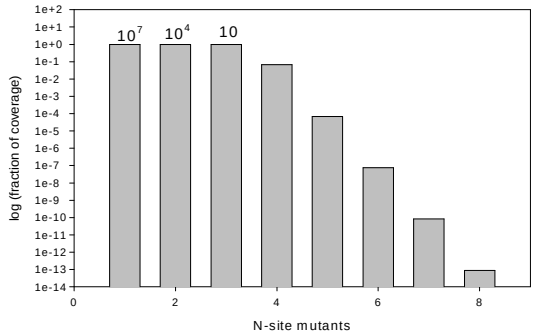
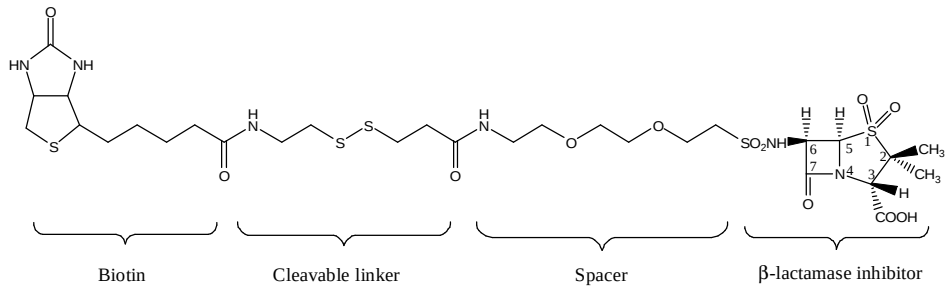
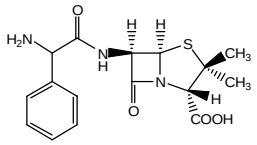


[B]

Figure 4.4 Structure of biotinylated penicillanic sulfone, a mechanism based inhibitor of β-lactamase. As a comparison, the structure of ampicillin, a substrate of β-lactamase, is also drawn.



biotinylated penicillanic acid sulfone



Ampicillin

4.2 Results and discussion

Several technical issues have impeded the accomplishment of this catalytic selection. For example, although the protocol for making small mRNA-peptide fusions is quite mature, the preparation of mRNA-lactamase fusion proved to be a new challenge due to relatively large size of β-lactamase (263 amino acid residues) and its encoding mRNA (814 oligonucleotides). The nature of large β-lactamase imposes three problems: 1) the secondary structure prevents the efficient ligation of mRNA with the short puromycin oligomer, 2) ligated mRNA is inseparable from unligated mRNA in polyacryamide gel, and 3) the fusion formation from ligated mRNA is inefficient. To enhance the ligation, the oligomer mixture in water was pre-heated to disrupt secondary structures. The sample was then cooled down in ice to allow the annealing between complementary sequences. The second problem can be solved by a two-step separation, gel purification followed by dT-cellulose affinity chromatography. Nevertheless, the optimization of conditions was unable to improve the fusion formation.

The activity assay for mRNA-linked β-lactamase was also problematic due to the limited amount of fusion. The standard nitrocefin assay was not sensitive enough to test such low amount of enzyme. Thus, whether the mRNA-linked β-lactamase is functional or not is still a mystery.

The purpose of this project is to address the catalytic selection with mRNA display library. The mRNA display β-lactamase is by far the largest mRNA protein fusion made in our lab. The lessons we have learnt include 1) the mRNA display technique is inefficient for large protein fusion, and 2) the appending mRNA template

may impair the activity of its encoding enzyme. Nevertheless, a recent similar research on ribosome display library indicates the catalytic selection with immobilized mechanism-based inhibitors is a powerful approach to identify functional mutants of enzymes. Future work of this unfinished project will focus on the identification of the "smallest" catalytic polypeptides from a fusion library containing ~100 amino acid residues.

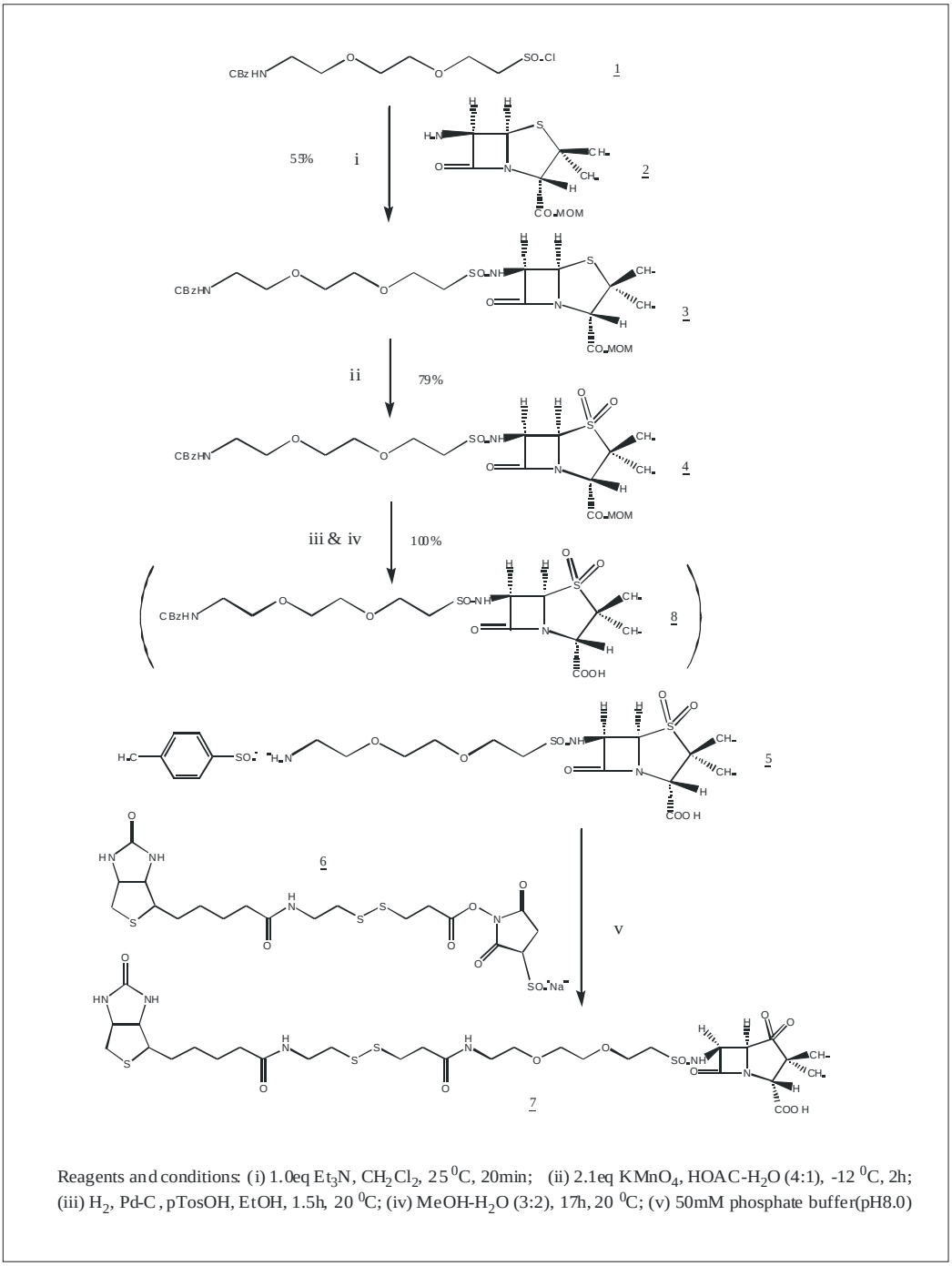
The existence of catalytic RNA has already shed a light on the early evolution of life. However, the evolution of enzymes remains enigmatic. Was the takeover of catalysis from RNA by proteins originally performed by peptides or were "modern"-sized proteins recruited to play this role? We hope to answer these questions by examining the abundance of short protein catalysts in future.

4.3 Materials and methods

1. Synthesis of biotinylated penicillanic acid sulfone

The synthesis of biotinylated penicillanic acid sulfone follows the procedure described previously (Figure 4.5). MOM (methoxymethyl) protected 6-aminopenicillanic acid 2 is used as the starting material to give penam derivative 3. Subsequent oxidation of 3 with $KMnO_4$ results in the protected penicillanic acid sulfone 4. MOM deprotection by mild hydrolysis quantitatively yields the free acid 8. Instead, hydrogenation of protected penicillanic acid sulfone 4, followed by deprotection of MOM, can give the total deprotected penam sulfone 5. This sulfone reacts with biotin-SS-NHS 6 to give the final product biotinylated penicillanic acid sulfone 7. Since the p-toluenesulfonate counter anion in the totally deprotected penam sulfone 5 has a high UV absorbance at

Figure 4.5 Synthesis pathway of biotinylated penam sulfone.



Reagents and conditions: (i) 1.0eq Et_3N, CH_2Cl_2, 25 ^{0}C, 20min; (ii) 2.1eq $KMnO_4$, HOAC-H_2O (4:1), -12 ^{0}C, 2h; (iii) H_2, Pd-C, pTosOH, EtOH, 1.5h, 20 ^{0}C; (iv) MeOH-H_2O (3:2), 17h, 20 ^{0}C; (v) 50mM phosphate buffer(pH8.0)

235nm that interferes with the inhibitory measurement, the partial deprotected penam sulfone 8 was used to examine its inhibitory competence. More than 70% activity of β-lactamase is lost after 3 hours incubation with inhibitor at pH 4.5 (Figure 4.6 A). However, the inhibitor is unable to inactive β-lactamase at pH 7.0 because the normal deacylation pathway is favored at high pH (Figure 4.6 B).

2. Preparation of mRNA-lactamase fusion

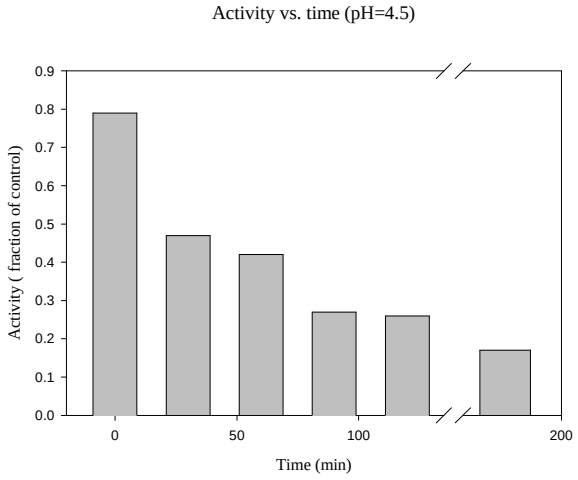
a. Preparation of b-lactamase DNA template

RTEM-1 β-lactamase DNA template is amplified from a plasmid pET22b (Novagene) by polymerase chain reaction (PCR), using two synthetic oligomers (P063a01 and P028a01) as primers. The composition of P063a01 includes a T7 promoter sequence, a sequence adopted from the 5' untranslated region (5'-UTR) of tobacco mosaic virus, and the first 18 nucleotides of β-lactamase open reading frame. The P028a01 is complementary to the last 28 nucleotides of β-lactamase. The PCR reaction buffer contains 10mM Tris-HCl (pH9.0 at 25°C), 50mM KCl, 0.1% Triton X-100, 1.5mM Mg^{2+}, 200 μM each dNTP, 1 μM each primer, 20ng pET22b, and 2.5U Taq polymerase in per 100 μl solution. The PCR reaction is conducted in a thermal cycler (MJ research) with the program: 3 mins at 94°C followed by 20 cycles of 1 min at 94°C, 1 min at 55°C, 2 mins at 72°C, and ended with 5 mins at 72°C.

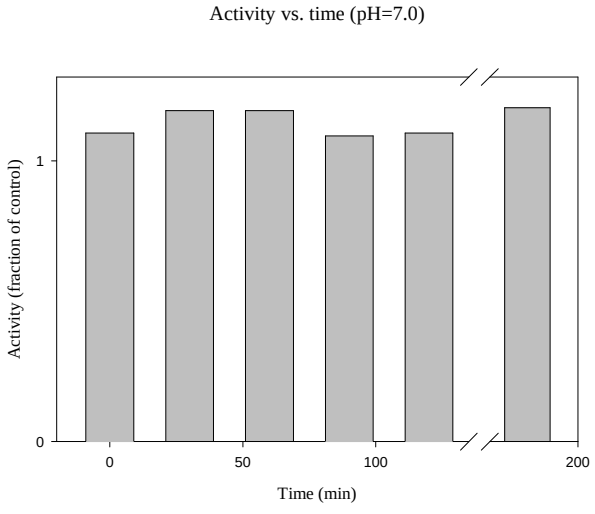
b. In vitro transcription of b-lactamase DNA template

The transcription reaction is accomplished in the buffer including 80mM HEPES-KOH (pH7.5), 40mM DTT, 25mM Mg^{2+}, 4mM each NTP, 0.2nM DNA template, and 10U/ml T7 RNA polymerase. The reaction mixture is incubated at 37°C for 3 hours,

Figure 4.6 [A]. Inhibition of β-lactamase by penicillanic acid sulfone at pH4.5. The remaining activity of β-lactamase is taken as the fraction of the activity of β-lactamase with inhibitor over that of β-lactamase without inhibitor. [B]. Inhibition of β-lactamase by penicillanic acid sulfone at pH7.0.



[A]



[B]

then 1/10 volume of EDTA (500mM, pH8.0) is added to dissolve magnesium pyrophosphate precipitate. The supernatant is extracted with phenol/chloroform (50:50) mixture and subjected to ethanol precipitation. The mixture is incubated at –70°C for 30 min or at –20°C for overnight. After spinning at 15000 RPM for 10 min at 4°C, the pellet is washed with 70% ethanol twice and dried in air, then dissolve in DEPC-treated water. The RNA template is transferred to a size exclusion column (NAP-25, Pharmacia Biotech) to remove nucleotides.

c. Ligation of b-lactamase mRNA with puromycin oligonucleotides

A short oligomer 30-P with a 3'-puromycin is synthesized from puromycin-CPG (Glen Research). The β-lactamase mRNA ligates with 30-P with T4 DNA ligase in the presence of a splint template (P030a02). The ligation reaction consists of 50mM Tris-HCl (pH 7.8 at 25°C), 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 25 μg/ml BSA, 15 μM 30-P, 15 μM mRNA, 30 μM P030a02, and T4 DNA ligase at a concentration of 2U per picomole of mRNA. The mixture of oligomers is heated at 90°C for 30 secs first and cooled down for 15 min at ice. After adding T4 DNA ligase and ligation buffer, the reaction is performed at room temperature for 1 hour.

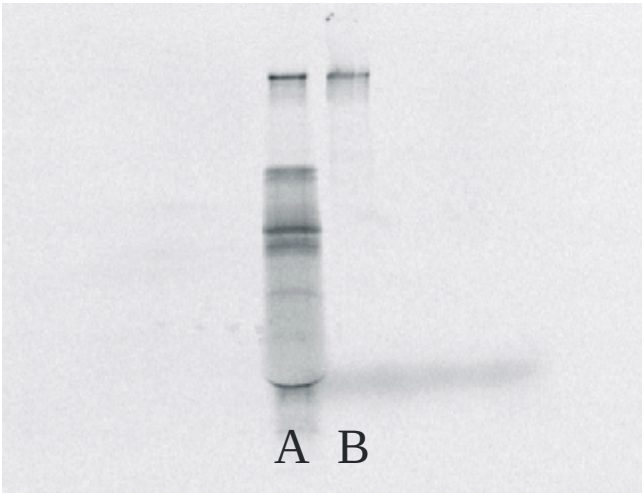
Two steps are required to purify ligated mRNA from unligated mRNA because they are indistinguishable even in 5% urea-PAGE (polyacrylamide gel electrophoresis). First, both ligated mRNA and unligated mRNA are separated from unreacted 30-P and P030a02 by PAGE. Then, ligated mRNA is purified with dT-cellulose (New England Biolab). The sample is shaken with dT-cellulose for 1 hour at 4 °C in binding buffer (1M NaCl; 20mM Tris-HCl, pH 8.0; 10mM EDTA; 1 mM DTT; 0.2% Triton X-100). After washing with 10 volume of binding buffer, following with 10 volume of washing buffer

(0.3 M NaCl; 20mM Tris-HCl, pH 8.0; 1 mM DTT), pure mRNA is eluted from dT-cellulose with water. A quick spin of elution in 0.2 μm filter unit easily removes the fine powder of dT-cellulose resin.

d. In vitro translation of b-lactamase

Translation reaction is performed in reticulocyte lysate (Novagene) by following conditions recommended by manufacturer. Upon the completion of translation, Mg^{2+} in mixture is adjusted to 50mM and K^{+} to 0.6M. The mixture is then kept at –20°C for 24 hours for dT-cellulose purification and SDS-PAGE analysis (Figure 4.7).

Figure 4.7 Preparation of mRNA-lactamase fusion. Lane A: fusion in rabbit reticulocyte lysate; Lane B: fusion after dT-oligo purification.



3. Inhibitory measurement of partial deprotected penicillanic acid sulfone 8

3 mg partial deprotected penicillanic acid sulfone 8 was dissolved in 500 μl mixture of 95% water and 5% DMSO. 25 μl inhibitor solution (10mM in 5% DMSO) was added to 75 μl reaction solution (NaOAC, 100mM, pH 4.5) containing β-lactamase at room temperature. 2 μl portions were taken out at an interval of 30 minutes and diluted into 100 μl of ampicillin solution (1mM; 50mM phosphate, pH 7.0). The enzyme activity was recorded for 5 minutes as the UV absorbance at 235nm decreases. As a control, another β-lactamase solution without the inhibitor was tested the same way. The remaining enzyme activity in the presence of inhibitor was calculated as the fraction of control enzyme's activity. All kinetic studies are conducted at 25°C.

Oligo sequences:

P063a01

5’-TAATACGACTCACTATAGGGACAATTACTATTTACAACTACCATGCACCC
AGAAACGCTGGTG

P028a01

5’-CCGCTCGAGCCAATGCTTAATCAGTGAG

P030a02

5’-TTTTTTTTTTTTTTNCCAATGCTTAATCAG

N: 50% T, 16.7% A, 16.7% G, 16.7% C

F30P

5’-d$A_{21}[C_9]_3$dACdCP;
C_9=triethylene glycol phosphate
P = puromycin

4.4 References

1. Thunnissen, M. M., Taddei, N., Lighri, G., Ramponi, G. and Nordlund, P. Crystal structure of common type acylphosphatase from bovine testis. *Structure* **5**, 69-79 (1997).

2. Hahn, K. W., Klis, W. A. and Stewart, J. M. Design and synthesis of a peptide having chymotrypsin-like esterase-activity. *Science* **248**, 1544-1547 (1990).

3. Atassi, M. Z. and Manshouri, T. Design of peptide enzymes (pepzymes) — surface-simulation synthetic peptides that mimic the chymotrypsin and trypsin active-sites exhibit the activity and pecificity of the respective enzyme. *Proc. Natl. Acad. Sci. USA* **90**, 8282-8286 (1993).

4. Arnold, F. H. Design by directed evolution. *Acc. Chem. Res.* **31**, 125-131 (1997).

5. Zhao, H. and Arnold, F. H. Combinatorial protein design : strategies for screening protein libraries. *Curr. Opin. Struct. Biol.* **7**, 480-485 (1997).

6. Lerner, R. A., Benkovic, S. J. and Schultz, P. G. At the crossroads of chemistry and immunology: Catalytic antibodies. *Science* **252**, 659-667 (1991).

7. Wagner, J., Lerner, R. A. and III, C. F. B. Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science* **270**, 1797-1800 (1995).

8. Wirsching, P., Ashley, J. A., Lo, C. H. L., Janda, K. D. and Lerner, R. A. Reactive Immunization. *Science* **270**, 1775-1782 (1995).

9. Janda, K. D. et al. Chemical Selection for catalysis in combinatorial antibody libraries. *Science* **275**, 945-948 (1997).

10. Jelsch, C., Mourey, L., Masson, J. M. and Samama, J. P. Crystal structure of *Escherichia coli* TEM1 β-lactamase at 1.8A resolution. *Proteins: Struct. Funct. Gene.* **16**, 364-383 (1993).

11. Knowles, J. R. Penicillin resistance: the chemistry of β-lactamase inhibitor. *Acc. Chem. Res.* **18**, 97-104 (1985).

12. Escobar, W. A., Tan, A. K. and Fink, A. L. Site-directed mutagenesis of β-lactamase leading to accumulation of a catalytic intermediate. *Biochemistry* **30**, 10783-10787 (1991).

13. Strynadka, N. C. J. et al. Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7A resolution. *Nature* **359**, 700-705 (1992).

14. Clarke, A. J., Mezes, P. S., Vice, S. F., Dmitrienko, G. I. and Viswanatha, T. Inactivation of *Bacillus cereus* 569/H β-lactamase by 6-b-trifluoromethane sulfonyl amidopenicillanic acid sulfone and its N-methyl dervative. *Biochimica. Biophysica. Acta* **748**, 389-397 (1983).

15. Dmitrienko, G. I. et al. Inhibition of β-lactamase by 6-b-sulfonamidopenicillanic acid sulfones: evidence for conformational change accompanying the inhibition process. *Bioorg. Chem.* **13**, 34-36 (1985).

16. Marchand-Brynaert, J., Bouchet, M., Touillaux, R., Beauve, C. and Fastrez, J. Design and synthesis of a bifunctional label for selection of β-lactamase displayed on filamentous bacteriophage by catalytic activity. *Tetrahedron* **52**, 5591-5606 (1996).

17. Vanwetswinkel, S., Fastrez, J. and Marchand-Brynaert, J. Synthesis of new sulfonylamido-penicillanic acid sulfones inhibitors of β-lactamase. *J. Antibiot.* **47**, 1041-1051 (1994).

18. Fisher, J., Charnas, R. L., Bradley, S. M. and Knowles, J. R. Inactivation of the RTEM β-lactamse from *E. Coli.*, interaction of penam sulfones with enzyme. *Biochemistry* **20**, 2726-2731 (1981).

19. Imtiaz, U., Billings, E. M., Knox, J. R. and Mobashery, S. A structure-based analysis of the inhibition of class A β-lactamases by sulbactam. *Biochemistry* **33**, 5728-5738 (1994).

20. Kemal, C. and Knowles, J. R. Penicillanic acid sulfone: interaction with RTEM β-lactamse from *Escherichia coli* at different pH values. *Biochemistry* **20**, 3688-3695 (1981).

21. Zaccolo, M., Williams, D. M., Brown, D. M. and Gherardi, E. An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. *J. Mol. Biol.* **255**, 589-603 (1996).

22. Zaccolo, M. and Gherardi, E. The effect of high-frequency random mutagenesis on *in vitro* protein evolution: a study on TEM-1 β-lactamase. *J. Mol. Biol.* **285**, 775-783 (1999).

23. Avalle, B., Vanwetswinkel, S. and Fastrez, J. *In vitro* selection for catalytic turnover from a library of β-lactamase mutants and penicillin binding proteins. *Bioorg. Med. Chem. Letters* **7**, 479-484 (1997).