Screening and Separation of β-Lactam Antibiotics Using Protein-engineered Enzymes[a]

HENRY Y. WANG,[b] MIN ZHANG,[b] AND TADAYUKI IMANAKA[c]

[b]*Department of Chemical Engineering*
University of Michigan
Ann Arbor, Michigan 48109

[c]*Department of Fermentation Technology*
Osaka University
Osaka 565, Japan

INTRODUCTION

With recent advances in biotechnology, it is now possible to synthesize a variety of valuable biologically active products from microbial sources. However, screening and economic recovery of these biosynthetically derived products poses a complex problem. The whole culture broth is an extremely heterogeneous aqueous mixture of biomass, nutrients, residual substrates, and other additives. In some cases, the desired product is generally present in very low concentrations (<0.1 wt %) in this heterogeneous colloidal suspension. Also, bioproducts are usually unstable and are prone to physical, chemical, or enzymatic degradation that limits the choice for separation and screening methods.

We have chosen β-lactam antibiotics as a representative small molecular weight (MW < 1000) bioproduct for investigating the problems associated with screening and processing small molecular weight bioactive compounds. The design and the construction of bioaffinity ligands and adsorbents for screening and processing whole culture broths containing β-lactam antibiotics have been examined. An attempt will be made to generalize the results to draw conclusions about the feasibility of using this scheme for other bioactive materials.

β-LACTAM ANTIBIOTICS

β-Lactams represent the largest group of commercially available antibiotics currently in use. Penicillins and cephalosporins are the two major classes of β-lactam antibiotics produced by fungi. Research and development efforts involving screening of other microbial cells for β-lactams have resulted in a boom of new discoveries in the past decade. Cephamycin was the first major β-lactam reported to be produced for

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Streptomyces species rather than fungi. Following this discovery, a number of new β-lactam antibiotics were found through volume screening of actinomycetes in many laboratories. Most of the β-lactam antibiotics produced by actinomycetes are structurally different penicillins and cephalosporins and are often referred to as nonclassic or atypical β-lactams (TABLE 1). Among the most exciting of these are a series of β-lactams designated as carbapenems.

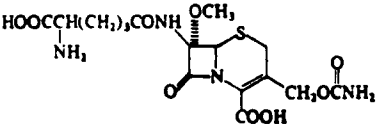
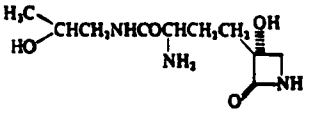
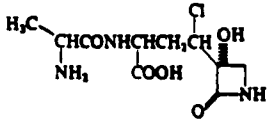
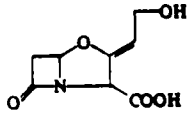
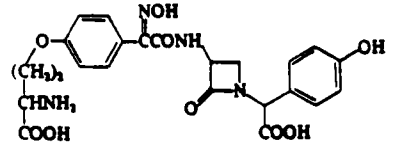
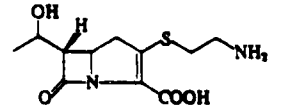
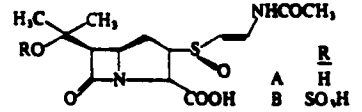
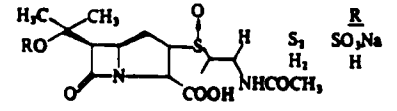
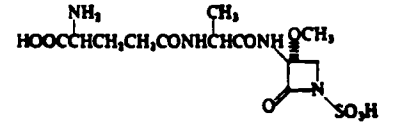
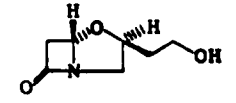
Carbapenems are the most potent of all the β-lactams known today. These compounds are characterized by an annealated five-membered ring containing a double bond. Thienamycin is the first and by far the most important member of this series.[1] Other members of the carbapenem family are epithienamycins, olivanic acids, PS-5, and carpetimycins. Most carbapenems display a broad spectrum of antimicrobial activity covering a wide variety of gram-positive, gram-negative, and anaerobic bacteria. Other atypical β-lactams include oxapenems and penems, which are natural extensions of carbapenems. In these compounds, the carbon atom at the 1-position is replaced by an oxygen or a sulfur atom. Most of these nonclassic β-lactams display an excellent resistance to degradation by a variety of β-lactamases. They also act as strong β-lactam inhibitors in the presence of substrates such as benzylpenicillin. We can safely assume that other, more potent β-lactams may exist in nature.

The question, though, is whether new screening methods can be developed to isolate and discriminate these new antibiotics from the existing antibiotics. The discovery of nonclassic β-lactams was soon followed by attempts to develop bioseparation methods that were suitable for processing large quantities of fermentation broth. However, economic recovery of these antibiotics presents a fairly complex engineering problem. The whole fermentation broth is an extremely heterogeneous colloidal suspension consisting of proteinaceous materials, a variety of other macromolecules, particulate solids, and cells. The antibiotic concentration in the broth is usually extremely low (~5–10 μg/mL). Most of the nonclassic β-lactams are stable only in low concentrations (<1.0 mg/mL) and in narrow ranges of pH.[1,2] Kahan *et al.*[1] in their pioneering work reported production of thienamycin in an overall recovery yield of less than 2%. Trieber *et al.*[3] subsequently reported an improved multistep process based on ion-exchange and reverse-phase chromatography. The overall yield by this process is still limited to 25–35%. The potential of ion-exchange chromatography for separation of thienamycin is quite limited due to the zwitterionic nature of the antibiotic. Furthermore, various amino acids and small peptides in the broth that follow a protonation pattern similar to thienamycin also get adsorbed nonspecifically to the ion-exchange resin. Similar problems are encountered in attempts to isolate other nonclassic β-lactams.[4]

AFFINITY ADSORPTION

Affinity adsorption due to its high degree of selectivity offers a promising alternative in the initial steps of a conventional biochemical product recovery scheme. It is a separation technique based on specific and reversible binding using biological interactions. Affinity adsorption is now frequently used in the purification steps, but it is seldom used in processing more complex solutions such as fermentation broth. This is mainly because of the need to have relatively clean solution devoid of any suspended

TABLE 1. Representative β-Lactam Antibiotics Produced in Actinomycetes

Compound	Structure	Organism
Cephamycin C		*S. clavuligerus*
Wildfire Toxin (Tabtoxin)		*Pseudomonas tabaci*
X–372A		*Streptomyces* sp. 372A
Clavulanic acid		*S. clavuligerus*
Nocardicin A		*Nocardia uniformis*
Thienamycin		*S. cattleya*
Carpetimycins		*Streptomyces* sp. KC-6643
C–19393 S_1 and H_2		*Streptomyces griseus* subsp. *cryophilus*
Sulfazecin		*Pseudomonas acidophila*
Hydroxyethyl-clavam		*Streptomyces antibioticus* subsp. *antibioticus*

solids, cell debris, or fouling proteins to perform affinity chromatography. Even a small fraction of solids carryover may lead to problems such as fouling and clogging of the affinity column, causing significant reduction in column performance.[5] Mechanical properties of adsorbent particles and reduced product adsorption under the conditions of high flow rates further limit the utility of affinity chromatography for large-scale applications.

One way of separating the bioproduct is by freely suspending the affinity adsorbent particles in the whole fermentation broth. Adsorbent particles consist of biospecific ligands covalently attached to an inert, porous, solid support. Many problems are encountered in this approach. Large adsorbent particle size is required to ensure easy handling in the broth. However, this leads to high internal mass-transfer resistance, which in turn significantly reduces the adsorption rate. Moreover, the presence of various organic macromolecules can lead to rapid fouling of the adsorbent particles. This can introduce the problems of extensive product contamination and reduced effectiveness of the affinity adsorbent. Affinity ligand itself can undergo enzymatic or chemical degradation due to free contact with proteolytic enzymes and other contaminants in the whole broth. The immobilized affinity adsorbents developed can potentially be used to isolate and screen new β-lactams during the drug discovery program.

We have been investigating the possibility of using affinity adsorbents entrapped in hydrogel beads or capsules for whole broth processing.[6,7] The hydrogel matrix can be provided by various reversible hydrogels. These hydrogels contain more than 95% water in their matrix. Consequently, additional resistance offered by the hydrogel matrix to the antibiotic diffusion is relatively insignificant. It is therefore possible to use relatively large beads or capsules (1–3 mm radius) to ensure easy recovery from the whole broth at the end of the screening and separation processes. These hydrogels can be dissolved by removing the multivalent cations used for cross-linking or by inducing temperature shifts to recover the adsorbed bioproduct. Most of the macromolecules and the proteolytic enzymes present in the broth are excluded from the immobilized bead because of pore size restriction. Undesired macromolecules that do penetrate foul the outer layer first. Most of the ligand distributed inside remains uncontaminated and can be recycled for further use. Preliminary research conducted in our laboratory using immobilized adsorbents has indicated significant advantages in terms of high adsorption rates, high selectivity, and reduced fouling characteristics.[7]

β-LACTAMASE AS AFFINITY LIGAND

β-Lactamases hydrolyze penicillins to produce penicilloates. Cephalosporates, on the other hand, are unstable in nature and decompose further. Most of the β-lactamases produced by gram-positive bacteria are inducible or exocellular.[8] β-Lactamases produced by gram-negative microorganisms can be either inducible or constitutive. In gram-negative microorganisms, β-lactamases are usually periplasmic or cell bound.[9] Several β-lactams have been reported to inhibit β-lactamase activity. Two major types of β-lactamase inhibitors have been identified:

(1) β-lactams that to some extent bind reversibly to block the active site of the enzyme and that display strong resistance to hydrolysis;

(2) β-lactams that inhibit β-lactamase activity by providing a competing substrate for the active site during the course of their normal hydrolytic reaction.

Most of the nonclassic β-lactams such as carbapenems belong to the first category. However, they may sometimes display a combination of both mechanisms during the course of the binding reaction.

The ability of β-lactams belonging to the first category to inhibit β-lactamases without undergoing significant hydrolysis makes them ideally suited for affinity extraction from fermentation broths using β-lactamase as the affinity ligand. Several research groups have recently reported cloning and expression of β-lactamase genes in a variety of host cells.[10,11] Large amounts of β-lactamase can be produced by these recombinant cells for use in production-scale separation applications. The enzyme can be separated and immobilized on a solid matrix such as controlled pore glass particles and it then can be entrapped in the hydrogel bead. Another possibility is to immobilize β-lactamase-producing cells directly in inert hydrogel beads or capsules, which will restrict the extracellular enzyme from leaking out into the broth. Immobilized cells can then be used as bioadsorbents for extracting β-lactam antibiotics directly from the broth. A major difficulty in using β-lactamases as affinity ligands is to elute the adsorbed antibiotic without hydrolyzing its active β-lactam ring. The hydrolytic activity of the enzyme can be significantly reduced by operating at low pH values (~4). However, our experience has indicated almost total degradation of the bound antibiotic even when extremely mild elution conditions such as shifts in pH or ionic strengths were employed.[12] Similarly, several investigators have reported significant degradation of β-lactam antibiotics in the reverse situation where they were employed as affinity ligands to purify small quantities of β-lactamase.

PROTEIN ENGINEERING OF β-LACTAMASE

Recent advances in protein engineering techniques have raised the exciting possibility of altering the amino acid sequence of this enzyme at its active site by making site-specific mutations. Several researchers have recently reported the changes observed in the catalytic characteristics of β-lactamase by effecting changes in the amino acid sequence at its active site.[13–15] The site-specific mutations can be used to "engineer" the enzyme such that it can only bind to the β-lactam reversibly without hydrolyzing it. The altered enzyme can then be used as an ideal ligand for affinity separation purposes.

The nucleotide sequence and restriction mapping of β-lactamase has already been reported in the literature.[15] This gene codes for a protein of 286 amino acid residues. The first 23 amino acids presumably form a signal for secretion because they do not appear to be in the mature enzyme whose partial amino acid sequence has been determined independently. The 3-D structure of *Staphylococcal* lactamase has been elucidated.[16] A computer-generated model of β-lactamase interacting with an ampicillin molecule is shown in FIGURE 1. Using various recombinant plasmids, β-lactamase genes from *Bacillus licheniformis* have been successfully cloned and expressed in *E. coli*, *Bacillus subtilis*, and *Bacillus stearothermophilus*.[10,11] Excess β-lactamase production has been observed in many cases. β-Lactamase catalyzes the hydrolysis of the amide bond of the lactam ring of penicillins and related antibiotics. The catalytic pathway includes an acyl-enzyme intermediate. A serine residue probably participates

in catalysis. It is speculated that Ser-70 and Thr-71 probably form a conserved dyad. Site-specific mutagenesis has been used to invert the order of the dyad from Ser-Thr to Thr-Ser (i.e., a double mutation: Ser-70 → Thr and Thr → Ser). The mutant bacteria show no β-lactamase activity, suggesting a functional defect in the enzyme.[17] Affinity extraction of β-lactam antibiotics that are resistant to the β-lactamase requires the elucidation of the structure of the enzyme's active site. Because the amino acid

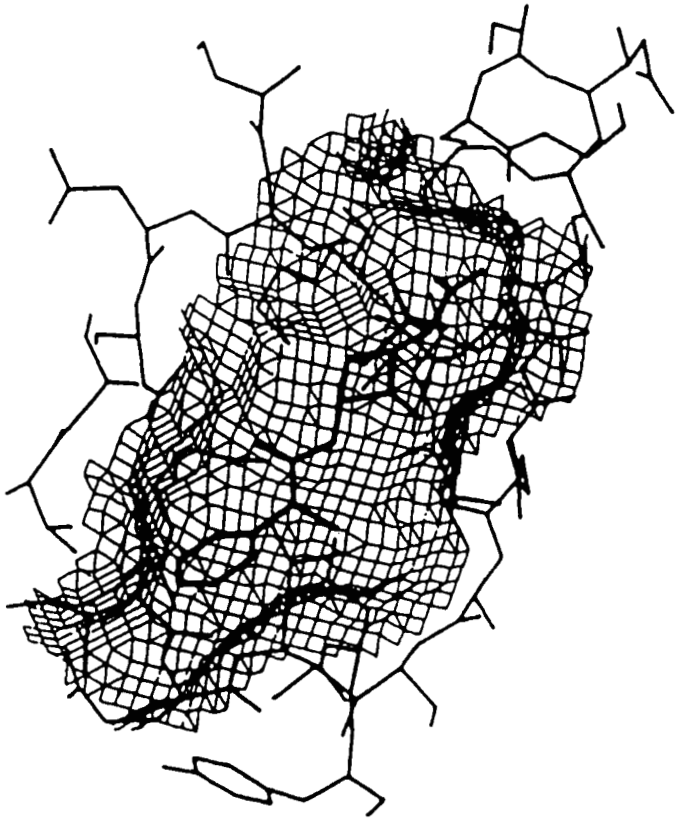


FIGURE 1. A computer-generated space filling model of the active site depression of β-lactamase. The proposed site is bound with an ampicillin molecule.

sequence of the β-lactamase is known, a rational approach to develop affinity ligands for β-lactam antibiotic extraction is possible.

The protein-engineered ligands were then used in subsequent experiments. In the initial phase, a gene bank of various β-lactamases was collected and used in the experiments. Oligonucleotide-directed site-specific mutagenesis was used initially to change the primary structures of the β-lactamases. The recombinant β-lactamases were tested for their ability to bind various β-lactams under different experimental conditions. Our initial success in developing first-generation recombinant proteins that

have these characteristics gives us the confidence that this is a viable approach.[18] We are now investigating the use of β-lactamase genes from *Pseudomonas aeruginosa* and other organisms that have much lower K_m values than those of the ones from *Bacillus* spp. and are comparing their binding abilities.

DEVELOPMENT OF MEMBRANE AFFINITY ADSORBENTS

We have developed the use of membrane-encapsulated affinity and other semispecific adsorbents for directly extracting the bioproduct from the crude solutions, bypassing the solids removal step.[6,7] Solid or liquid phase adsorbents can be encapsulated in membranes made of hydrogels such as calcium alginate, agarose, chitosan, or their mixtures (FIGURE 2). The membrane serves as a protective barrier against fouling. The membrane can also be used as a molecular sieve to increase the selectivity towards bioproducts having smaller molecular size by restricting the diffusion of larger contaminants. The membrane-encapsulated adsorbents can be directly contacted with crude solutions containing suspended solids. Due to their relatively large size (0.5–1.5 mm diameter), encapsulated adsorbents can be easily retrieved from the crude solution by sieving or gravity settling. If continued or repeated use of encapsulated adsorbent leads to fouling of the protective membrane, the adsorbent material can be recovered by disrupting or dissolving the hydrogel membrane. Recovered adsorbent can be reencapsulated for further use.

Three basic types of encapsulated affinity adsorbents can be prepared:

(a) Membrane-encapsulated polymer/affinity ligand derivative: Affinity ligand (recombinant β-lactamase) can be bound to high molecular weight soluble polymers such as dextrans, polyethylene glycol, or polyacrylonitrile. These polymer ligand derivatives can be encapsulated in membrane capsules.
(b) Membrane-encapsulated colloidal affinity ligand: It is possible to chemically fix cells of recombinant *Bacillus stearothermophilus* with overexpressed recombinant β-lactamase. A suspension of these cells was membrane encapsulated to serve as affinity adsorbent for β-lactam antibiotics (FIGURE 3).
(c) Membrane-encapsulated porous support/affinity ligand derivative: Commercial

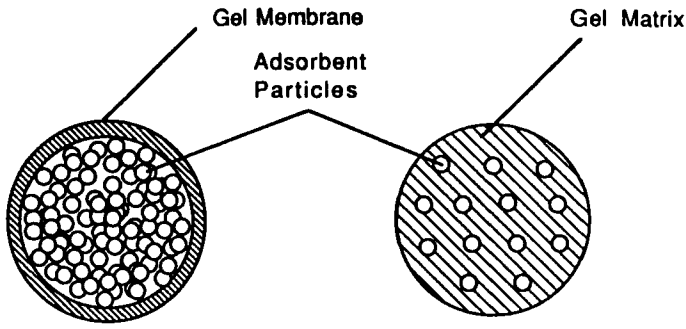


FIGURE 2. Schematic drawings of various membrane-encapsulated affinity adsorbents used for screening and separation.

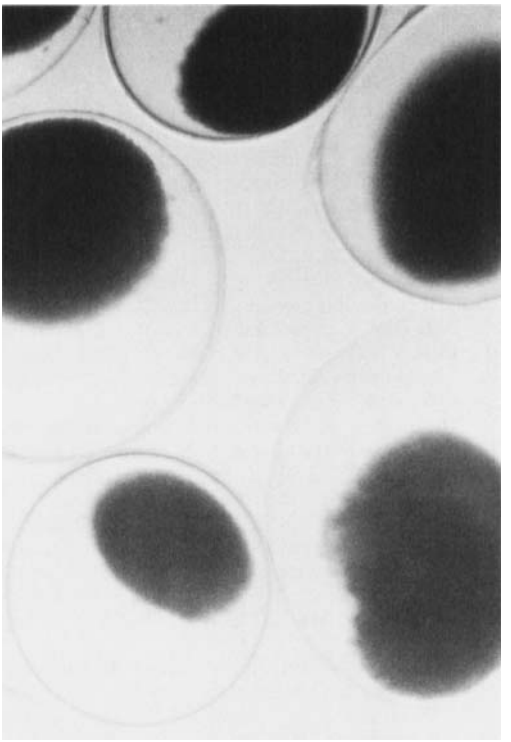


FIGURE 3. A microscopic photograph of membrane-encapsulated recombinant *Bacillus stearothermophilus* that overexpresses β-lactamase. These adsorbents have been used to isolate thienamycin from the fermentation broth.[20]

affinity adsorbents containing recombinant β-lactamase covalently bound to various solid supports can be membrane encapsulated for use in crude solutions. A similar approach has been developed in our laboratories to isolate and purify *Staphylococcal* protein A from cell homogenates.[16]

The optimization of batch adsorption time, capacity, and selectivity needs to be carried out by changing various design parameters of encapsulated adsorbents. A mathematical model similar to the one developed in reference 19 can be utilized in conjunction with experiments to relate the effect of different design parameters on binding kinetics, capacity, and selectivity. We are currently evaluating all of these possible designs of membrane-encapsulated protein-engineered enzymes and other ligands for various bioseparation and bioscreening purposes.

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