A Fluorescein-Modified β-Lactamase as Biosensor for β-Lactam Antibiotics

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Declaration

I hereby declare that the thesis summarizes my own work carried out since my registration for the Degree of Master of Philosophy in October, 2000, and that it has not been previously included in a thesis, dissertation or report submitted to this or any other institution for a degree, diploma or other qualification.

Chan Pak Ho

August, 2002

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Abstract

β-Lactam antibiotics (e.g. cephalosporins and penicillins) and β-lactamase inhibitors (e.g. sulbactam and clavulanate) are clinically important antibacterial agents widely used in the clinical treatment of bacterial infections. Because of their effective antibacterial activities, a great variety of β-lactam antibiotics and β-lactamase inhibitors are produced in the pharmaceutical industry and they are used routinely in the clinical treatment of human beings and livestock. Therefore, a new sensor that can effectively detect these drugs is highly desired. Such a sensor will be of importance in drug screening, discovery of new β-lactamase inhibitors and routine measurement of antibiotic concentration in, for example, contaminated food samples.

In our study, a new biosensor for β-lactam antibiotics and β-lactamase inhibitors has been prepared by labeling a mutant of β-lactamase I (E166C) with the environment-sensitive fluorescent probe fluorescein-5-maleimide. The mutant was constructed by site-directed mutagenesis in which a particular amino acid (Glu-166) on the Ω-loop of the enzyme was replaced with a thiol-containing cysteine residue. The cysteine residue was then labeled with the thiol-reactive fluoresein-5-maleimide via the formation of thioether to give a fluorescein-labeled β-lactamase I (E166Cf).

As the wild-type β-lactamase I contains no cysteine residue, this single-point mutation allows the attachment of the thiol-reactive fluorophore to a specific site on the enzyme. The Glu-166 residue was chosen as the labeling site not only of its closeness to the active site, but also of the flexibility of the Ω-loop, which allows the fluorophore to move when the antibiotic enters the active site.

Spectrophotometric assays showed that the activity of the E166Cf enzyme is conserved after the labeling reaction. Fluorescence measurements indicated that the fluorescence intensity of the E166Cf enzyme increases with the concentration of penicillins (penicillin G, penicillin V and ampicillin) and cephalosporins (cefuroxime, cefoxitin and moxalactam). The fluorescence enhancement is attributed to the conformational change in the active site upon binding of the antibiotic. Such a conformational change causes the fluorescein label to depart from the active site, thus enhancing the fluorescence quantum yield of the fluorescein molecule. Time-resolved fluorescence measurements revealed that the E166Cf enzyme can detect β-lactam antibiotics at trace level (10^{-7} M). Moreover, the E166Cf enzyme is capable of distinguishing penicillin antibiotics from cephalosporin antibiotics. With penicillin G, penicillin V and ampicillin as substrates, the fluorescence intensity of the E166Cf enzyme increases and then declines slowly. In the presence of cefuroxime, cefoxitin

and moxalactam, the fluorescence signal of the labeled enzyme increases slowly but does not decline. The E166Cf enzyme also exhibits different fluorescence signals when incubating with the β-lactamase inhibitors (sulbactam and clavulanate).

The use of the E166Cf enzyme in screening bacteria for β-lactamases against a panel of β-lactam antibiotics has been investigated. β-Lactamase II, penPC β-lactamase, penP β-lactamase and TEM-1 β-lactamase secreted by *Bacillus subtilis* and *Escherichia coli* were screened against penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam using the E166Cf enzyme as a reporting tool. The fluorescence signals from the E166Cf enzyme allow one to distinguish antibiotics that are resistant to the hydrolytic activities of the bacterial β-lactamases from those that are unstable towards the bacterial enzymes.

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Chapter 1

Introduction

1.1 Background

Penicillins and cephalosporins are the two important classes of β-lactam antibiotics widely used in antimicrobial therapies and as health promoting agents in animal feedstuffs. These two classes of antibiotics share a common structural characteristic of four-membered β-lactam ring which is a highly reactive moiety. The four-membered cyclic structure of the β-lactam ring induces a high angle strain which makes the ring readily to be opened via the cleavage of amide bond. Moreover, unlike a normal amide group which has a partial double bond character, the amide resonance on the β-lactam ring is significantly reduced due to the non-planar structure of the bicyclic moiety (Figure 1.1). As a result, the β-lactam carbonyl carbon is susceptible to nucleophilic attacks [1]. The highly reactive β-lactam ring plays an important role in antibacterial mechanism, and therefore this moiety is strictly conserved in all penicillin and cephalosporin antibiotics. Figure 1.2 shows the structures of some penicillin and cephalosporin antibiotics.

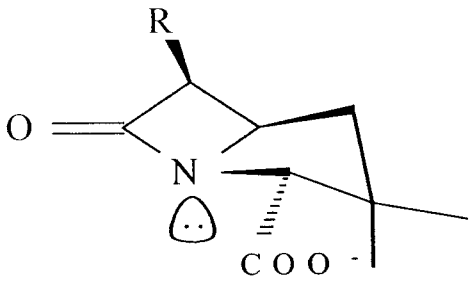


Figure 1.1 Structure of a penicillin molecule. The non-planar structure of the bicyclic moiety reduces the amide resonance on the β-lactam ring [1].

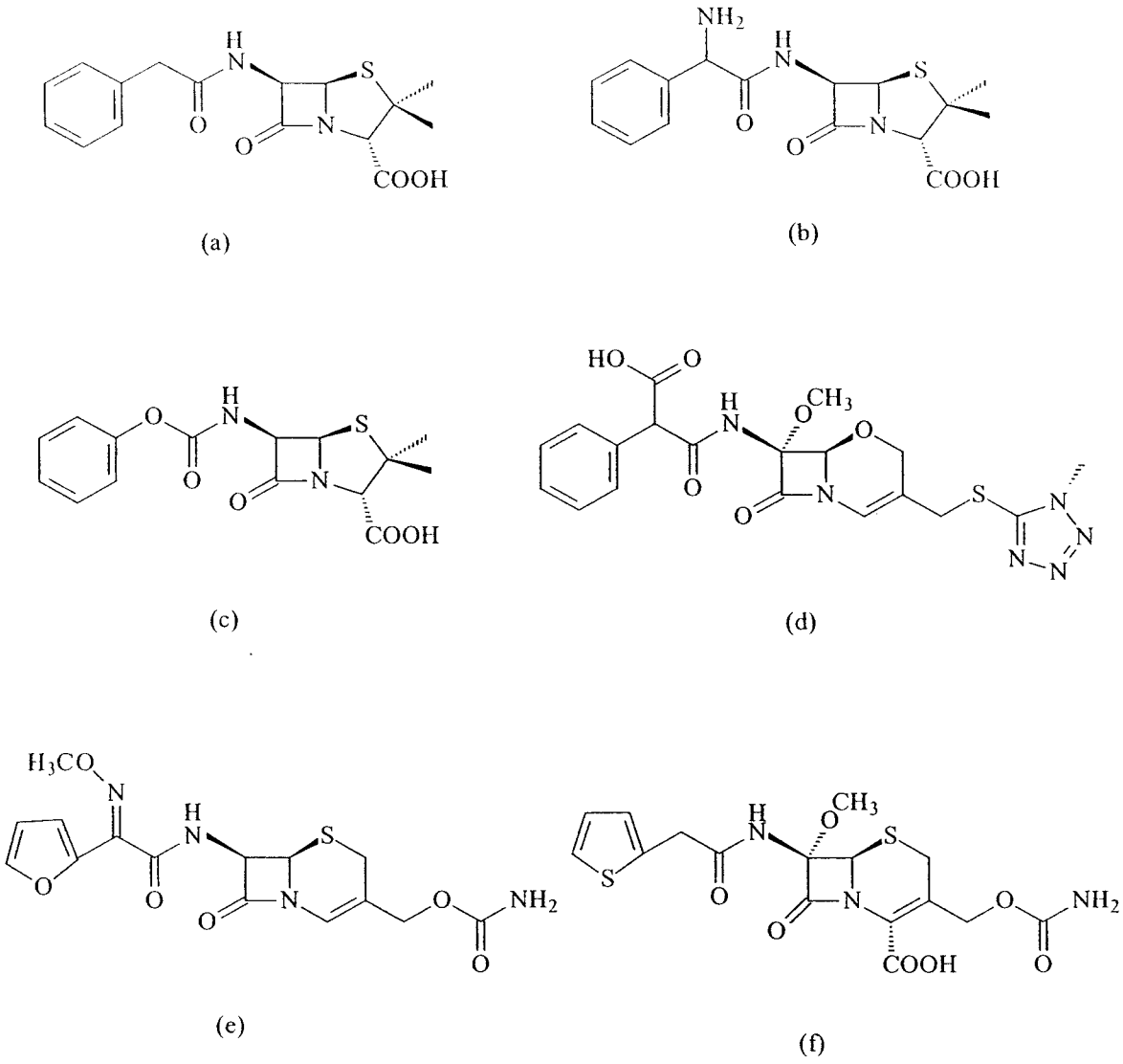
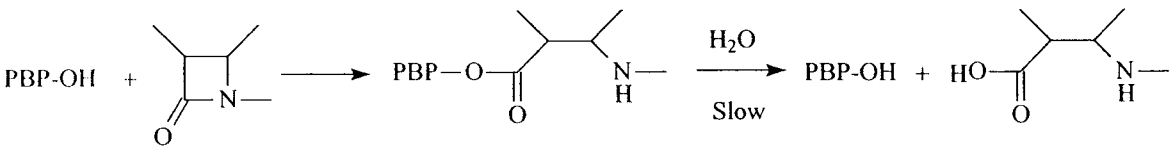


Figure 1.2 Structure of some penicillin antibiotics: penicillin G (a), ampicillin (b), penicillin V (c) and cephalosporin antibiotics: moxalactam (d), cefuroxime (e), cefoxitin (f).

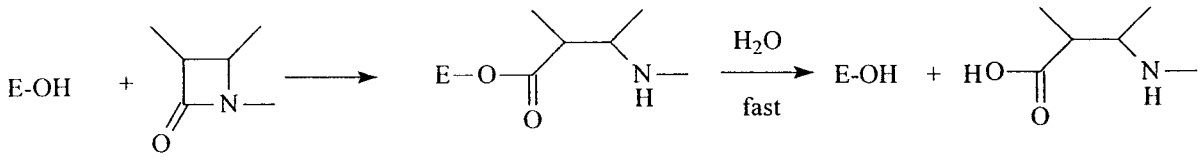
The physiological targets of β-lactam antibiotics are bacterial DD-transpeptidases (also called penicillin-binding proteins). These membrane-bound proteins are active-site-serine enzymes which are responsible for synthesizing bacterial cell wall [2]. Therefore, inactivation of these enzymes will lead to cell death. The antibacterial mechanism of β-lactam antibiotics toward the penicillin-binding proteins involves the acylation of the serine residue in the active site to form an acyl-enzyme complex. This complex, however, is very stable and resists the deacylation to regenerate the free enzyme (Scheme 1.1). As a consequence, the activity of the penicillin-binding protein to synthesize cell wall is impaired which, in turn, leads to cell death [3].

Scheme 1.1



Because of their effective antibacterial activities, β-lactam antibiotics are indispensable tools to antibacterial therapies. The clinical importance of β-lactam antibiotics, however, has been challenged by the emergence of bacteria which are capable of producing β-lactamases [4-6]. The secreted β-lactamases can protect their parent bacteria by catalyzing the hydrolysis of β-lactam rings to inactive carboxylic acids efficiently (Scheme 1.2) [6-8].

Scheme 1.2



1.2 Catalytic properties of class A and class B β-lactamases

To date, more than 200 β-lactamases have been identified [9], and they are divided into four classes (A, B, C and D) based on their different primary sequences [10, 11]. The members of class B (also called β-lactamase II) are metalloenzymes which utilize Zn^{2+} ions as a cofactor [12, 13]. The metal ion plays a dual role in the hydrolysis of β-lactam antibiotics. The Zn^{2+} ion can polarize the coordinated water molecule to give a hydroxide ion which, in turn, nucleophilically attacks the β-lactam carbonyl carbon. In addition, the Zn^{2+} ion can bind and stabilize the tetrahedral oxyanion formed after the nucleophilic attack on the β-lactam carbonyl carbon via metal-ligand coordination [14-16]. A major characteristic of class B β-lactamases is that they can inactivate many penicillin and cephalosporin antibiotics used for therapeutic purposes [17-21]. Therefore the emergence of these enzymes leads to a worrying clinical problem.

Unlike class B β-lactamases which utilize metal ions to catalyze β-lactam hydrolysis [12, 13], the members of class A, C and D are active-site-serine enzymes and can be distinguished based on their primary sequences [10, 11]. The catalytic mechanism of these enzymes involves the acylation of the serine residue in the active site by the β-lactam ring to form a covalent acyl-enzyme complex. This complex is very unstable and undergoes deacylation in the presence of water to regenerate the free enzyme and to give an inactive carboxylic acid [7, 8]. The catalytic pathway of the active-site-serine β-lactamases follows a three-step model (Scheme 1.3) [22].

Scheme 1.3

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$

where E is the free enzyme, S the β-lactam substrate, ES a noncovalent complex, ES^* a covalent acyl-enzyme complex and P the inactive carboxylic acid.

Of all the active-site-serine β-lactamases, the members of class A (also called β-lactamase I) have been intensively studied because they constitute a major proportion of all known β-lactamases [8]. The structures of some class A β-lactamases have been resolved by X-ray crystallography [23-28]. In general, the tertiary structure of class A β-lactamases consists of two globular domains. One domain is composed of α-helices while the other consists of a five-stranded antiparallel β-sheet. The active site is located at the interface between the two domains. The bottom of the catalytic pocket is closed by an Ω-loop. Figure 1.3 shows the tertiary structure of β-lactamase I [29].

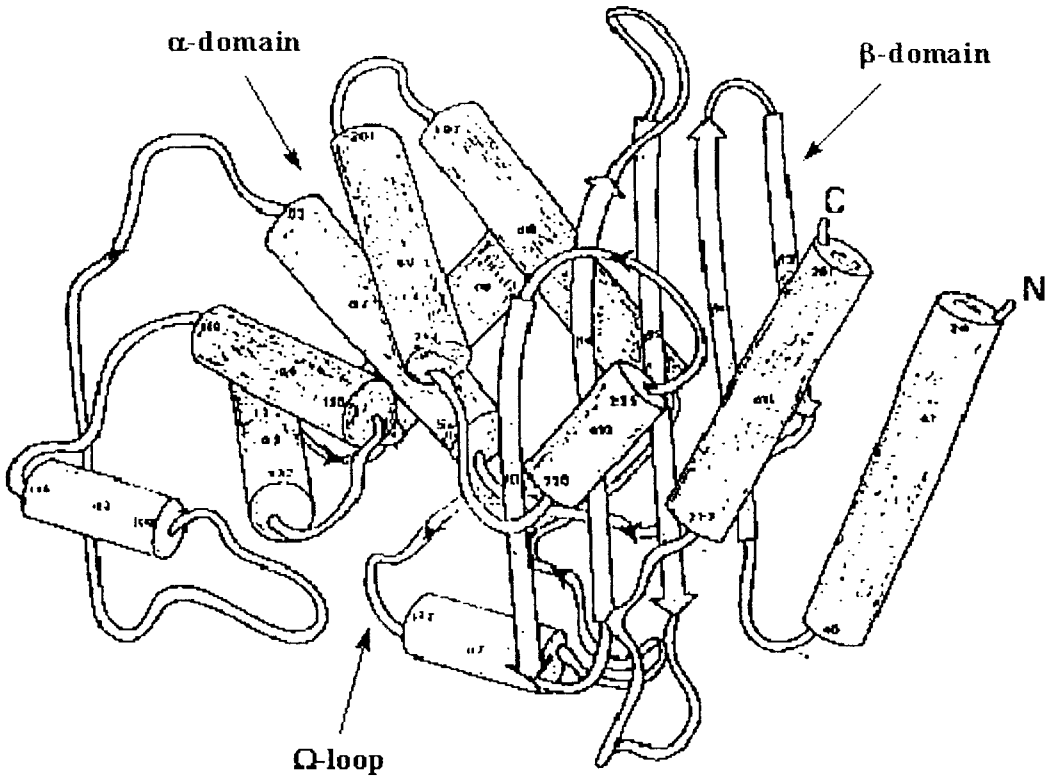


Figure 1.3 Tertiary structure of β-lactamase I [29].

Unlike class B β-lactamases, the members of class A have more specific substrate profiles. In general, they catalyze the hydrolysis of penicillins very efficiently (high k_{cat}/K_m, where k_{cat} is the turnover number which is defined as the number of moles of substrates converted to products per mole of enzyme per second, and K_m is the Michaelis constant which is defined as the substrate concentration that yields one-half of the maximum velocity of the enzymatic reaction) whereas they exhibit poor catalytic efficiency with cephalosporins (low k_{cat}/K_m) [7, 8]. The catalytic mechanism of class A β-lactamases involves several strictly conserved amino acid residues at the active site: Ser-70, Lys-73, Ser-130 and Glu-166 [8]. Figure 1.4 shows the putative mechanism for the formation of covalent acyl-enzyme complex [7]. The catalytic process is first initiated by abstracting the hydroxyl proton of Ser-70 via a water bridge which is hydrogen-bonded with both Ser-70 and Glu-166. The activated O_γ of Ser-70 attacks the β-lactam carbonyl carbon to form an acyl-enzyme complex. The abstracted proton is then delivered back to the β-lactam nitrogen via a network of hydrogen bonds involving the amino groups of Lys-73 and Lys-234, a second water molecule and the hydroxyl group of Ser-130 [30, 31]. Afterwards, the acyl-enzyme complex is deacylated when a hydroxide ion (formed by deprotonation of a water molecule by Glu-166) attacks the ester bond to give a carboxylic acid. The free enzyme is then regenerated by protonation of Ser-70 [30].

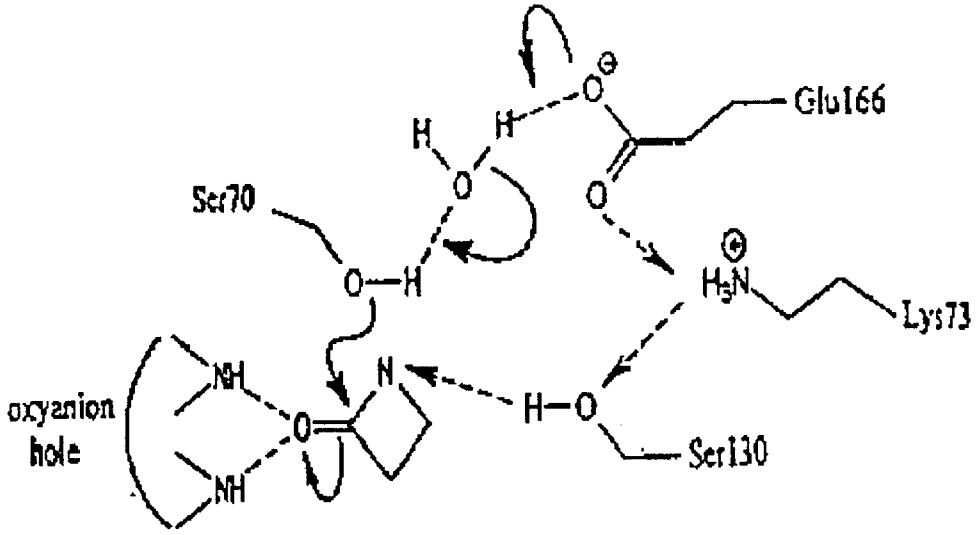
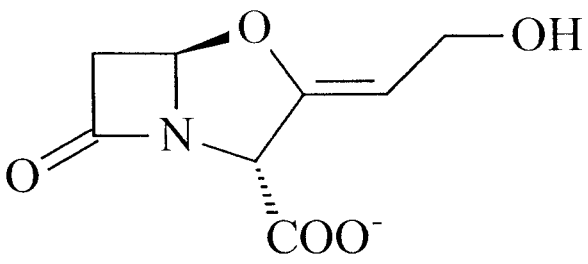


Figure 1.4 Putative mechanism for the formation of the tetrahedral intermediate in the acylation of class A β-lactamases [7].

1.3 Recent developments on assays for β-lactamase activity

The emergence of new or modified β-lactamases with extended substrate profiles due to the widespread use and sometimes abuse of β-lactam antibiotics [8] causes the pharmaceutical industry to produce new β-lactam antibiotics with stronger resistance toward β-lactamases (low k_{cat}/K_m). In addition, β-lactamase inhibitors such as clavulanate and sulbactam have been produced for antibacterial therapies (Figure 1.5). These compounds serve as sacrificial agents to block the active sites of bacterial β-lactamases via covalent modification [32, 33]. Therefore, when they are used in combination with other β-lactam antibiotics, the antibiotics can reach their bacteria targets without being inactivated [34].

(a)



(b)

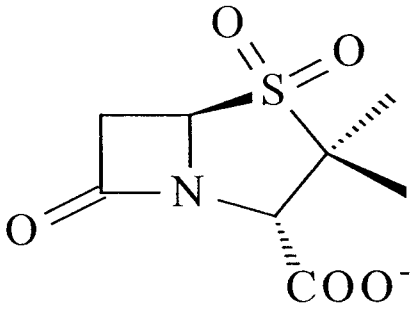


Figure 1.5 Structures of clavulanate (a) and sulbactam (b).

Although a great variety of β-lactam antibiotics are available for antibacterial therapies, clinical treatment of β-lactamase-producing infections is still problematic because a wide variety of β-lactamases have been identified and most of them can inactivate unique subsets of many available β-lactam antibiotics [9]. In addition, β-lactamase encoding genes can be transferred from one strain to another [5]. Therefore, identification of the infecting bacterial species is not a reliable approach to search for effective β-lactam antibiotics. In order to choose a potent antibiotic for a patient confidently, the substrate specificity of the β-lactamase produced by the infecting bacterial species must be known. Therefore, a sensitive method for detecting β-lactamase activity toward various β-lactam antibiotics is required in order to search for potent antibiotics.

The conventional method of detecting β-lactamase activity is based on the monitoring of bacterial growth on an agar plate containing a β-lactam antibiotic and a β-lactamase-producing bacterial strain [35, 36]. When the antibiotic is inactivated by the bacterial β-lactamase, bacterial growth will be observed. Although this method is sensitive, it is time-consuming because overnight incubation is usually required.

Apart from the microbiological method, a number of colorimetric assays such as iodometric [37-39] and acidimetric [40-42] methods have been developed. The iodometric method utilizes iodine/starch complexes as an indicator of β-lactamase activity. When β-lactam hydrolysis occurs, carboxylic acids will be produced and they will reduce the iodine molecules to iodide ions. This results in a decrease in visible absorption at 620 nm. For the acidimetric method, the carboxylic acid produced by the hydrolytic action of β-lactamase causes a decrease in pH of an unbuffered solution, and this can be detected by pH indicators such as phenol red and bromocresol purple. Although the iodometric and acidimetric methods are simple and convenient, they suffer some disadvantages. For example, the sensitivity of the iodometric and acidimetric methods is relatively low, and therefore a high concentration of β-lactam antibiotic is required in order to obtain a detectable signal. In addition, the reaction conditions of the iodometric and acidimetric assays must be carefully controlled to

ensure that the positive results are due to the β-lactamase activities instead of other side reactions. Thus, positive and negative controls must be run in parallel in order to obtain reliable results.

Recently, a new colorimetric assay of β-lactamase activity has been devised [43]. The principle of this assay is that when a β-lactamase hydrolyzes a β-lactam antibiotic to a carboxylic acid, the acid produced will reduce phosphomolybdic acid to a blue reaction product which absorbs visible light at 750 nm. This assay can be applied to β-lactamase-producing microbial cultures because the unique visible absorption of the blue reaction product is well separated from that of the microbial culture. However, the formation of blue reaction product in the absence of β-lactamase was also observed. Therefore, false positive results may be obtained when unstable β-lactam antibiotics are assayed.

Spectrophotometric assays of β-lactamase activity based on measuring the UV spectral changes due to β-lactam hydrolysis have also been developed [44]. Although this method is simple and convenient, it cannot be applied to complex samples such as bacterial culture and crude biological fluid because of their strong interference on the UV spectral measurement.

Detection of β-lactamase activity based on electrochemiluminescence (ECL) method has been developed recently [45, 46]. The principle of this method is that when both ruthenium(II) (tris)bipyridine complex $[Ru(bpy)_3]^{2+}$ and β-lactam antibiotic (coreactant) are oxidized electrochemically, the unstable antibiotic radical will donate one of its electrons to $Ru(bpy)_3^{3+}$ to give excited $Ru(bpy)_3^{2+}*$. This species then returns to its ground state by emitting visible light at 620 nm. When chemically or enzymatically hydrolyzed β-lactam antibiotics were used as coreactants, dramatic changes in ECL signal were observed. In general, hydrolyzed penicillins cause the ECL signal to increase whereas hydrolyzed cephalosporins suppress the ECL signal [45]. However, some exceptional cases were reported. For example, when cefaclor and moxalactam (both are cephalosporins) were tested, the ECL signals were increased [45].

Recently, fluorescence spot tests have been developed for detecting β-lactamase activity [47-49]. The principle of this assay is that when a β-lactam antibiotic is hydrolyzed to carboxylic acid by β-lactamase, the acid formed will react to form a fluorogenic product after heating. The sensitivity of this method was further improved by using fluorescence developers to enhance the fluorescence intensity of the hydrolyzed β-lactam antibiotics [47, 49]. This method is sensitive, convenient and

specific to β-lactamases. However, it is only applicable to the β-lactam antibiotics having an α-amino group and an α-phenyl group on their acyl side chains [47]. Some clinically important β-lactam antibiotics such as penicillin G and cefoxitin, which do not meet the structural requirements, are unable to form fluorogenic products [47]. Therefore, their clinical effectiveness cannot be investigated by this method.

Detection of β-lactamase activity based on calorimetric method has been developed [50]. This method involves the use of an enzyme thermistor and a Chelating Sepharose Fast Flow column immobilized with β-lactamases. When β-lactam antibiotics are hydrolyzed by the immobilized β-lactamases, temperature changes are resulted and these can be detected by the enzyme thermistor. This method, however, is difficult to be developed as a high-throughput assay for detecting β-lactamase activity because the β-lactamase-immobilized column cannot be fed with different β-lactam antibiotics simultaneously.

Recently, Chambers and coworkers [51] have reported an antibody-based assay for detecting β-lactamase activity. In this assay, IFRN 2104, a monoclonal antibody capable of binding to the β-lactamase's active site, is used to probe the resistance of β-lactam antibiotics towards the hydrolytic activity of β-lactamases. When a

β-lactamase is incubated with a poor β-lactam antibiotic (slow β-lactam hydrolysis), a stable ES complex will be formed and therefore the antibody can no longer bind to the β-lactamase. Thus, the inhibition of binding between the β-lactamase and antibody indicates that the antibiotic candidate is resistant to the β-lactamase activity. Although this method can be developed as a high-throughput assay for detecting β-lactamase activity, it is time-consuming because a lengthy incubation of β-lactamases with antibodies is required.

1.4 Detection of β-lactam antibiotics in food samples

As β-lactam antibiotics are effective antibacterial agents, they have been widely used as health promoting agents for livestock (e.g. diary cows). The widespread use and sometimes abuse of β-lactam antibiotics lead to antibiotic contamination of food (e.g. milk), which in turn results in serious health consequences on mankind (e.g. the evolution of antibiotic-resistant bacteria and allergic reactions). For these reasons, the European Union has established the maximum residue levels for a number of β-lactam antibiotics in food (e.g. 4 μg/L for penicillin G in milk) [52]. Therefore, a rapid and convenient method that can detect β-lactam antibiotics at trace levels is highly desired.

Walt and coworkers [53] have reported the preparation of optical biosensors for penicillins. These sensors are prepared by immobilizing both the pH-sensitive fluorophores (e.g. fluorescein and hydroxypyrenetrisulfonate) and β-lactamases on a polymer film which is subsequently attached to the tip of an optical fiber. When the immobilized β-lactamases hydrolyze β-lactam antibiotics to carboxylic acids, a decrease in pH is resulted and hence the fluorescence intensity of the immobilized fluorophore is suppressed. These sensors, however, can only detect β-lactam

antibiotics at 10^{-5} M, which is above the allowed maximum limit of 10^{-8} M β-lactam antibiotics in milk as regulated by the European Union [52]. A number of methods for detecting β-lactam antibiotics have been also developed in recent years, including liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) [54], chemiluminescence (CL) [55, 56], electrochemiluminescence (ECL) [45], fluorescence immunoassay [57] and microbial test [58]. These methods usually involve the use of sophisticated and expensive instruments, and require well-trained crews to perform the tests. Moreover, tedious extraction steps are required in some methods (e.g. LC-ESI-MS/MS [54]). Thus, a sensor that can detect β-lactam antibiotics in liquid samples (e.g. milk) at regulated levels before subjecting to a test for confirmation and quantification is highly desired. Such a sensor should be simple, inexpensive and easy to use.

1.5 Aims and objectives of this project

As noted from the previous section, intensive efforts have been made to develop new assays for detecting β-lactamase activity. Despite this, the assays mentioned previously suffer some disadvantages such as long detection time (e.g. microbiological method [35, 36]) and poor sensitivity (e.g. iodometric [37-39] and acidimetric methods [40-42]). These disadvantages may limit their applications in drug screening. Moreover, a sensor that can detect β-lactam antibiotics at trace levels is highly desired for the routine measurement of antibiotics in liquid samples (e.g. milk).

Because there are great demands for a rapid and high-throughput drug screening method and a sensor for detecting β-lactam antibiotics, we are interested in developing a novel fluorescent biosensor that can sensitively detect β-lactam antibiotics and β-lactamase inhibitors, and can conveniently screen bacteria for β-lactamases against a panel of antibiotics. As a variety of fluorophore-labeled biosensors for small ligands and biomolecules have been successfully developed [59-68], we reasoned that a fluorescent biosensor for β-lactam antibiotics and β-lactamase inhibitors could be developed by labeling a β-lactamase with an

environment-sensitive fluorophore. In this project, we have constructed a mutant of β-lactamase I (E166C) with its Glu-166 on the Ω-loop replaced with a thiol-containing cysteine residue by site-directed mutagenesis. This mutant was then labeled with the thiol-reactive fluorescein-5-maleimide via the formation of thioether (E166Cf). The E166Cf enzyme can serve as a reagentless tool to detect β-lactam antibiotics and β-lactamase inhibitors. In addition, the E166Cf can specifically detect β-lactam antibiotics and β-lactamase inhibitors because the mutant serves as a recognizing element. The preparations of the E166C and E166Cf mutants will be discussed in Chapter 2 of this thesis.

As the fluorescein molecule is attached to the active site of the E166C mutant, the fluorescein label is able to detect the conformational change in the active site upon binding of β-lactam antibiotics. Therefore, we have investigated the fluorescence behavior of the E166Cf enzyme with some penicillins, cephalosporins and β-lactamase inhibitors as substrates. In addition, we have investigated the use of the E166Cf enzyme in screening bacteria for β-lactamases against a panel of antibiotic candidates. The results will be discussed in Chapter 3 and 4 respectively.

Chapter 2

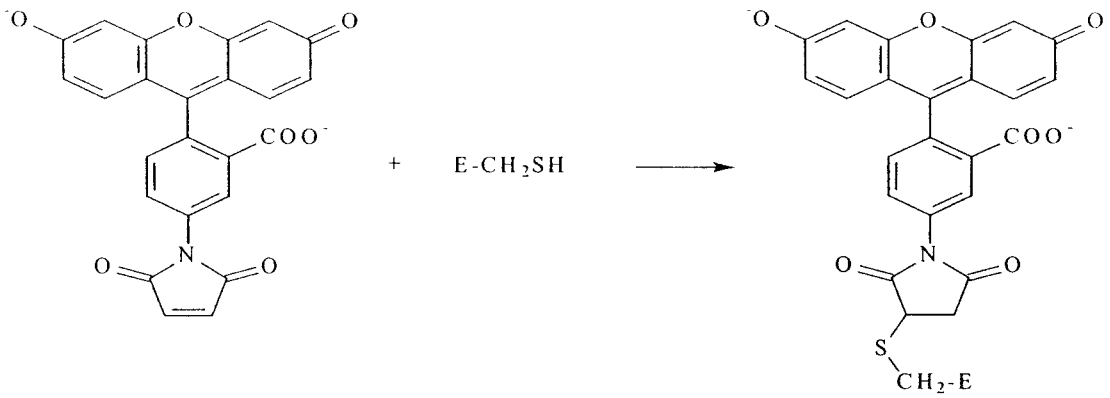
Preparation and characterization of fluorescein-labeled β-lactamase I

2.1 Introduction

Among the 20 amino acids, cysteine is a relatively reactive amino acid because it contains a thiol group (-SH). The thiol group is highly reactive to some organic linkers such as maleimidyl [61, 64] and iodoacetyl [59, 62, 63, 66] groups, and therefore it can be specifically labeled with a fluorophore containing a thiol-reactive linker. As most proteins contain only a few or no cysteines, incorporation of a cysteine residue into a protein by site-directed mutagenesis would allow site-specific fluorophore labeling. This approach has been widely used to label proteins with fluorophores [59-66].

In this project, we have constructed a fluorescein-labeled β-lactamase I (E166Cf) as a biosensor for β-lactam antibiotics and β-lactamase inhibitors using the same approach. A mutant of β-lactamase I (E166C) was first constructed in which a particular amino acid residue (Glu-166) on the Ω-loop was replaced with a cysteine residue by site-directed mutagenesis. The mutant was then labeled with thiol-reactive fluorescein-5-maleimide via the formation of thioether (Scheme 2.1).

Scheme 2.1



As the wild-type β-lactamase I contains no cysteine [69], single-point mutation of this enzyme with a cysteine residue allows the attachment of the fluorescein molecule to the specific site of the enzyme. The Glu-166 was chosen as the labeling site not only of its closeness to the active site, but also of the flexibility of the Ω-loop [70, 71] which allows the fluorescein label to move when a β-lactam substrate enters the active site.

The use of the fluorescein-labeled enzyme as a biosensor for β-lactam antibiotics and β-lactamase inhibitors offers a number of advantages. First, the labeled enzyme is a reagentless tool and therefore no additional reagent is required to give a fluorescence signal. Second, as the labeled enzyme is used as a recognizing element, specific detection of β-lactam antibiotics and β-lactamase inhibitors can be achieved. This is

particularly important when complex samples such as crude biological fluids and bacterial cultures are tested. Third, as the environment-sensitive fluorescein molecule is attached closely to the active site, the labeled enzyme can detect β-lactam antibiotics and β-lactamase inhibitors sensitively by sensing the conformational change in the active site upon binding of antibiotics.

Apart from the E166Cf enzyme, we have also constructed a S130C mutant in which the Ser-130 residue was replaced with a cysteine. This mutant was then labeled with fluorescein-5-maleimide (S130Cf) for comparison with the E166Cf enzyme. The Ser-130 residue is an amino acid located opposite to the Glu-166 residue at the enzyme's active site [29]. When fluorescein-5-maleimide is placed at this position, it may detect a different environmental change when the antibiotic binds to the active site and hence exhibits a different fluorescence signal.

In this chapter, we report the preparation and characterization of wild-type β-lactamase I as well as the E166C, S130C, E166Cf and S130Cf mutants.

2.2 Experimental section

2.2.1 Materials

All chemicals used were of analytical grade. Penicillin G, penicillin V, ampicillin, guanidine hydrochloride (GuHCl), maleic anhydride, chloramphenicol, sodium citrate and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were purchased from Sigma Chemical Co. Potassium dihydrogenphosphate (KH_2PO_4), potassium bromide (KBr) and zinc chloride ($ZnCl_2$) were obtained from Aldrich Chemical Co. and were used as received. 5-Aminofluorescein was purchased from Fluka Chemical Co. Brain heart infusion, nutrient agar and yeast extract were obtained from OXOID Ltd. Celite 545 was purchased from BDH Laboratory Supplies. Deionized water was purified by a Milli-Q water purification system (Millipore).

2.2.2 Physical measurements

^1H NMR measurements were performed using a Bruker DPX-400 (400MHz) spectrometer with tetramethylsilane (TMS) as the internal standard. Infrared spectra were obtained as KBr pellets using a Bruker Vector 22 FT-IR spectrometer. UV/VIS

spectral measurements were performed on a Hewlett Packard 8452A diode array spectrophotometer.

Fluorescence measurements were carried out using a Perkin Elmer LS50B spectrofluorimeter. Excitation wavelength was set at 494 nm. Both excitation and emission slit widths were set at 5 nm. All fluorescence measurements were taken at room temperature.

Circular dichroism (CD) measurements were performed using a Jasco J810 Spectropolarimeter (Jasco Co.). The spectropolarimeter was flushed thoroughly with N_2 gas before each measurement. A quartz cuvette with path length of 1 mm was used in all measurements. All CD measurements were performed at room temperature.

Spectrophotometric assays of the β-lactamase activities were performed using a Perkin Elmer Lambda Bio20 UV/Vis spectrometer. Substrate hydrolysis was monitored at fixed wavelength: 235 nm for ampicillin, 232 nm for penicillin G and penicillin V. A quartz cuvette with path length of 1 cm was used in all measurements. All spectrophotometric assays were taken at 20 °C.

Electrospray ionization mass spectra were obtained with a VG Platform mass spectrometer (Micromass) equipped with an electrospray interface. Protein samples (20 µl) were injected into the electrospray source via a loop injector as solution in H_2O/CH_3CN (1:1) containing formic acid (0.2 %, v/v).

2.2.3 Synthesis of fluorescein-5-maleimide

Fluorescein-5-maleimide was synthesized as described in the literature [72].

Synthesis of fluorescein-5-maleamic acid

5-Aminofluorescein (174 mg, 0.5 mmol) was dissolved in 50 ml of acetic acid. The solution was filtered by suction to remove any undissolved 5-aminofluorescein. Maleic anhydride (49 mg, 0.5 mmol) was added to the filtrate, and the mixture was stirred at room temperature for 4 h. The yellow solid precipitated was filtered by suction, washed with ethyl acetate (100 ml) and then dried in a vacuum oven. Yield: 80 %. ^{1}H NMR (DMSO-d_6): δ = 12.92 (br, 1H), 10.76 (s, 1H), 10.11 (s, 2H), 8.31 (s, 1H), 7.83 (dd, J = 8.4, 1.6 Hz, 1H), 7.23 (d, J = 8.3 Hz, 1H), 6.50-6.65 (m, 7H), 6.34 (d, J = 12.0, 1H).

Synthesis of fluorescein-5-maleimide

$ZnCl_2$ (37 mg, 0.27 mmol), HMDS (100 µl, 0.54 mmol) and fluorescein-5-maleamic acid (60 mg, 0.13 mmol) were added to a solvent mixture of benzene (9 ml) and

dimethylformamide (1 ml). The mixture was refluxed for 2.5 h and then cooled to room temperature. The solution was concentrated using a rotatory evaporator. About 13 ml of ice-cold water was added to the residual DMF fraction. The pH of the solution was adjusted to 6.0 with 0.1M HCl. Upon cooling in an ice bath, an orange solid precipitated. The solid was filtered by suction and then dried in a vacuum oven. Yield: 86 %. IR (KBr): ν = 1720, 1600, 1460, 1390, 1310 and 1210 cm^{-1}. ^1H NMR (DMSO-d_6): δ = 10.27 (s, 2H), 8.08 (d, J = 1.1 Hz, 1H), 7.89 (dd, J = 8.2, 1.6 Hz, 1H), 7.52 (d, J = 8.2 Hz, 1H), 7.37 (s, 2H), 6.59-6.78 (m, 6H).

2.2.4 Expression and purification of wild-type β-lactamase I, E166C and S130C mutants

Expression and purification of wild-type β-lactamase I, E166C and S130C mutants were performed as described previously [73] with slight modifications. Both wild-type β-lactamase I, E166C and S130C mutants were expressed in *B. subtilis* ϕ105MU331. A bacterial strain was streaked on an agar plate containing 5 μg/ml chloramphenicol, and the plate was incubated at 37 °C for 24 h. A single bacterial colony from the agar plate was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) containing 5 μg/ml chloramphenicol, which was then incubated at 37 °C with shaking at 300 rpm overnight. About 2 ml of overnight inoculum was added to each of four conical flasks containing 100 ml of sterilized BHY medium. The inoculated media were then incubated at 37 °C with shaking at 300 rpm. When the OD_{600} reached 3.5-4.0, the bacterial cultures were heated in a water bath at 51 °C for 5 min and then incubated at 37 °C with shaking at 300 rpm for a further 6 h. The supernatant of the bacterial cultures was collected by centrifugation (9000 rpm) at 4 °C for 25 min, and adjusted to pH 7.0 with conc. HCl. The β-lactamases were extracted by mixing the supernatant with 40 g of celite 545 for 30 min in an ice bath. After discarding the supernatant, the celite was washed three to

four times with 300 ml of deionized water. The β-lactamases were collected by mixing the celite three times with 100 ml of protein elution buffer (100 mM Tris-HCl, 2 M NaCl and 100 mM tri-sodium citrate, pH 7.0). The protein solution was filtered by suction and then concentrated to 10 ml at 4 °C using a Amicron concentrator equipped with a piece of YM-1 membrane (MWCO = 1,000). The concentrated protein solution was exchanged with 20 mM NH_4HCO_3 and then freeze-dried. The enzyme powder was stored at –80 °C. About 15 mg of wild-type β-lactamase I, 20 mg of E166C mutant and 12 mg of S130C mutant were obtained by the above procedures.

The expression and purification of the wild-type β-lactamase I, E166C and S130C mutants were monitored by SDS/PAGE (Figures 2.1 to 2.6).

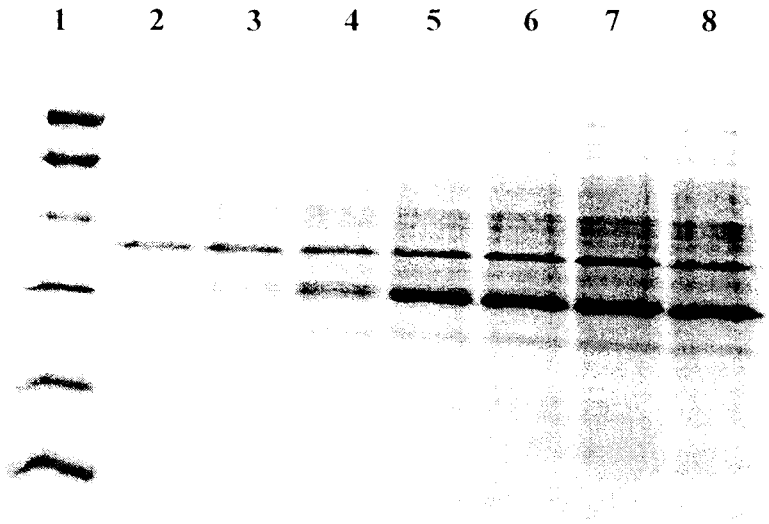


Figure 2.1 SDS/PAGE showing the expressions of the wild-type β-lactamase I after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

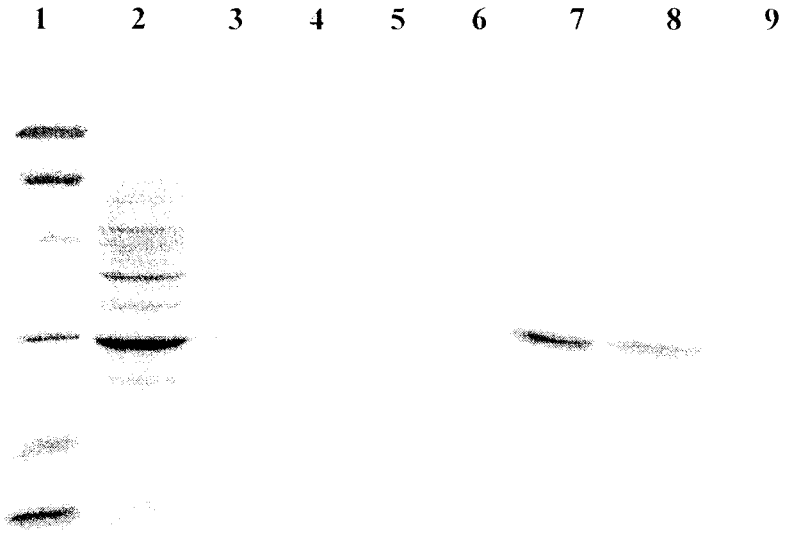


Figure 2.2 SDS/PAGE showing the purification of the wild-type β-lactamase I. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: culture supernatant before mixing with the celite; lane 3: culture supernatant after mixing with the celite; lane 4-6: deionized water after rinsing the celite; lane 7-9: protein elution buffer.

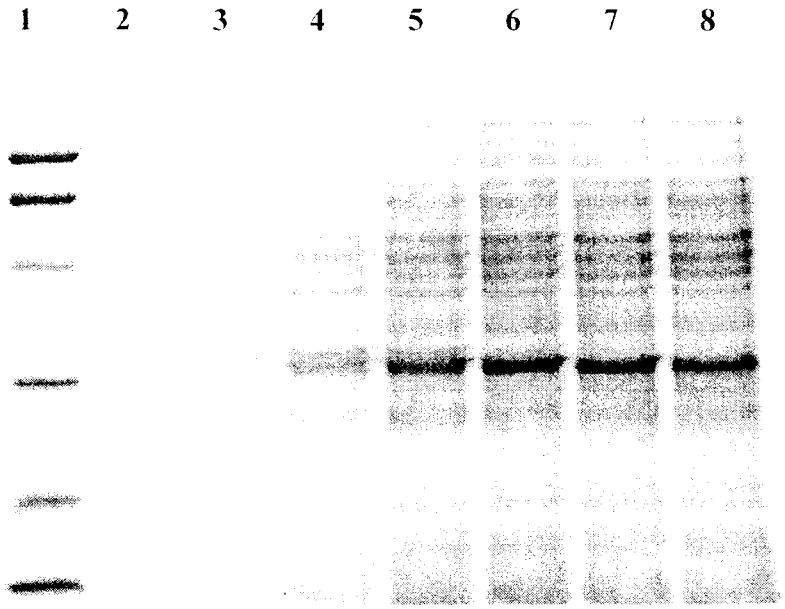


Figure 2.3 SDS/PAGE showing the expressions of the E166C mutant after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

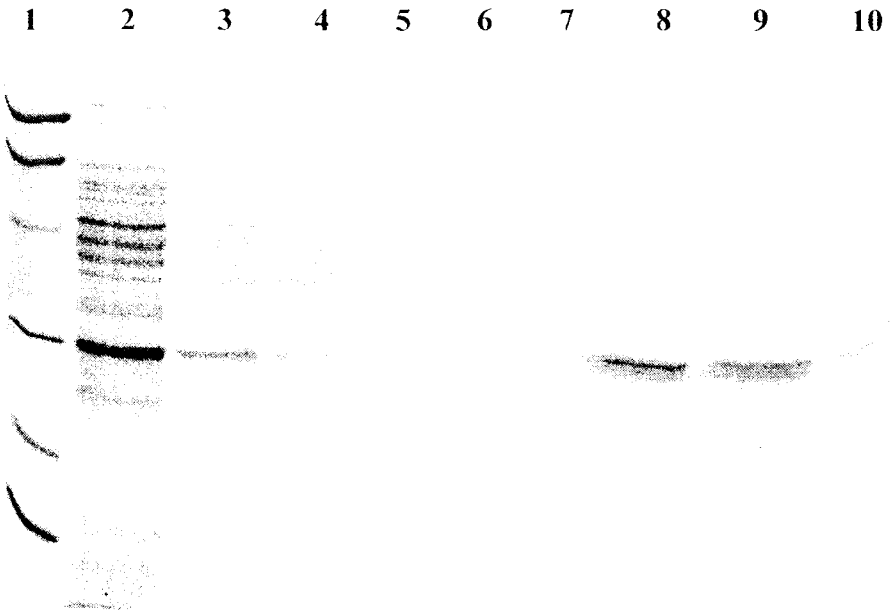


Figure 2.4 SDS/PAGE showing the purification of the E166C mutant. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: culture supernatant before mixing with the celite; lane 3: culture supernatant after mixing with the celite; lane 4-7: deionized water after rinsing the celite; lane 8-10: protein elution buffer.

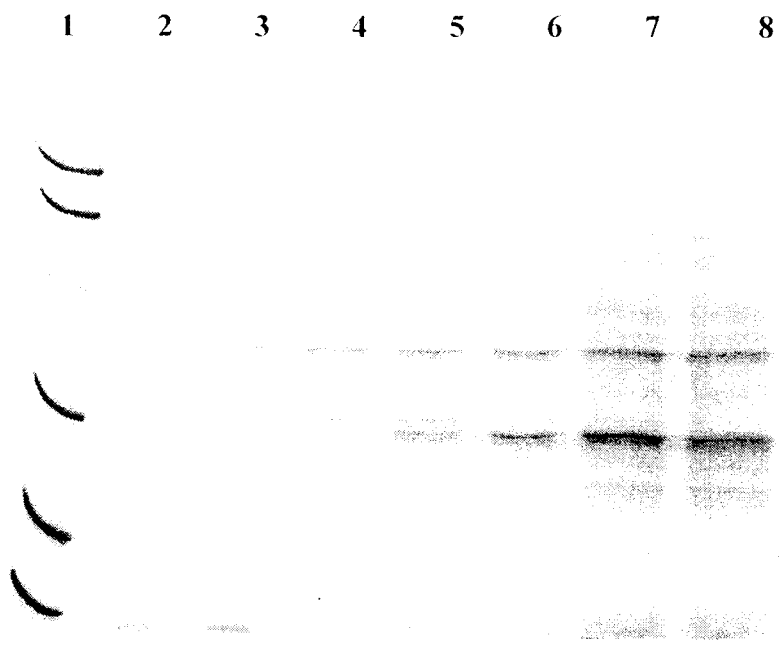


Figure 2.5 SDS/PAGE showing the expressions of the S130C mutant after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

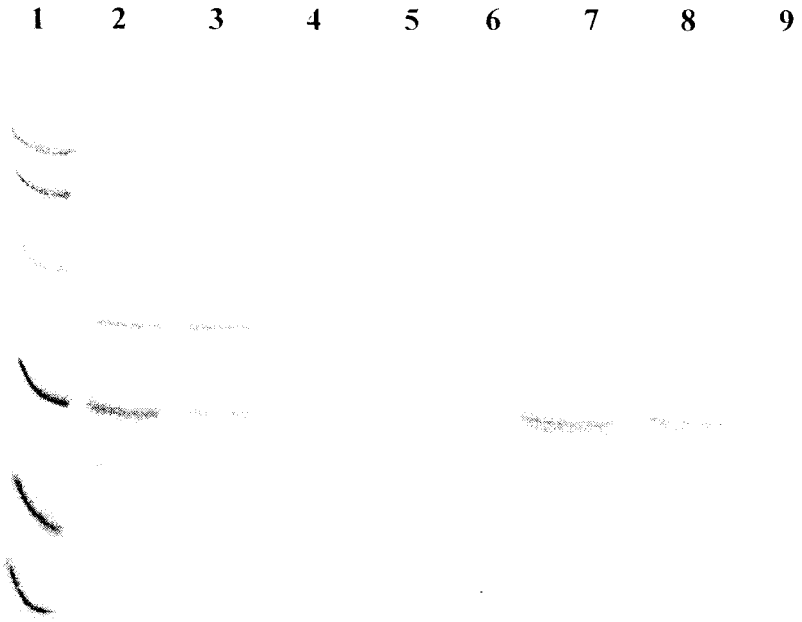


Figure 2.6 SDS/PAGE showing the purification of the S130C mutant. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: culture supernatant before mixing with the celite; lane 3: culture supernatant after mixing with the celite; lane 4-6: deionized water after rinsing the celite; lane 7-9: protein elution buffer.

2.2.5 Protein labeling

About 2.5 mg of E166C mutant was dissolved in 4 ml of 6 M guanidine hydrochloride. The protein solution was incubated at room temperature for 30 min to unfold the mutant. A ten-fold molar excess of fluorescein-5-maleimide (dissolved in dimethylsulfoxide) was added to the protein solution, and the pH of the mixture was adjusted to 7.5 with 0.2 M NaOH. The mixture was stirred at room temperature for 2 h in dark and then dialyzed with a dialysis tubing (MWCO = 12,000) against 1 L of 20 mM NH_4HCO_3 (pH 7.0) at 4 °C for about 3 days to remove the free dyes. Buffer exchanges were carried out regularly during dialysis. After dialysis, the labeled mutant (E166Cf) was freeze-dried and stored at –80 °C.

The labeling of the S130C mutant with fluorescein-5-maleimide was performed by the same procedures.

2.3 Results and discussion

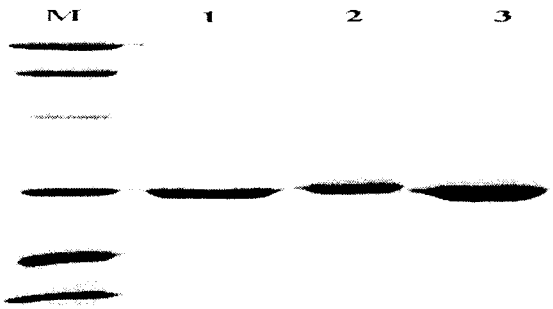
2.3.1 Labeling of the E166C and S130C mutants with fluorescein-5-maleimide

The labeling reaction with fluorescein-5-maleimide is pH-dependent. Maleimide will only react specifically with the thiol group of the cysteine residue when the amino groups of all other amino acids are protonated [74]. In this study, we tried to label the E166C and S130C mutant with fluorescein-5-maleimide at pH 7.5 because all the amino groups on the protein are protonated while the cysteine residue remains unprotonated at this pH. A negative control was set up in which the wild-type β-lactamase I (which contains no cysteine) [69] was subjected to the same labeling reaction. After dialysis, both samples were analyzed by SDS/PAGE. Figure 2.7a shows the polyacrylamide gel stained with Coomassie blue. The protein bands in lane 2 and 3 correspond to the E166Cf enzyme and the wild-type β-lactamase I respectively. Before staining with Coomassie blue, the same gel was illuminated with long-wavelength UV radiation for a fluorescence image. As shown in Figure 2.7b, only one fluorescent band corresponding to the E166Cf enzyme was observed (lane 2). Since the wild-type β-lactamase I contains no cysteine [69] whereas the E166C mutant contains a cysteine residue on its Ω-loop, the fluorescein molecule is

specifically attached to the cysteine residue of the E166C mutant at pH 7.5.

We failed to refold the S130Cf mutant to its native tertiary structure after labeling the unfolded S130C mutant with fluorescein-5-maleimide. This was confirmed by the spectrophotometric assay which showed that the hydrolytic activity of the S130Cf enzyme towards penicillin G is lost. Unlike the E166Cf mutant in which fluorescein is attached to its flexible Ω-loop, the cysteine residue of the S130C mutant lies in a relatively rigid region such that the attached fluorescein molecule may hinder the unfolded polypeptide chain from refolding to its native tertiary structure.

(a)



(b)

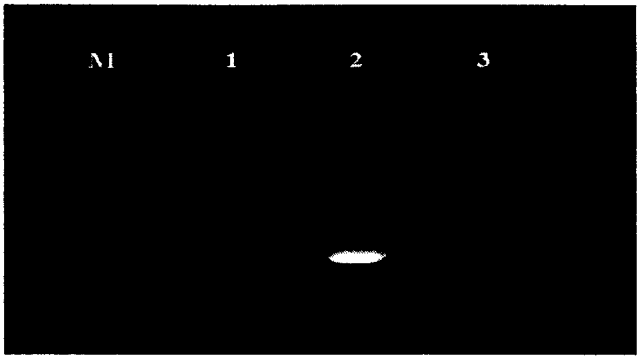


Figure 2.7 SDS/PAGE of the wild-type β-lactamase I, E166C and E166Cf mutants. M: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 1: E166C; lane 2: E166Cf; lane 3: wild-type β-lactamase I subjected to the same labeling reaction (a). Fluorescence image of the same polyacrylamide gel (b).

The labeling reaction was performed with the protein unfolded such that the cysteine residue is exposed to the outside environment. Under this condition, the E166C mutants can be completely labeled with fluorescein-5-maleimide. After removal of any free dyes, the extent of the protein labeling was determined by the Bradford method (which indicates protein concentration) [75] and UV/Vis measurement (which indicates fluorescein concentration). The UV/Vis spectrum of the E166Cf enzyme is shown in Figure 2.8. The peak at 494 nm corresponds to the visible absorption of the fluorescein label. The concentration of the fluorescein molecule was determined to be 1.16×10^{-5} M according to a calibration curve constructed with various concentrations of fluorescein. The protein concentration of the E166Cf enzyme was determined to be 1.18×10^{-5} M by the Bradford method [75]. The results from the Bradford assay and UV/Vis measurement indicate that the concentration of the fluorescein label to that of the E166C mutant is about 1:1. Therefore, the E166C mutants were completely labeled with fluorescein-5-maleimide under unfolding condition.

The excitation and emission spectra of the E166Cf enzyme are shown in Figure 2.9.

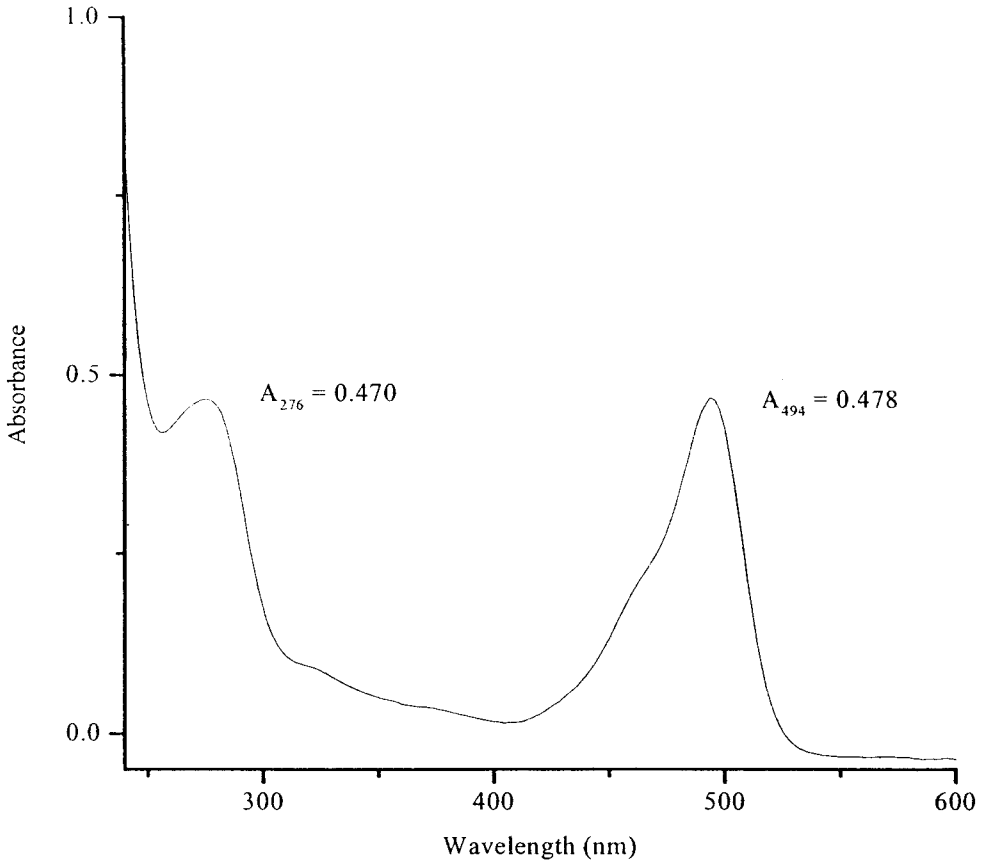


Figure 2.8 UV/Vis spectrum of the E166Cf enzyme in 50 mM phosphate buffer (pH 7.0)

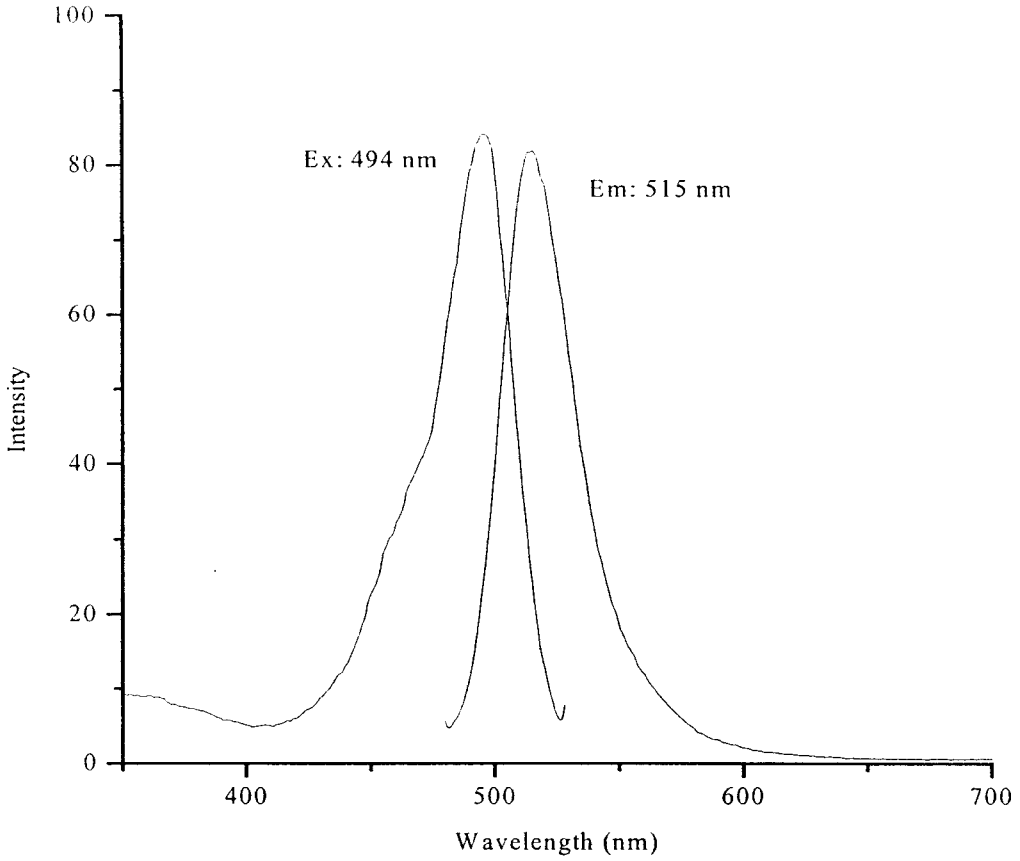


Figure 2.9 Excitation and emission spectra of the E166Cf enzyme in 50 mM phosphate buffer (pH 7.0)

2.3.2 Characterization of the wild-type β-lactamase I, E166C and E166Cf mutants by electrospray ionization mass spectrometry (ESI/MS)

Electrospray ionization mass spectroscopy is a powerful tool to identify an unknown protein by measuring its molecular mass accurately [76]. In addition, this technique can be used to determine the purity of a protein sample. The molecular mass of the wild-type β-lactamase I, E166C and E166Cf mutants were measured by ESI/MS. Figures 2.10 to 2.12 show the ESI mass spectra of the wild-type β-lactamase I, E166C and E166Cf mutants respectively. The molecular mass of the wild-type β-lactamase I, E166C and E166Cf mutants determined by ESI/MS together with the calculated value based on their primary sequences are summarized in Table 2.1. As shown in the table, the molecular mass of the wild-type β-lactamase I and E166C mutant determined by ESI/MS are consistent with the calculated value. The molecular mass of the E166Cf enzyme measured by ESI/MS is higher than that obtained from calculation by 23. This mass difference is likely due to the association of a sodium ion with the E166Cf enzyme. The sodium ion may be associated with the negatively charged fluorescein or with the negatively charged side groups on the protein. It is interesting to note from Figure 2.12 that almost all the E166C mutants were labeled with fluorescein-5-maleimide. This is consistent with the result obtained from the Bradford

method [75] and UV/Vis measurement.

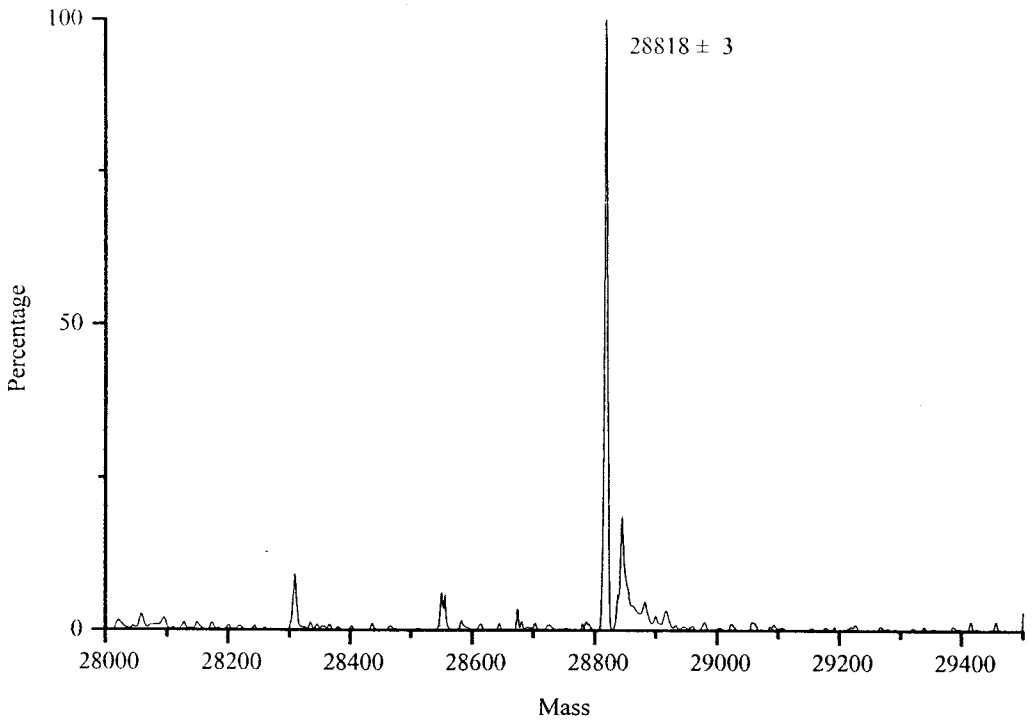


Figure 2.10 ESI mass spectum of the wild-type β-lactamase I

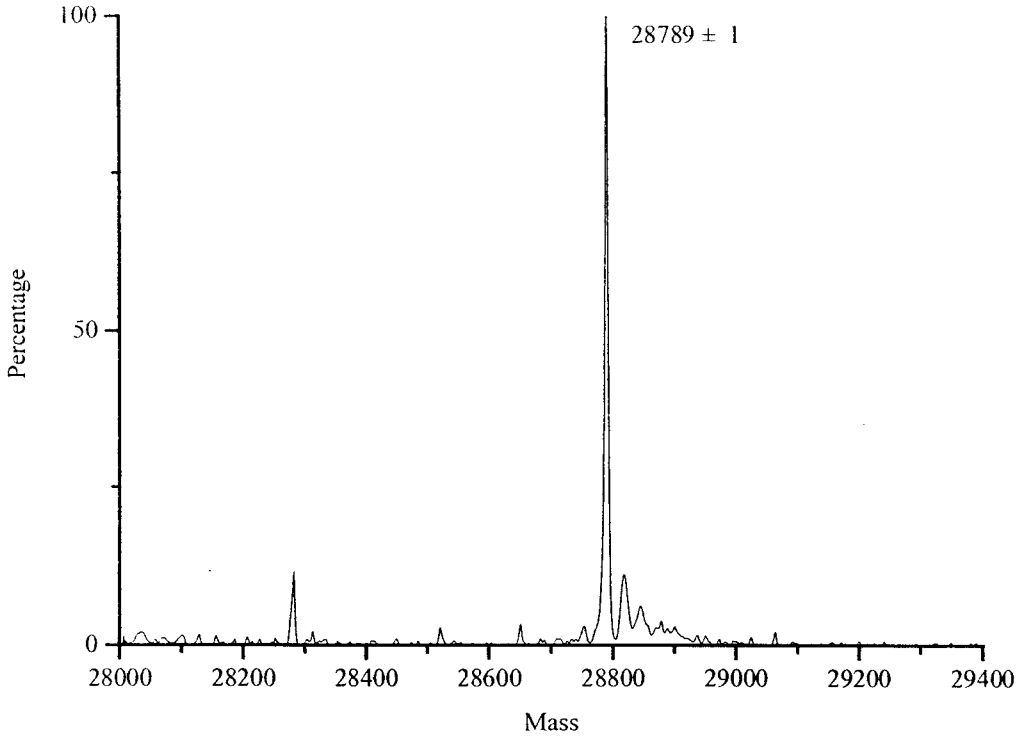


Figure 2.11 ESI mass spectum of the E166C mutant

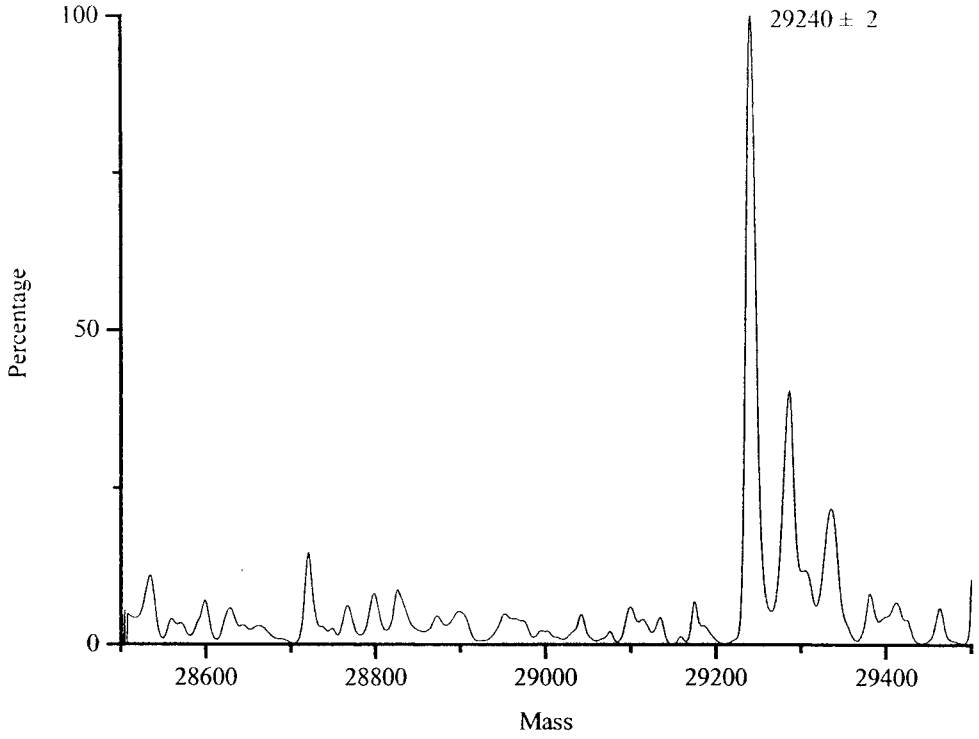


Figure 2.12 ESI mass spectum of the E166Cf enzyme

Table 2.1 Comparison of the calculated and measured mass of the wild-type β-lactamase I, E166C and E166Cf mutants

	Calculated mass	Measured mass
Wild-type β-lactamase I	28815	28818 ± 3
E166C	28789	28789 ± 1
E166Cf	29216	29240 ± 2

2.3.3 Characterization of the wild-type β-lactamase I, E166C and E166Cf mutants by circular dichroism (CD)

Circular dichroism spectropolarimetry is a valuable tool that can provide information on the secondary structure of a protein by measuring the CD signal in the far UV region (180-250 nm). The basic chromophore of a polypeptide chain is the amide group which exhibits two electronic absorptions: π-π^* and n-π^* transitions. These transitions become CD active (optically active) under the influence of the different substituents on the asymmetric α-carbon in a free amino acid amide (Figure 2.13). In the polypeptide chain, the amide groups interact with each other to give a CD spectrum which is characteristic of the amide-amide orientation. Thus, different secondary structures (e.g. α-helix and β-sheet) exhibit different CD spectra [77].

The CD spectra of the wild-type β-lactamase I, E166C and E166Cf mutants at the same concentration (6.0×10^{-6} M) in 50 mM phosphate buffer (pH 7.0) were recorded and compared. As shown in Figure 2.14, the CD signals exhibit no significant difference. This indicates that the secondary structure of the E166Cf enzyme is conserved after labeling with fluorescein. The fractions of various secondary structures of the wild-type β-lactamase I, E166C and E166Cf mutants as determined

by Jasco Secondary Structure Estimation (a software provided by Jasco Co.) are summarized in Table 2.2.

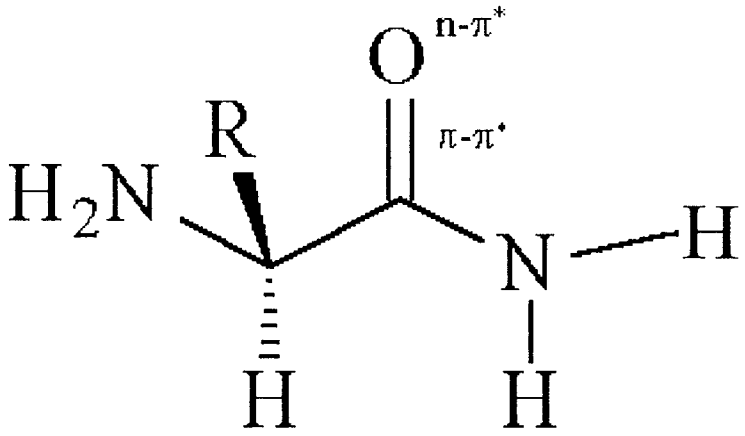


Figure 2.13 Structure of a free amino acid amide. The optical activity is induced by the different groups attached to the asymmetric α-carbon [77].

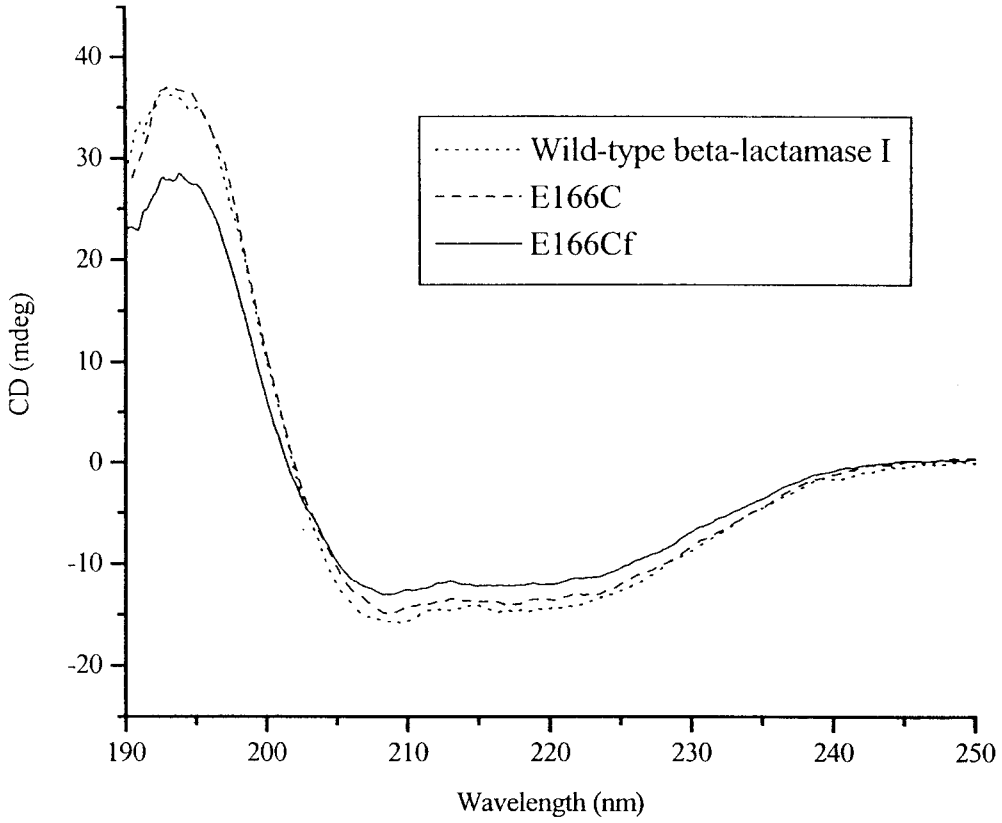


Figure 2.14 CD spectra of the wild-type β-lactamase I, E166C and E166Cf mutants at the same concentration (6.0×10^{-6} M) in 50 mM phosphate buffer (pH 7.0)

Table 2.2 Comparison of the secondary structures of the wild-type β-lactamase I, E166C and E166Cf mutants

	Wild-type	E166C	E166Cf
α-Helix	50.1 %	54.4 %	41.4 %
β-Sheet	35.6 %	35.3 %	41.2 %
Turn	0 %	0 %	0 %
Random coil	14.3 %	10.3 %	17.4 %

2.3.4 Spectrophotometric assays of the activities of wild-type β-lactamase I, E166C and E166Cf mutants

The hydrolytic activities of the wild-type β-lactamase I, E166C and E166Cf mutants towards penicillin G, penicillin V and ampicillin were monitored by the spectrophotometric method [44]. Substrate hydrolysis was monitored at a fixed wavelength: 235 m for ampicillin, 232 nm for penicillin G and penicillin V. The initial rate of substrate hydrolysis occurring within 5 min was determined in duplicate at each of 6 different substrate concentrations in 50 mM potassium phosphate buffer (pH 7.0) at 20 °C. The initial rates determined were then fitted to the program of Stanislawski [78] to calculate the Michaelis constants (K_m) and turnover numbers (k_{cat}) using non-linear regression analysis (equation 2.1):

Equation 2.1

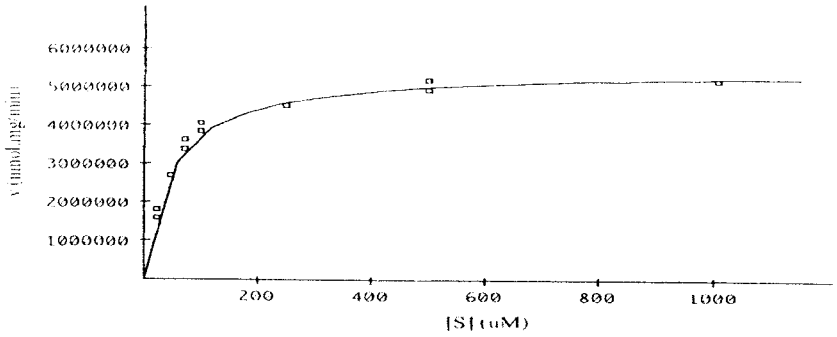
$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where v is the initial rate of substrate hydrolysis, V_{max} the maximum rate of reaction, [S] the initial substrate concentration, K_m the Michaelis constant and

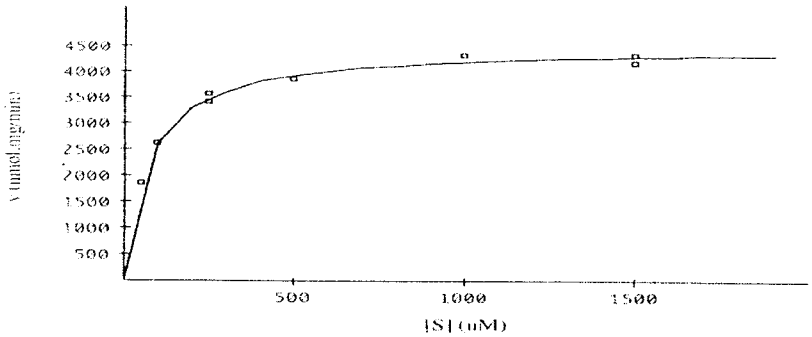
$k_{cat} = V_{max}/ [Enzyme]$.

Figure 2.15 shows some of the plots obtained with the wild-type β-lactamase I, E166C and E166Cf mutants. The plots indicate that the wild-type β-lactamase I, E166C and E166Cf mutants follow the Michaelis-Menten kinetics. The measured steady-state kinetic parameters for hydrolysis of penicillin G, penicillin V and ampicillin by the wild-type β-lactamase I, E166C and E166Cf mutants are summarized in Table 2.3. In general, the catalytic efficiencies (k_{cat}/K_m) of the wild-type β-lactamase I with penicillin G, penicillin V and ampicillin as substrates are about 2000 times higher than those of the E166C and E166Cf mutants. This can be attributed to the absence of the catalytically important Glu-166 residue [7, 8, 30, 31] in the E166C and E166Cf mutants. The catalytic efficiencies (k_{cat}/K_m) of the E166Cf enzyme are comparable to those of the E166C mutant, indicating that the hydrolytic activity of the labeled enzyme is conserved after the labeling reaction. The K_m values of the E166Cf enzyme are generally greater than those of the E166C mutant, and this may be ascribed to the steric hindrance induced by the fluorescein label.

(a)



(b)



(c)

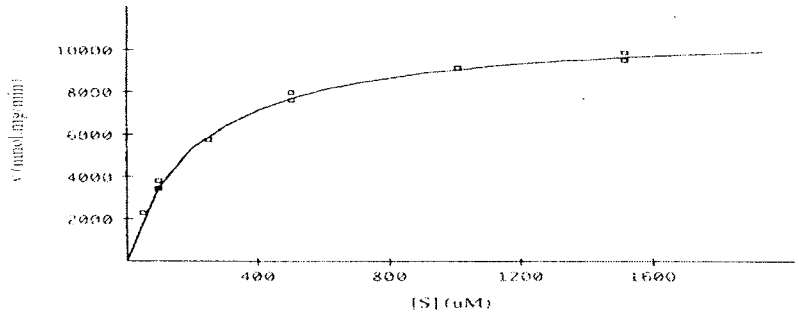


Figure 2.15 Michaelis-Menten plots of the wild-type β-lactamase I (a), E166C (b) and E166Cf (c) with penicillin G as substrate. Substrate hydrolysis was monitored in 50 mM potassium phosphate buffer (pH 7.0) at 20 °C.

Table 2.3 Steady-state kinetic parameters for hydrolysis of penicillin G, penicillin V and ampicillin by the wild-type β-lactamase I, E166C and E166Cf mutants

	K_m (μM)		
	Wild-type	E166C	E166Cf
Penicillin G	48 ± 3	72 ± 3	213 ± 11
Penicillin V	52 ± 4	71 ± 6	117 ± 10
Ampicillin	142 ± 8	306 ± 30	262 ± 31

	k_{cat} (s^{-1})		
	Wild-type	E166C	E166Cf
Penicillin G	2612 ± 320	2.07 ± 0.02	5.28 ± 0.09
Penicillin V	2109 ± 4	1.53 ± 0.03	2.97 ± 0.06
Ampicillin	5213 ± 275	4.1 ± 0.1	6.2 ± 0.2

	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)		
	Wild-type	E166C	E166Cf
Penicillin G	54 ± 10	0.029 ± 0.002	0.025 ± 0.002
Penicillin V	41 ± 4	0.021 ± 0.002	0.025 ± 0.003
Ampicillin	37 ± 4	0.013 ± 0.002	0.023 ± 0.004

2.4 Concluding remarks

The E166C mutant has been successfully prepared and labeled with fluorescein-5-maleimide at the cysteine residue on the Ω-loop. The Bradford assay [75], UV/Vis measurement and ESI mass measurements indicated that almost all the E166C mutants were labeled with fluorescein. In contrast, the S130C mutant was unable to refold to its native tertiary structure after the labelling reaction. This may be attributed to the presence of the bulky fluorescein molecule at a location which hinders the unfolded S130C mutant from refolding to its native structure. Circular dichroism spectropolarimetry showed that the secondary structure of the E166Cf enzyme is conserved after the labeling reaction. As demonstrated by the spectrophotometric assays, the catalytic efficiencies of the E166Cf enzyme with penicillin G, penicillin V and ampicillin as substrates are comparable to those of the E166C mutant, indicating that the hydrolytic activity of the E166Cf enzyme is conserved even in the presence of the fluorescein label. The K_m values of the E166Cf enzyme are generally higher than those of the E166C mutant, which may be attributed to the steric hindrance induced by the fluorescein label.

Chapter 3

Studies on the fluorescence behavior of the fluorescein-labeled β-lactamase I (E166Cf) in the presence of some penicillins, cephalosporins and β-lactamase inhibitors

3.1 Introduction

In recent years, a number of optical biosensors for small ligands and biomolecules have been developed by labeling an allosteric protein molecule with an environment-sensitive luminescent probe [61, 63, 66]. Upon substrate binding, the protein molecule undergoes conformational changes which can be detected by the luminescent probe. Thus, these biosensors can sensitively detect their substrates at low concentration. For example, a biosensor that can detect Ca^{2+} ion at nanomolar concentration has been reported [61]. This sensor was constructed by labeling a calcium-binding protein, calmodulin, with the environment-sensitive probe N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide which is responsible for detecting the conformational change upon binding to a calcium ion. Interestingly, a generic biosensor for beta-lactamase inhibitory protein (BLIP) has been reported recently [79]. This sensor consists of a β-lactamase enzyme and a green fluorescent protein which are linked together by genetic engineering. The green fluorescent protein is responsible for detecting the conformational change when the BLIP binds to the β-lactamase enzyme.

The above approach to construct fluorescent biosensors, however, only works for allosteric proteins which undergo a relatively large change in structure upon substrate binding. We reasoned that placing an environment-sensitive label close to the active site of an enzyme might also serve the same purpose as the active site will usually undergo a conformational change upon substrate binding. However, care must be taken as the fluorescent label might block the approach of the substrate molecule. The fluorescein-labeled β-lactamase I (E166Cf) is a good test of this concept as the fluorescent label is located on a flexible Ω-loop close to the active site of the enzyme [70, 71]. Such a fluorescent enzyme would serve as a sensitive reagentless biosensor for the detection of β-lactam antibiotics. In this chapter, we report the results of fluorometric studies on the E166Cf enzyme in the presence of penicillin G, penicillin V and ampicillin. In addition, the fluorescence changes of the labeled enzyme with cefuroxime, cefoxitin and moxalactam which are resistant to the hydrolytic activity of β-lactamase I were also investigated. Furthermore, the fluorescence behavior of the E166Cf enzyme in the presence of two β-lactamase inhibitors (sulbactam and clavulanate) was monitored.

3.2 Experimental section

3.2.1 Materials

All chemicals used were analytical grade reagents. Penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam were obtained from Sigma Chemical Co. Potassium dihydrogenphosphate (KH_2PO_4) was purchased from Aldrich Chemical Co. Deionized water was purified by a Milli-Q water purification system (Millipore).

3.2.2 Molecular modeling

Based on molecule A of the structure of β-lactamase from *B. licheniformis* 749/C as the starting model (Protein Data Bank code 4blm) [80], two models were generated: that of E166C mutant labeled with fluorescein-5-maleimide (E166Cf) and that of E166Cf covalently linked with penicillin G via the active-site Ser-70 (E166Cf-penicillin G). The E166C mutation was engineered using the program *O* [81] with the $\chi 1$ angle preserved. The coordinate model of fluorescein-5-maleimide was generated with the Dundee PRODRG server [82].

The penicillin-bound structure was modeled based on the structure of the acyl-enzyme intermediate of *E. coli* RTEM-1 β-lactamase (Protein Data Bank code 1fqg) [80]. The manually built models were subjected to two cycles of refinement in order to relax the poor geometry. Each cycle contains a simulated annealing step employing torsional molecular dynamics [83] followed by conjugate gradient minimization. These computational refinements were performed with the program *CNS* [84]. The topology and parameter files for fluorescein-5-maleimide and penicillin G used in the refinement were generated with the PRODRG server (see above). During refinement, all hydrogen atoms were included but all solvent molecules were excluded. The final models of the E166Cf and E166Cf-penicillin G enzymes have good stereochemistry which is comparable in quality to the starting model (data not shown). Solvent accessibility was calculated with the program *NACCESS* [85] with hydrogen atoms and solvent molecules excluded. The two pictures of the models were created with *BOBSCRIPT* [86] and rendered with *Raster3D* [87]. The molecular surface was generated with GRASP [88].

3.2.3 Physical measurements

Fluorometric studies were performed on a Perkin Elmer LS50B spectrofluorimeter. For time-resolved fluorescence measurement, excitation and emission wavelengths were set at 494 and 515 nm respectively. Both excitation and emission slit widths were set at 5 nm. All fluorescence measurements were performed at room temperature.

For fluorescence measurements in which automatic injection of antibiotic solution was required, a FLUOstar microplate reader (BMG Labtechnologies) equipped with two sample injectors was used. The injection speed of the sample injector was set at 310 μl/s. Excitation and emission filters of 485 and 520 nm respectively were used. The samples were placed in a 96-well microtiter plate (Corning Costar) in all measurements.

Circular dichroism (CD) measurements were performed on a Jasco J810 Spectropolarimeter (Jasco Co.). The spectropolarimeter was flushed thoroughly with N_2 gas before measurement. A quartz cuvette with path length of 1 mm was used. All CD measurements were performed at room temperature.

Spectrophotometric assays of the activity of E166Cf enzyme were performed on a Perkin Elmer Lambda Bio 20 UV/Vis spectrometer. Substrate hydrolysis was monitored at a fixed wavelength: 235 nm for ampicillin, 232 nm for penicillin G and penicillin V. A quartz cuvette with path length of 1 cm was used in all measurements.

3.3 Results and discussion

3.3.1 Fluorometric studies of the E166Cf enzyme towards penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam

The effects of penicillin and cephalosporin antibiotics on the fluorescence behavior of the E166Cf enzyme were investigated by measuring the fluorescence signals of the labeled enzyme in the presence of various concentrations of β-lactam antibiotics. With penicillin G as substrate, the fluorescence signal of the E166Cf enzyme (1.2×10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) increases as a function of the antibiotic concentration (Figure 3.1). As shown in the figure, the E166Cf enzyme is capable of detecting β-lactam antibiotic down to 10^{-7} M. The time-resolved fluorescence signals of the E166Cf enzyme at 515 nm were also measured with various concentrations of penicillin G (Figure 3.2). At low antibiotic concentration (1.0×10^{-7} and 1.0×10^{-6} M), the fluorescence intensity of the E166Cf enzyme increases gradually and then declines. At high antibiotic concentration (1.0×10^{-5} and 1.0×10^{-4} M), the fluorescence signal increases instantaneously and levels off to a plateau. The fluorescence signal stays at the plateau for a certain length of time and then declines afterwards. Similar results were obtained with penicillin V and

ampicillin (Figures 3.3 and 3.4).

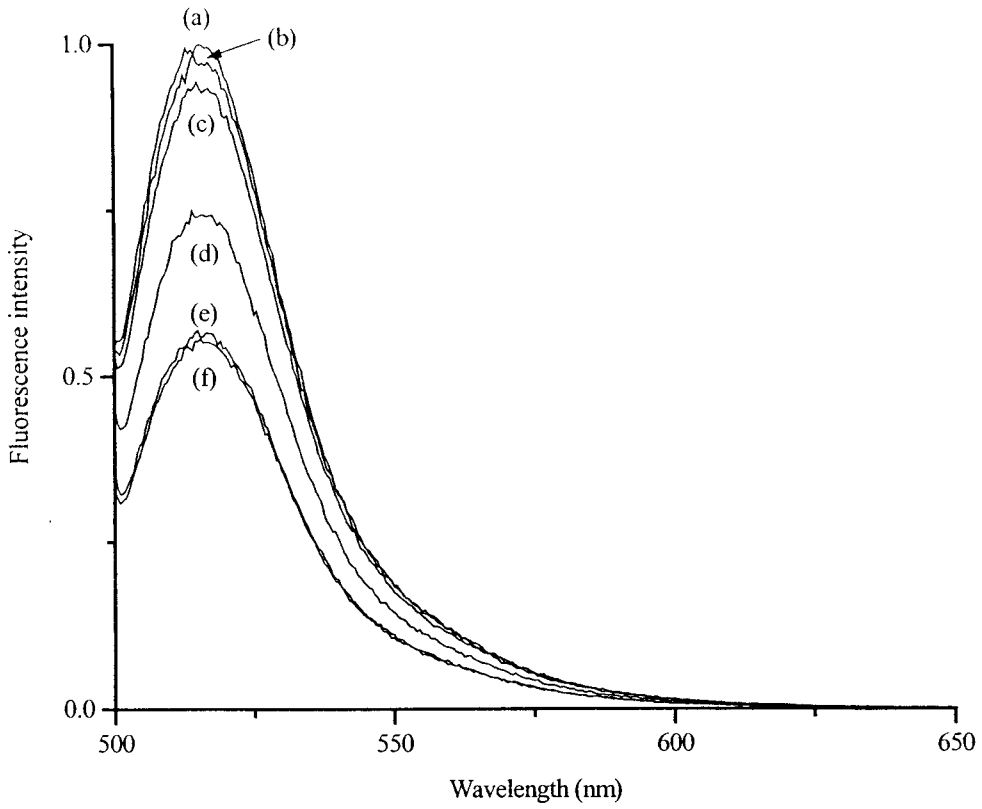


Figure 3.1 Fluorescence spectra of the E166Cf enzyme (1.2×10^{-7} M) in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0×10^{-4} M penicillin G (a), 1.0×10^{-5} M penicillin G (b), 1.0×10^{-6} M penicillin G (c), 1.0×10^{-7} M penicillin G (d), 1.0×10^{-8} M penicillin G (e) and 0 M penicillin G (f). The E166Cf enzymes were incubated with various concentrations of penicillin G for 130 s at room temperature before measurement. Excitation wavelength: 494 nm.

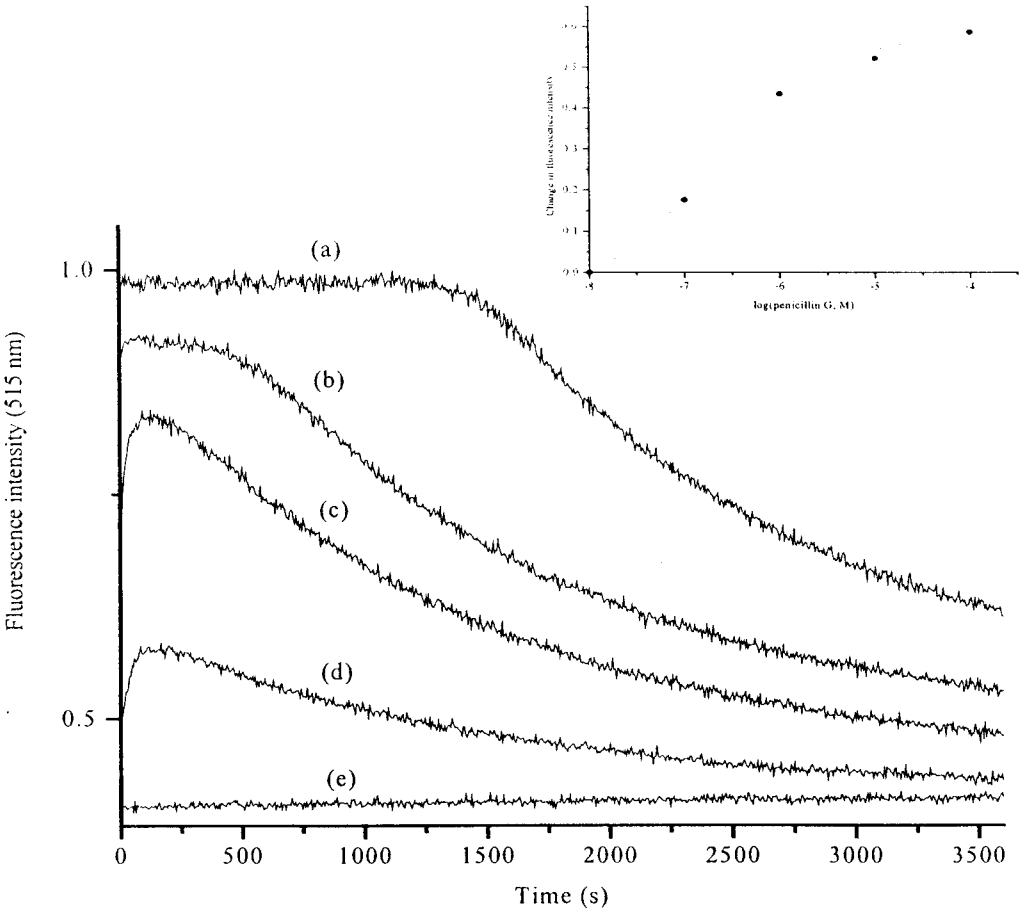


Figure 3.2 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M penicillin G (a), 1.0 x 10^{-5} M penicillin G (b), 1.0 x 10^{-6} M penicillin G (c), 1.0 x 10^{-7} M penicillin G (d) and 1.0 x 10^{-8} M penicillin G (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (penicillin G, M).

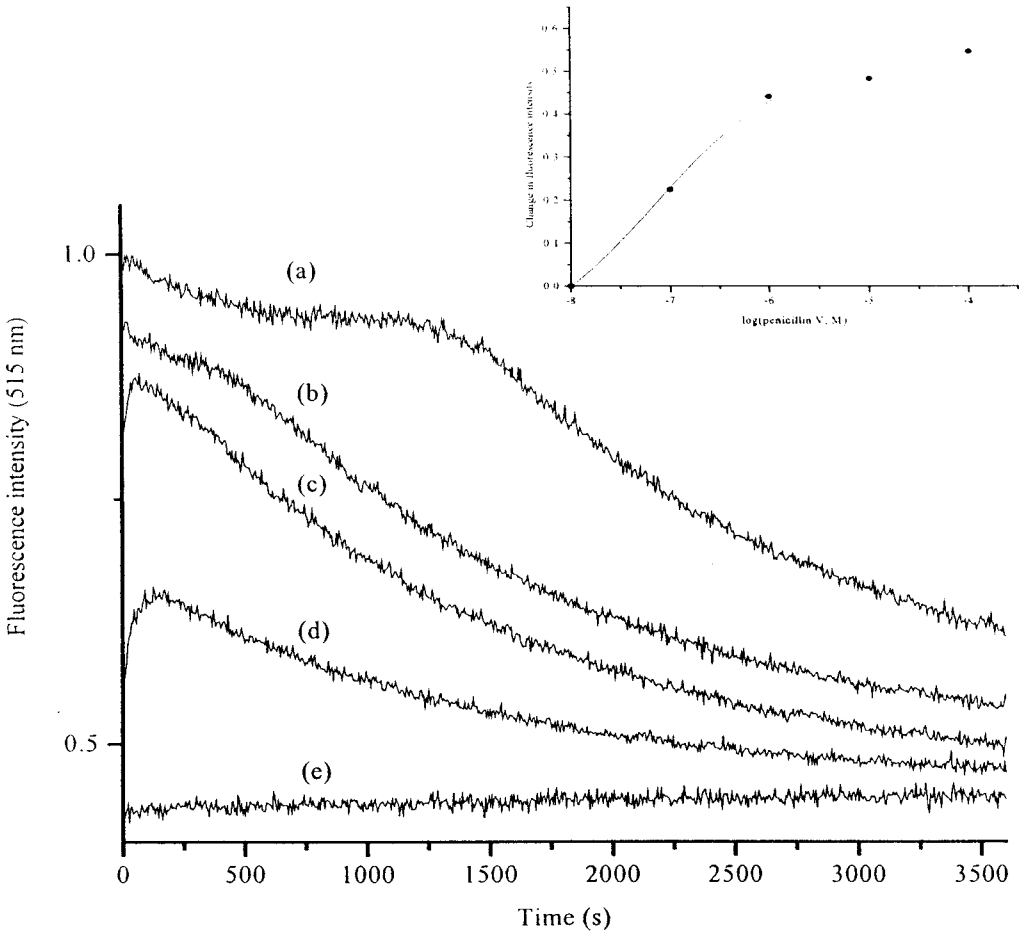


Figure 3.3 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M penicillin V (a), 1.0 x 10^{-5} M penicillin V (b), 1.0 x 10^{-6} M penicillin V (c), 1.0 x 10^{-7} M penicillin V (d) and 1.0 x 10^{-8} M penicillin V (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (penicillin V, M).

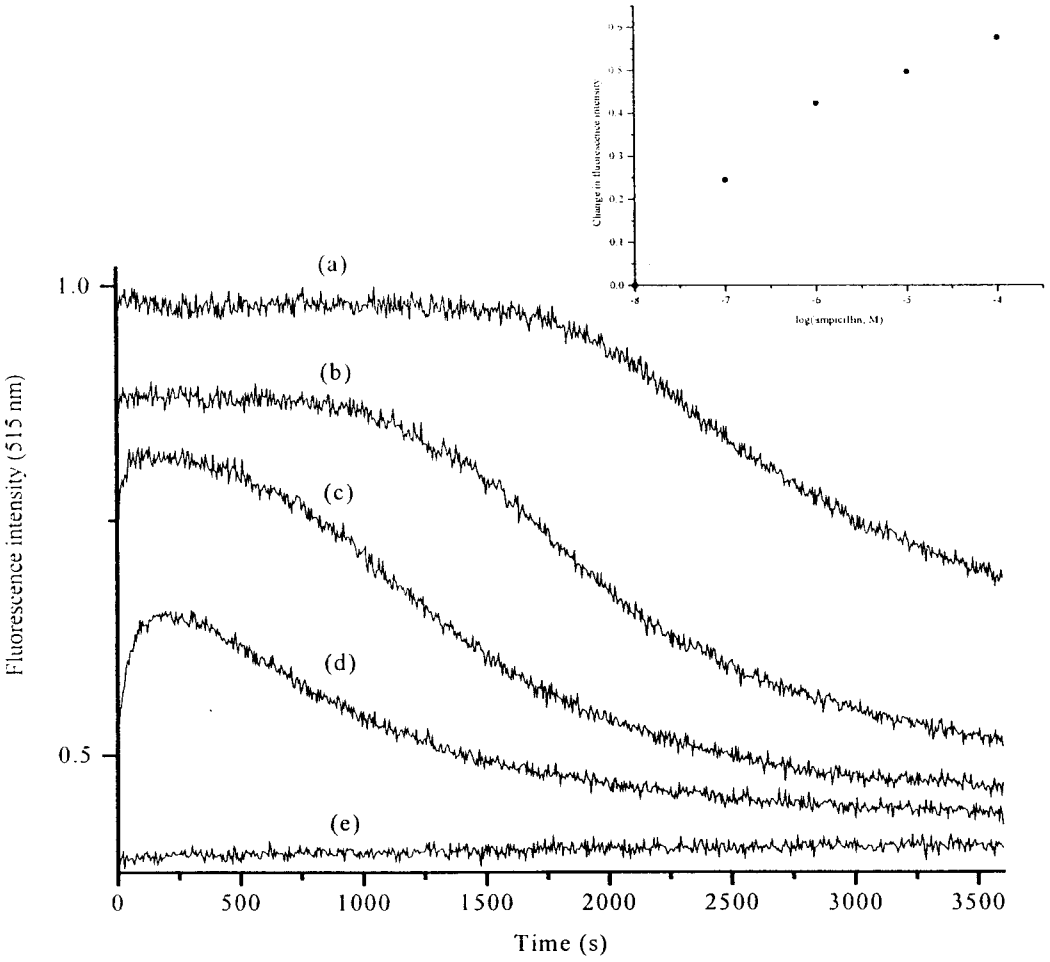


Figure 3.4 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M ampicillin (a), 1.0 x 10^{-5} M ampicillin (b), 1.0 x 10^{-6} M ampicillin (c), 1.0 x 10^{-7} M ampicillin (d) and 1.0 x 10^{-8} M ampicillin (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (ampicillin, M).

With cefuroxime, cefoxitin and moxalactam as substrates, the fluorescence intensities of the E166Cf enzyme (1.2×10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) also increase as a function of antibiotic concentration (Figures 3.5 to 3.7). The E166Cf enzyme exhibited increasing fluorescence signals with antibiotic concentration, but no subsequent decline in intensity was observed even after one hour. Moreover, the rate of increase in fluorescence intensity increases with the antibiotic concentration.

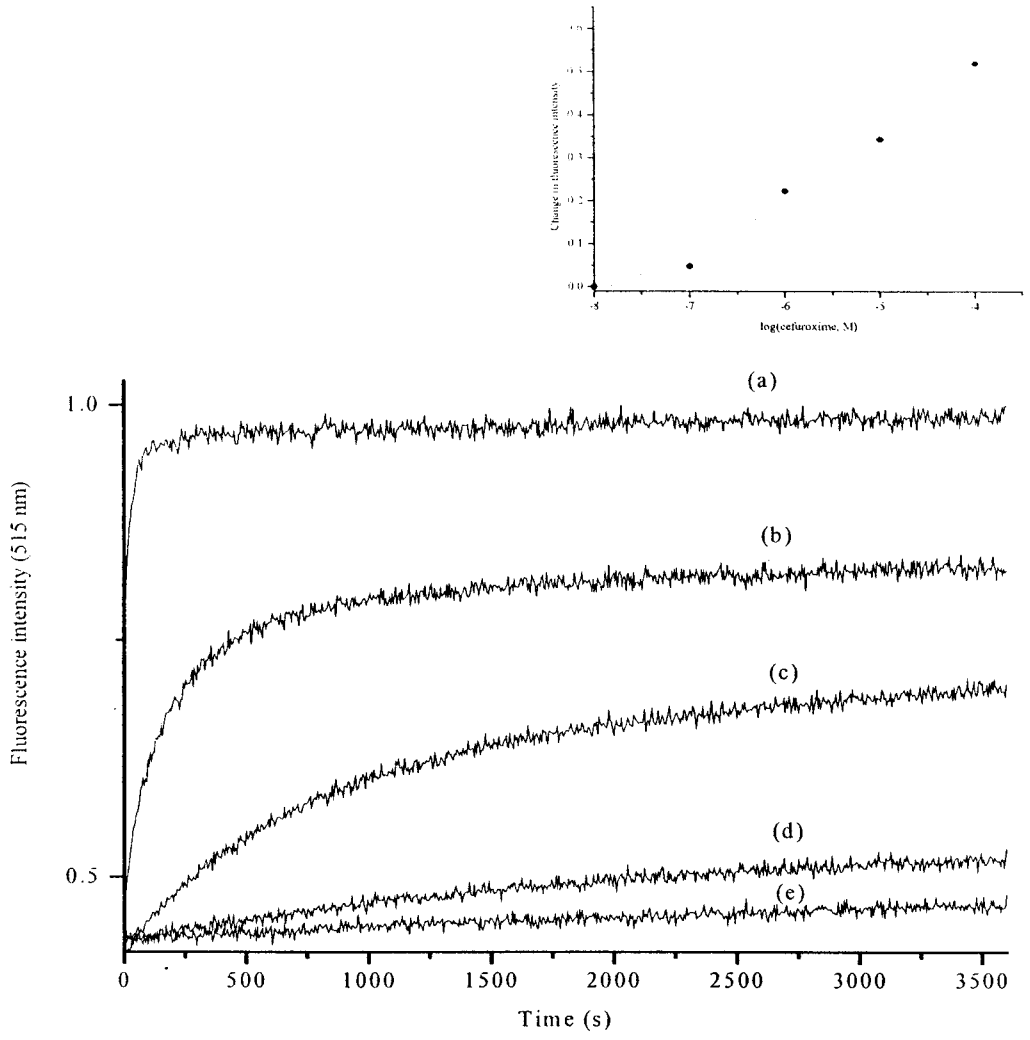


Figure 3.5 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M cefuroxime (a), 1.0 x 10^{-5} M cefuroxime (b), 1.0 x 10^{-6} M cefuroxime (c), 1.0 x 10^{-7} M cefuroxime (d) and 1.0 x 10^{-8} M cefuroxime (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (cefuroxime, M).

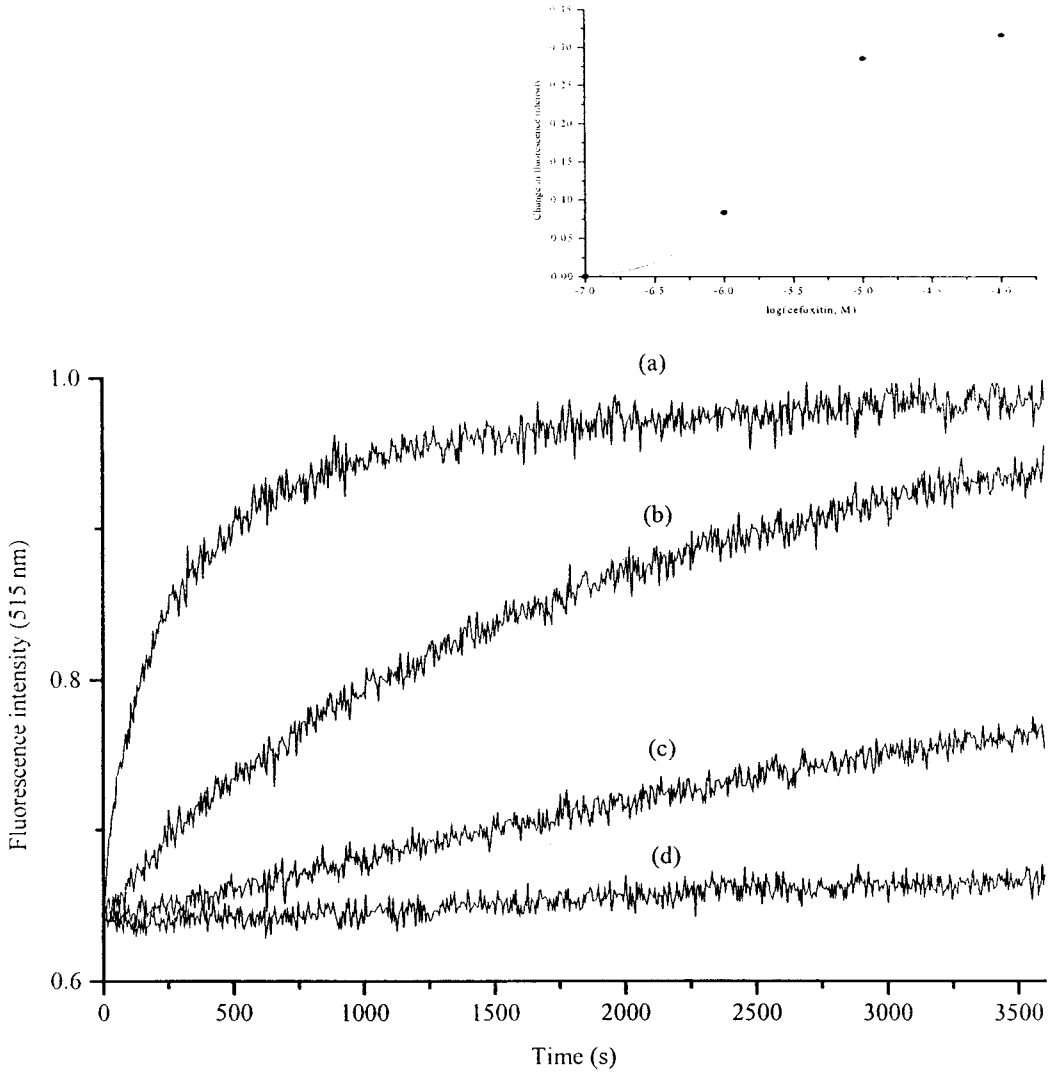


Figure 3.6 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M cefoxitin (a), 1.0 x 10^{-5} M cefoxitin (b), 1.0 x 10^{-6} M cefoxitin (c) and 1.0 x 10^{-7} M cefoxitin (d). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (cefoxitin, M).

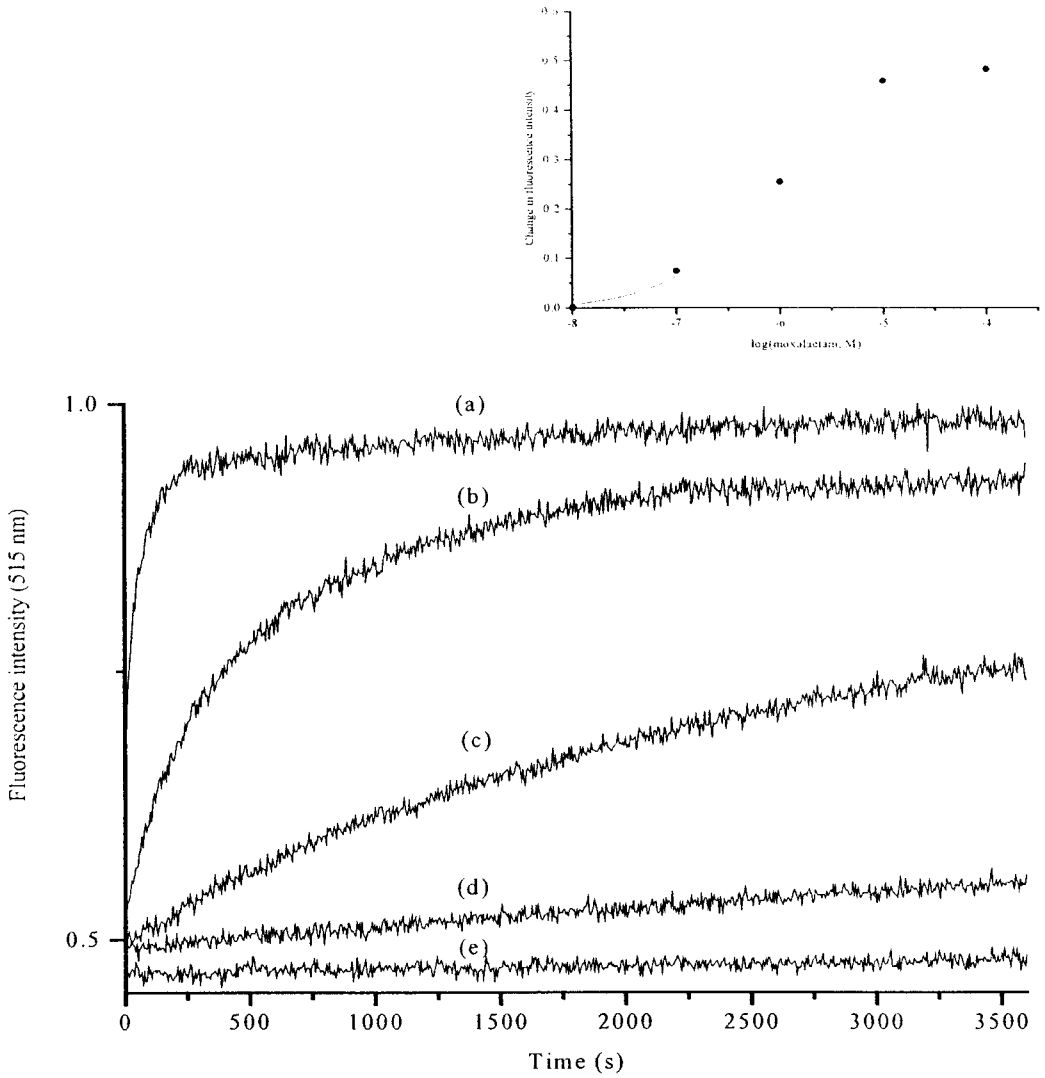


Figure 3.7 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M moxalactam (a), 1.0 x 10^{-5} M moxalactam (b), 1.0 x 10^{-6} M moxalactam (c), 1.0 x 10^{-7} M moxalactam (d) and 1.0 x 10^{-8} M moxalactam (e). Excitation wavelength: 494 nm. The inset shows the plot of the change in fluorescence intensity (at the maxima) as a function of log (moxalactam, M).

3.3.2 Effect of the binding of substrate on the fluorescence of the E166Cf enzyme

As indicated in the time-resolved fluorescence spectra, both penicillin and cephalosporin antibiotics are capable of enhancing the fluorescence intensity of the E166Cf enzyme. To investigate whether such fluorescence enhancement is due to the interaction between the fluorescein label and antibiotic, the fluorescence intensities of free fluorescein (1.2×10^{-7} M) at 512 nm in 50 mM phosphate buffer (pH7.0) in the presence of various concentrations of penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0×10^{-5} M) were monitored. As shown in Figures 3.8 to 3.13, the fluorescence intensities of the fluorescein molecule remain unchanged with increasing antibiotic concentration from 0 to 1.0×10^{-4} M. This indicates that the β-lactam antibiotics have neither enhancing nor quenching effect on the fluorescence intensity of the fluorescein molecule.

As the fluorescence intensity of fluorescein is known to change with pH [89, 90], investigations were also made to see if the observed fluorescence changes were a result of the change in pH upon hydrolysis of the antibiotics by the labeled enzyme. First of all, the fluorescence intensity of fluorescein is known to decrease when the pH is lowered [89, 90]. So the observed increase in fluorescence intensity in the

presence of antibiotics is not consistent with the generation of carboxylic acids upon hydrolysis by the enzyme. After the completion of hydrolysis, the overall change in pH of the bulk phosphate buffer solution as monitored by a pH electrode was less than 0.5 pH unit for penicillin G (1.0×10^{-5} M). When the fluorescence intensity of free fluorescein (1.2×10^{-7} M) in 50 mM phosphate buffer (pH 7.0) was measured with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0×10^{-5} M) in the presence of unlabeled E166C enzyme (1.2×10^{-7} M), it remains almost unchanged throughout the whole experiment (Figures 3.14 to 3.16) while the E166C enzyme was hydrolyzing the penicillin antibiotics (Figure 3.17). Moreover, cephalosporins, which are known to be poor substrates for the E166C enzyme [6-8], also enhance the fluorescence signals. So the possibility that the increase in fluorescence signal of the E166Cf enzyme is due to the change in pH of the bulk solution can be eliminated. Furthermore, the possibility that the observed change in fluorescence signal is due to a change in local pH at the enzyme's active site can also be eliminated because it is unlikely that such a change in pH at the active site takes hundreds to thousands of seconds as shown in the time-resolved fluorescence measurements.

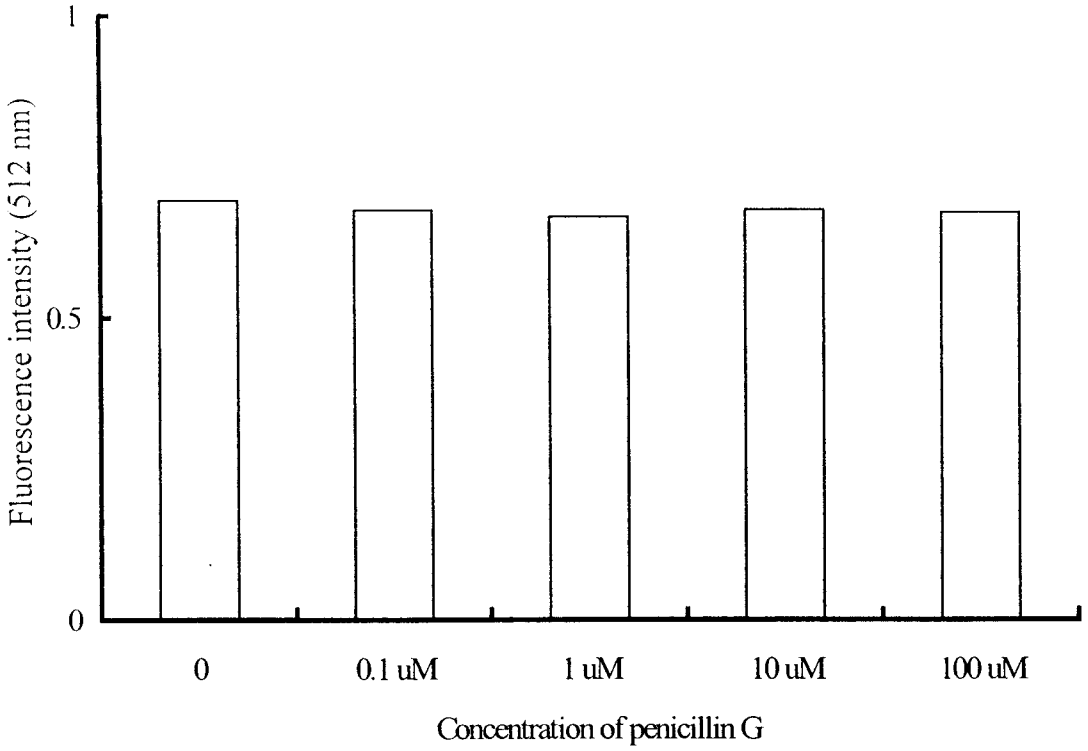


Figure 3.8 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of penicillin G

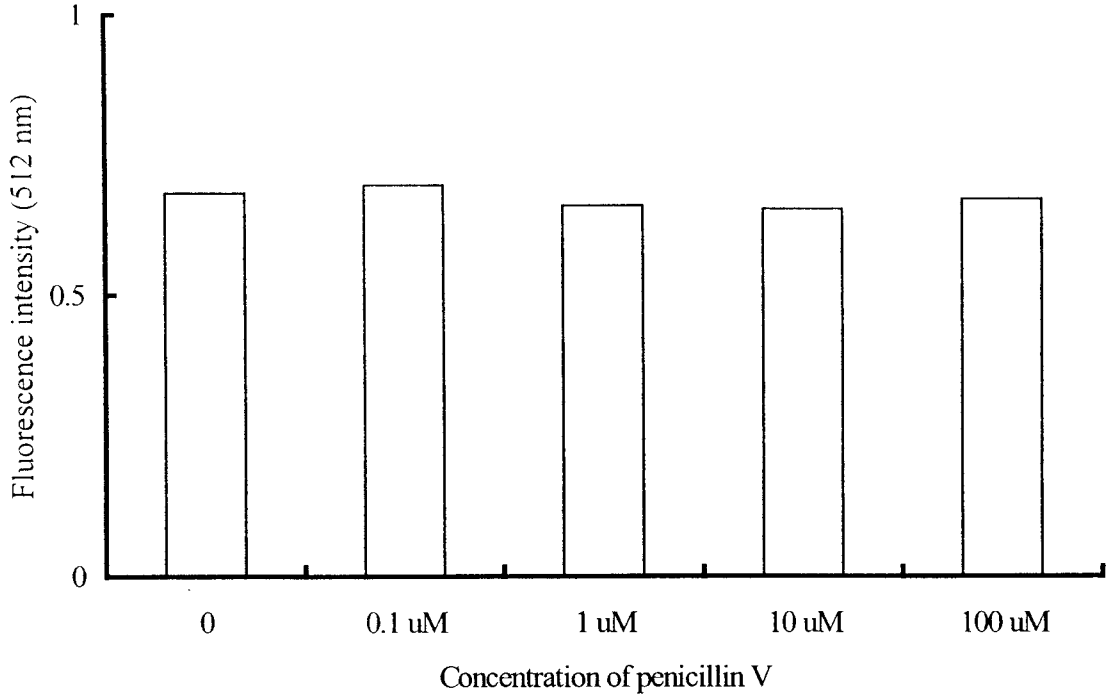


Figure 3.9 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of penicillin V

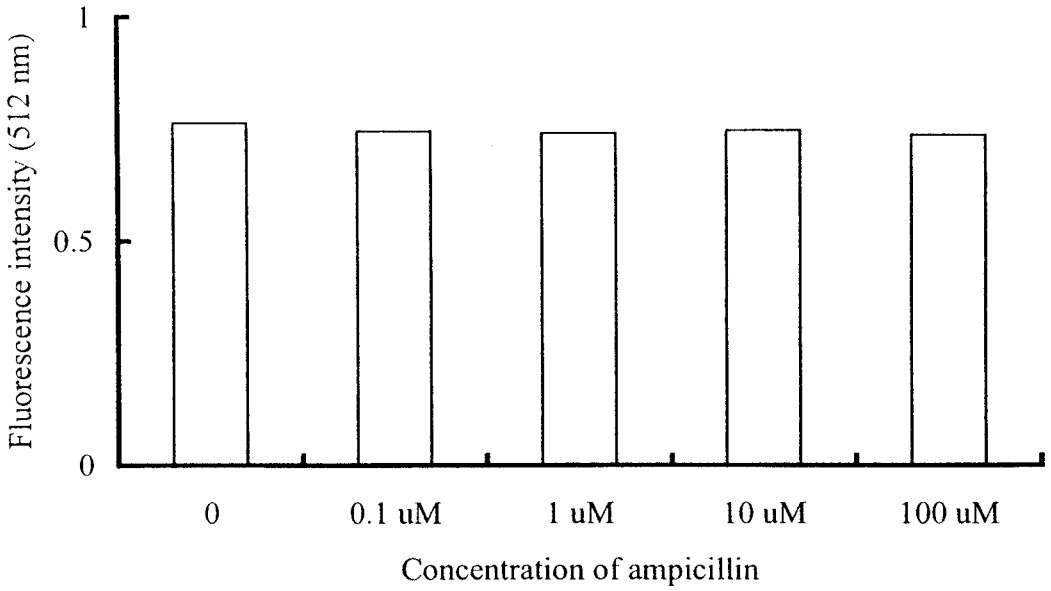


Figure 3.10 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of ampicillin

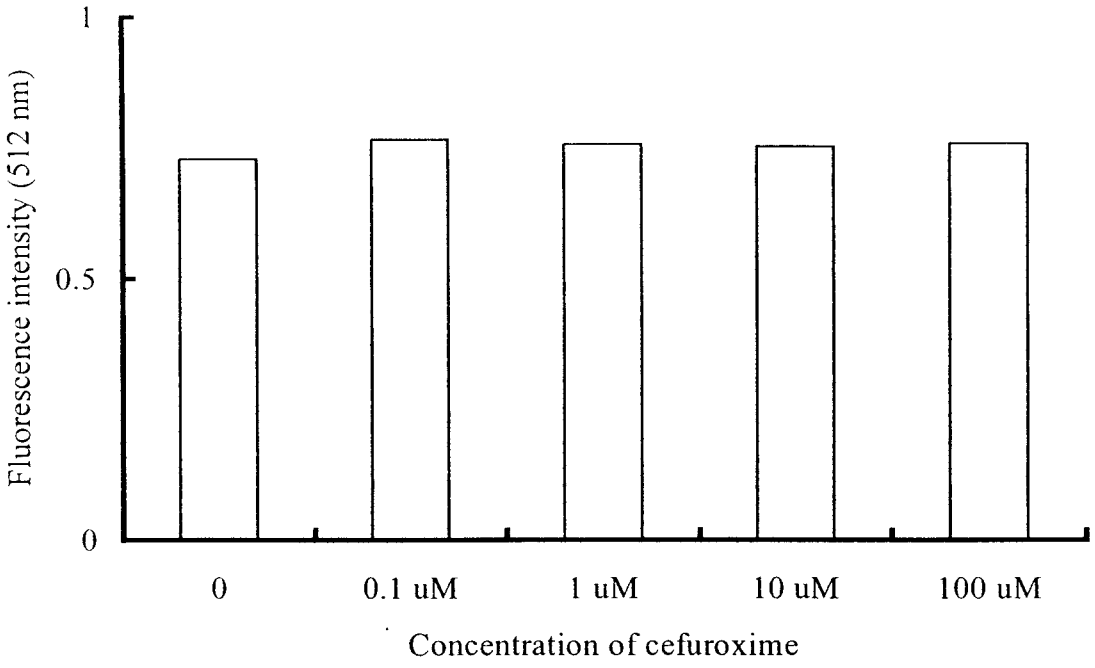


Figure 3.11 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of cefuroxime

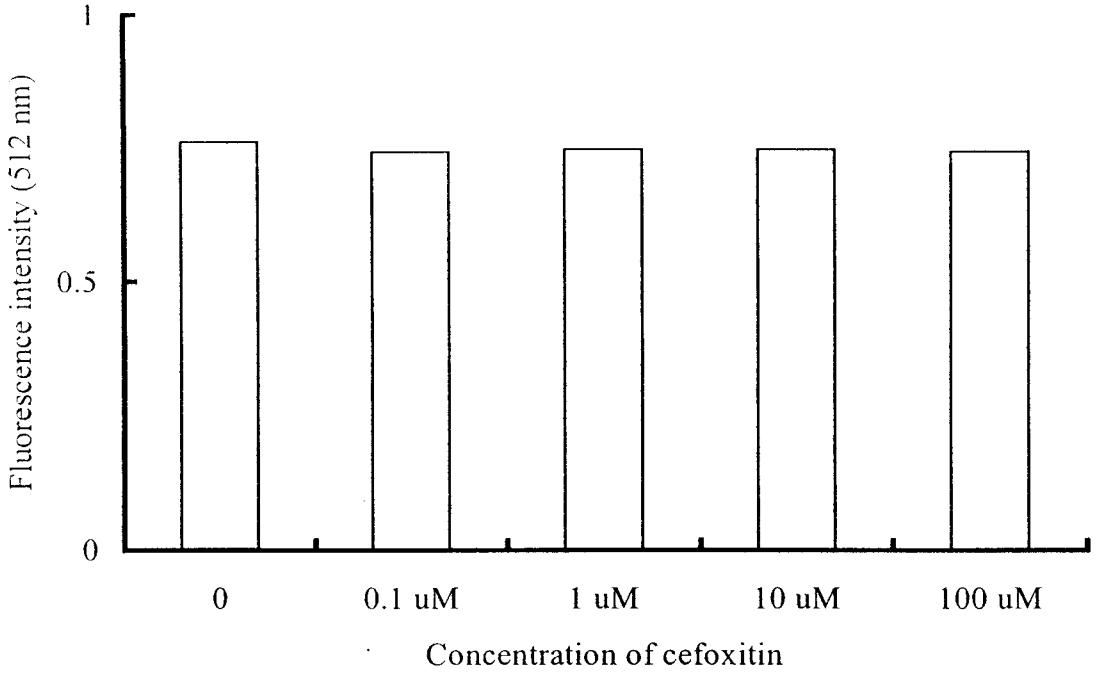


Figure 3.12 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of cefoxitin

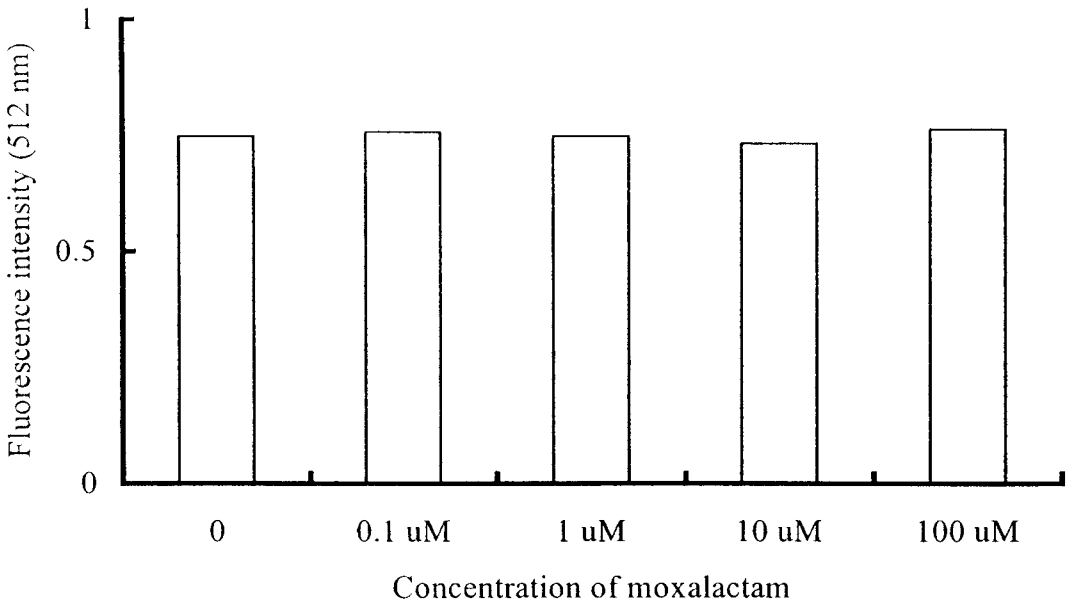
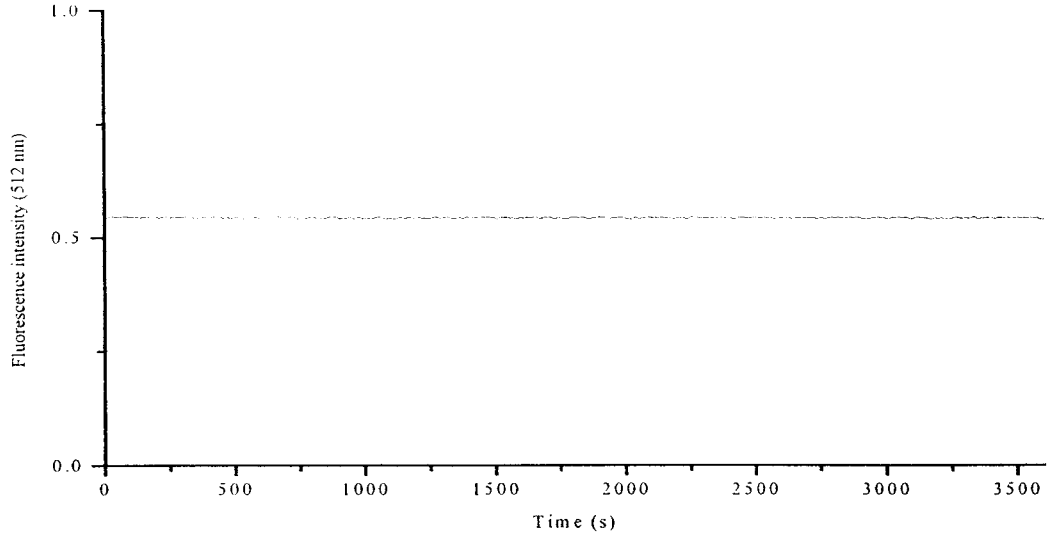


Figure 3.13 Fluorescence intensities of free fluorescein in 50 mM phosphate buffer (pH 7.0) in the presence of various concentrations of moxalactam

(a)



(b)

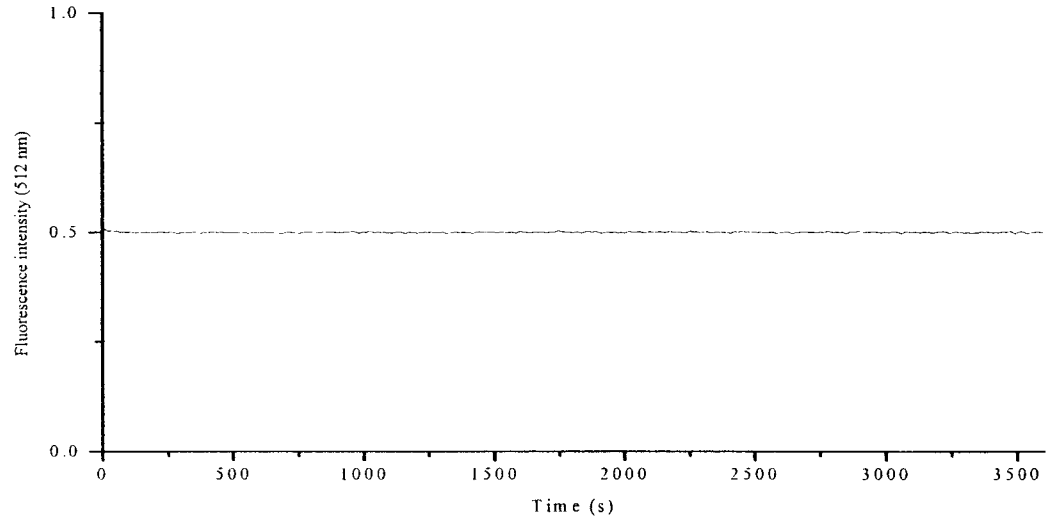
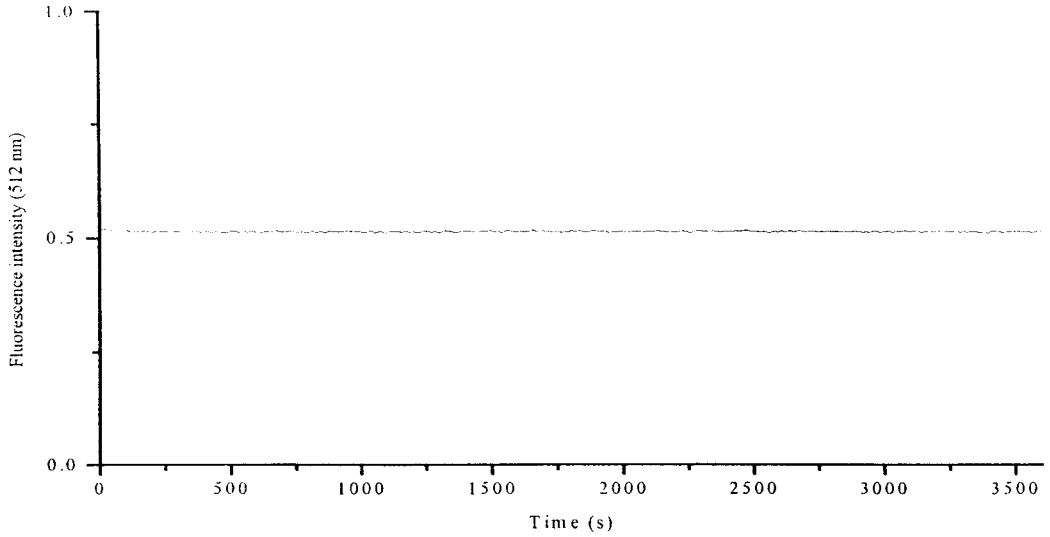


Figure 3.14 Time-resolved fluorescence measurements of free fluorescein (1.2 x 10^{-7} M) at 512 nm in 50 mM phosphate buffer (pH 7.0) in the presence of unlabeled E166C enzyme (1.2 x 10^{-7} M) with 1.0 x 10^{-5} M penicillin G (a) and 1.0 x 10^{-5} M penicillin V (b) as substrates. Excitation wavelength: 494 nm.

(a)



(b)

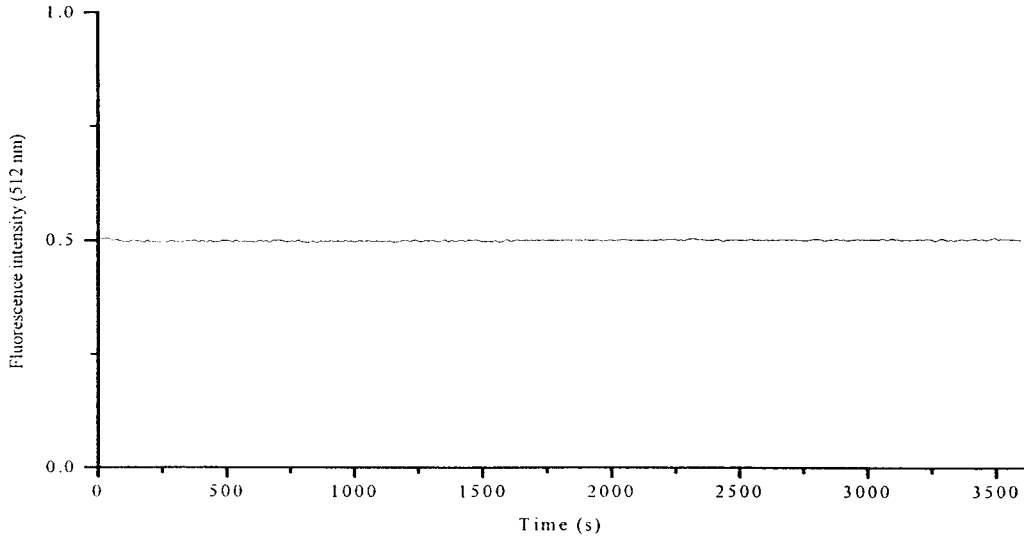
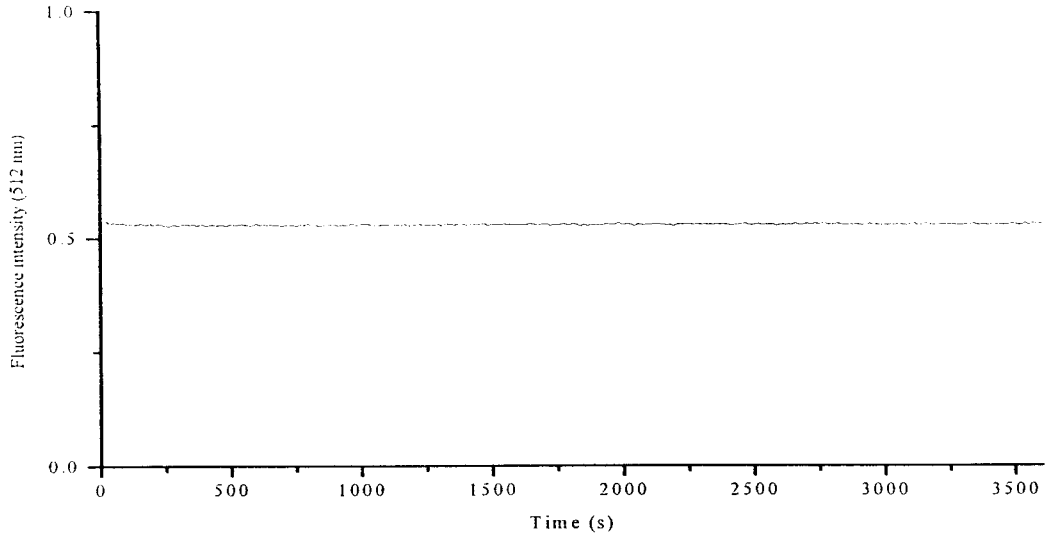


Figure 3.15 Time-resolved fluorescence measurements of free fluorescein (1.2 x 10^{-7} M) at 512 nm in 50 mM phosphate buffer (pH 7.0) in the presence of unlabeled E166C enzyme (1.2 x 10^{-7} M) with 1.0 x 10^{-5} M ampicillin (a) and 1.0 x 10^{-5} M cefuroxime (b) as substrates. Excitation wavelength: 494 nm.

(a)



(b)

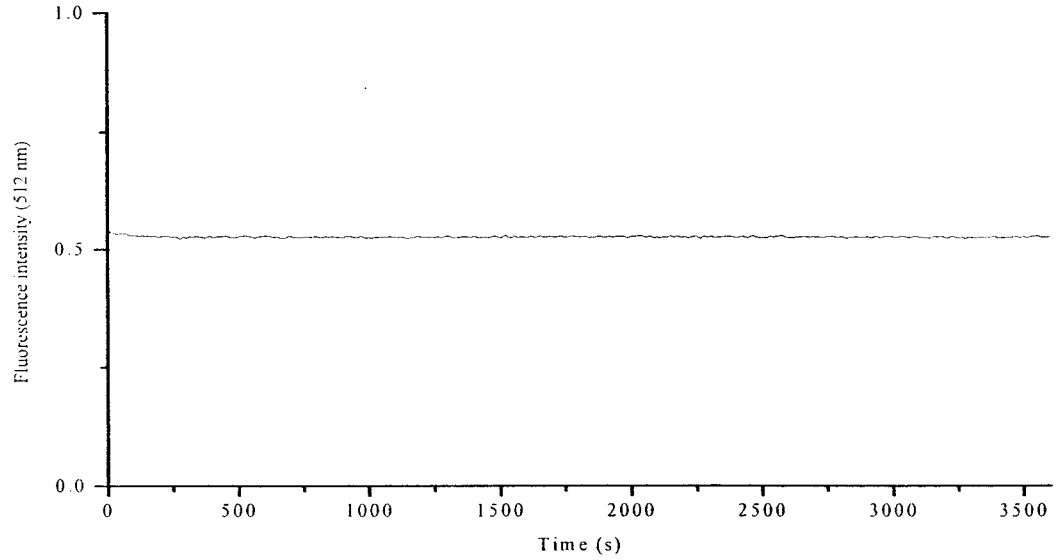
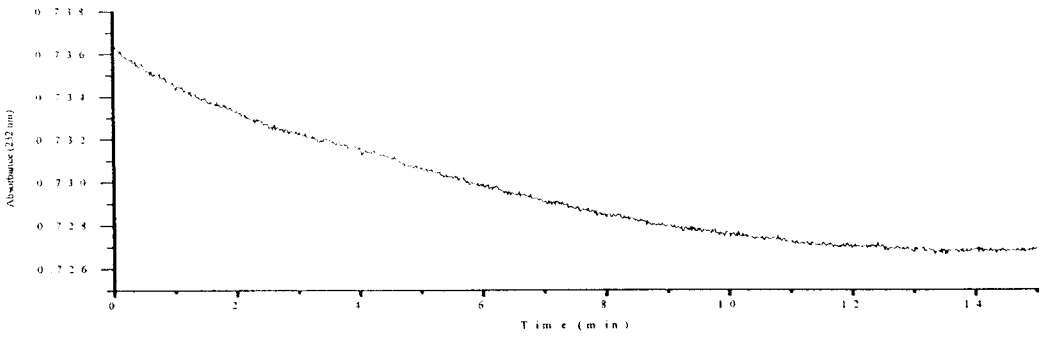
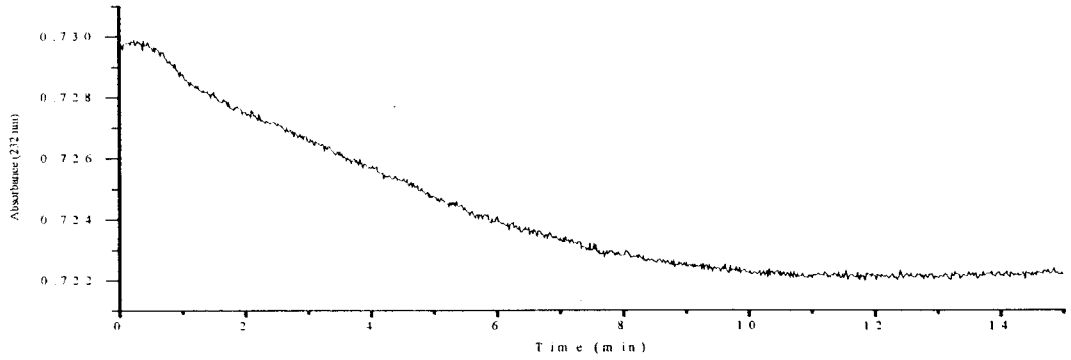


Figure 3.16 Time-resolved fluorescence measurements of free fluorescein (1.2 x 10^{-7} M) at 512 nm in 50 mM phosphate buffer (pH 7.0) in the presence of unlabeled E166C enzyme (1.2 x 10^{-7} M) with 1.0 x 10^{-5} M cefoxitin (a) and 1.0 x 10^{-5} M moxalactam (b) as substrates. Excitation wavelength: 494 nm.

(a)



(b)



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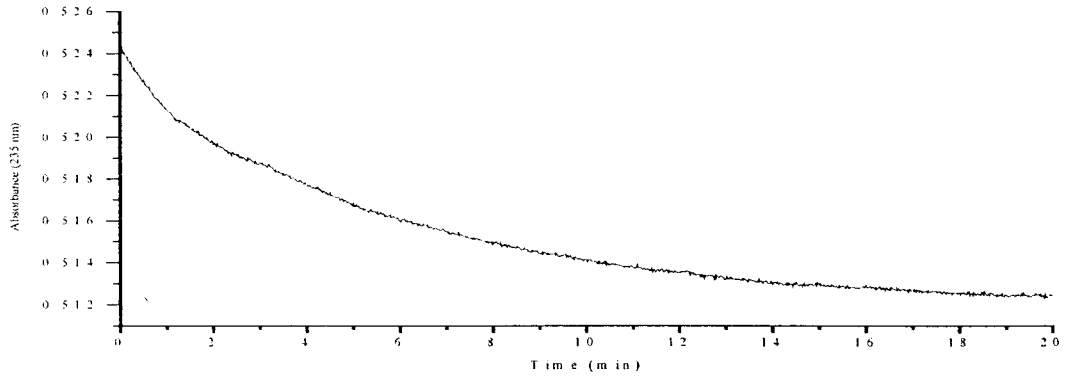
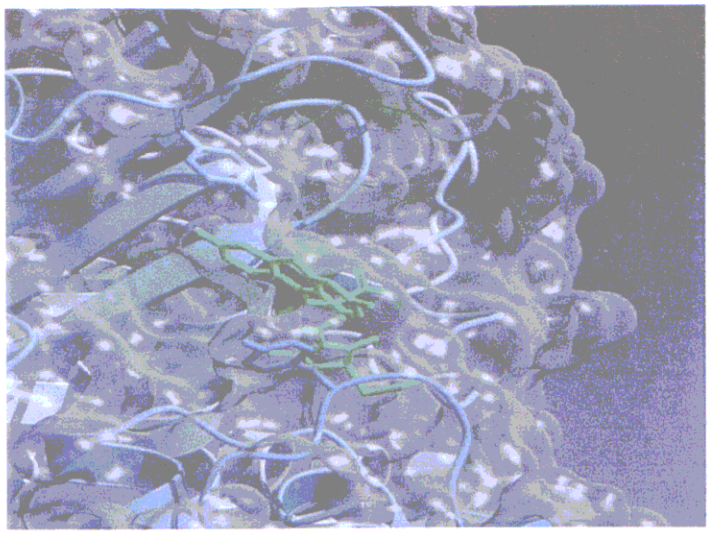


Figure 3.17 Spectrophotometric assays of the unlabeled E166C enzyme (1.2×10^{-7} M) in the presence of free fluorescein (1.2×10^{-7} M) and 1.0×10^{-5} M penicillin G (a), 1.0×10^{-5} M penicillin V (b) and 1.0×10^{-5} M ampicillin (c).

We speculate that the observed fluorescence signals are due to a change in the conformation of the active site upon substrate binding. The conformation of the active site of the E166Cf enzyme before and after binding a penicillin G substrate were simulated by molecular modeling (Figure 3.18). In the absence of penicillin G, the fluorescein label (the green molecule) is buried in the active site. When the labeled enzyme accommodates a penicillin G substrate (the red molecule) into its active site, the fluorescein label attached to the Ω-loop moves away from the catalytic pocket and becomes more exposed to the bulk water. This motion is possible because of the flexibility of the Ω-loop [70, 71]. The departure of the fluorescein label from the active site might separate the fluorescein molecule from the quenchers (amino acids) in the active site, thus restoring the fluorescence of the fluorescein label.

(a)



(b)

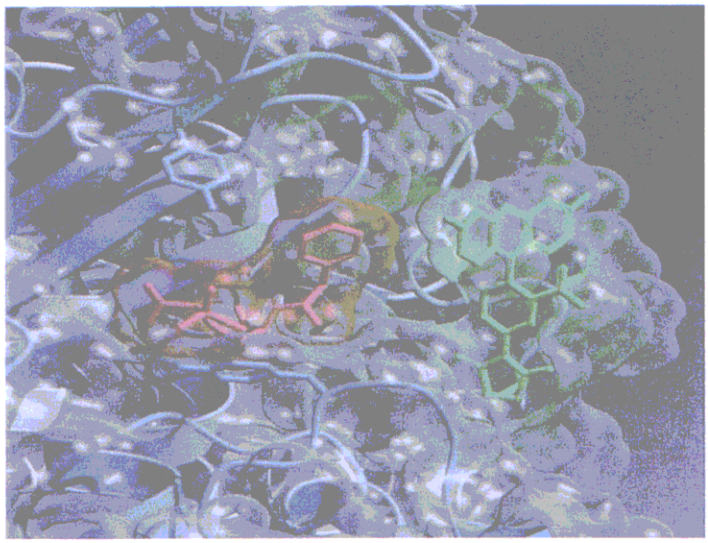


Figure 3.18 Stereoview of the active site of the E166Cf enzyme showing the positions of the fluorescein label (the green molecule) before (a) and after binding a penicillin G substrate (the red molecule) (b).

3.3.3 Effects of structure and concentration of β-lactam antibiotic on the fluorescence signal of the E166Cf enzyme

The time-resolved fluorescence signals of the E166Cf enzyme obtained in the presence of penicillin and cephalosporin antibiotics at various concentrations can be rationalized by the following three-step model [22]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$

where E is the free β-lactamase enzyme, S is the β-lactam substrate, ES is a noncovalent enzyme-substrate complex, ES^* is an acyl-enzyme complex and P is the carboxylic acid.

At low substrate concentration (1.0×10^{-7} and 1.0×10^{-6} M), the binding between the E166Cf enzyme (E) and penicillin substrate (S) would lead to the formation of the ES complex and hence the enhancement in the fluorescence signal. As hydrolysis of antibiotic proceeds, the majority of the penicillin substrates are converted to free carboxylic acids (P). As a result, most of the E166Cf enzymes return to their substrate-free conformation (E) and hence their original weak fluorescence signals

were restored (Figure 3.19). This can explain the slow decline in fluorescence signal with time after the peak. At this stage, addition of more penicillin G to the buffer solution containing the labeled enzyme would cause the fluorescence signal to increase again due to the formation of the ES complexes (Figure 3.20).

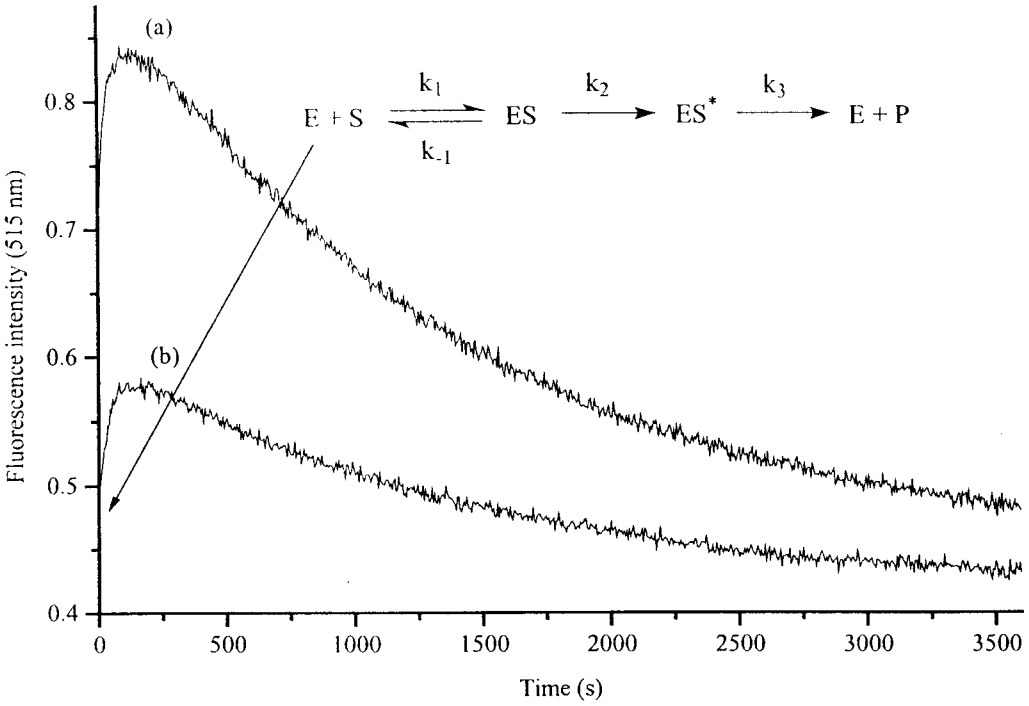


Figure 3.19 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-6} M penicillin G (a) and 1.0 x 10^{-7} M penicillin G (b). Excitation wavelength: 494 nm.

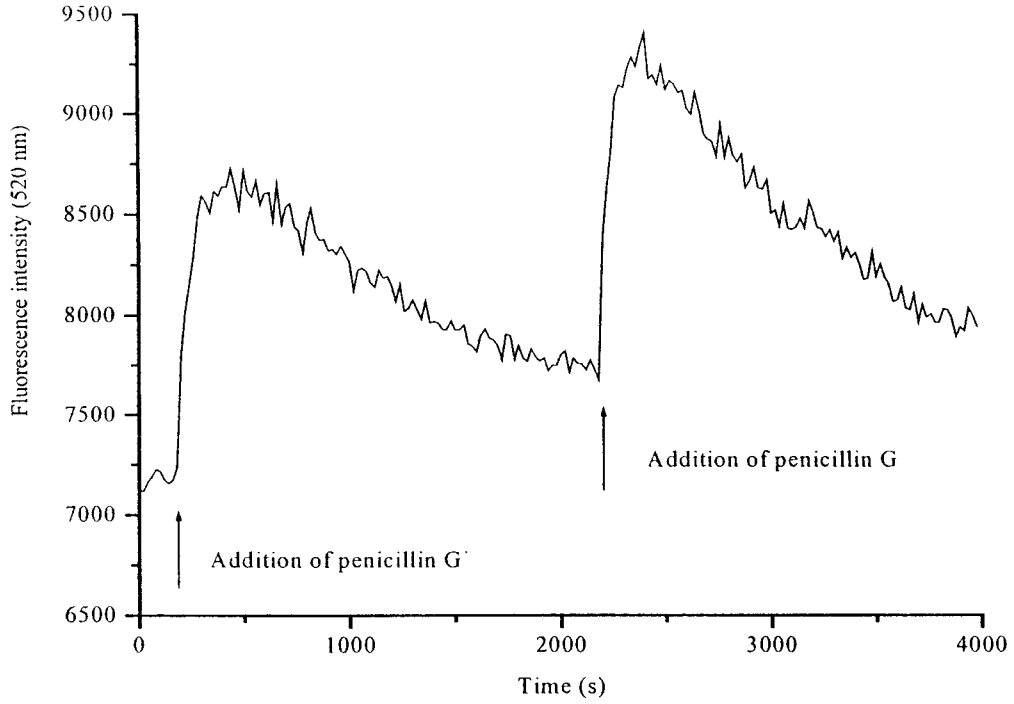


Figure 3.20 Time-resolved fluorescence measurement of the E166Cf enzyme (1.2 x 10^{-7} M) at 520 nm in 50 mM phosphate buffer (pH 7.0) injected with penicillin G at different time points. Excitation wavelength: 494 nm.

At high substrate concentration (1.0×10^{-5} and 1.0×10^{-4} M), the penicillin substrates rapidly occupy the active sites to form the ES complexes, thus switching on the fluorescence of the fluorescein labels instantaneously. Because of the antibiotic concentration is high, the continuous hydrolysis of antibiotics would maintain the E166Cf enzymes in the ES^* state for a while, and hence the fluorescence signal is leveled off to a plateau (Figure 3.21). This can be verified by the fact that the 'plateau time' (8 and 22 min for 1.0×10^{-5} and 1.0×10^{-4} M penicillin G respectively) is consistent with the 'substrate hydrolysis time' determined by spectrophotometric assay (7 and 20 min for 1.0×10^{-5} and 1.0×10^{-4} M penicillin G respectively) (Figure 3.22). When most of the penicillin substrates have been hydrolyzed to carboxylic acids, the fluorescence signal of the labeled enzyme will decline subsequently.

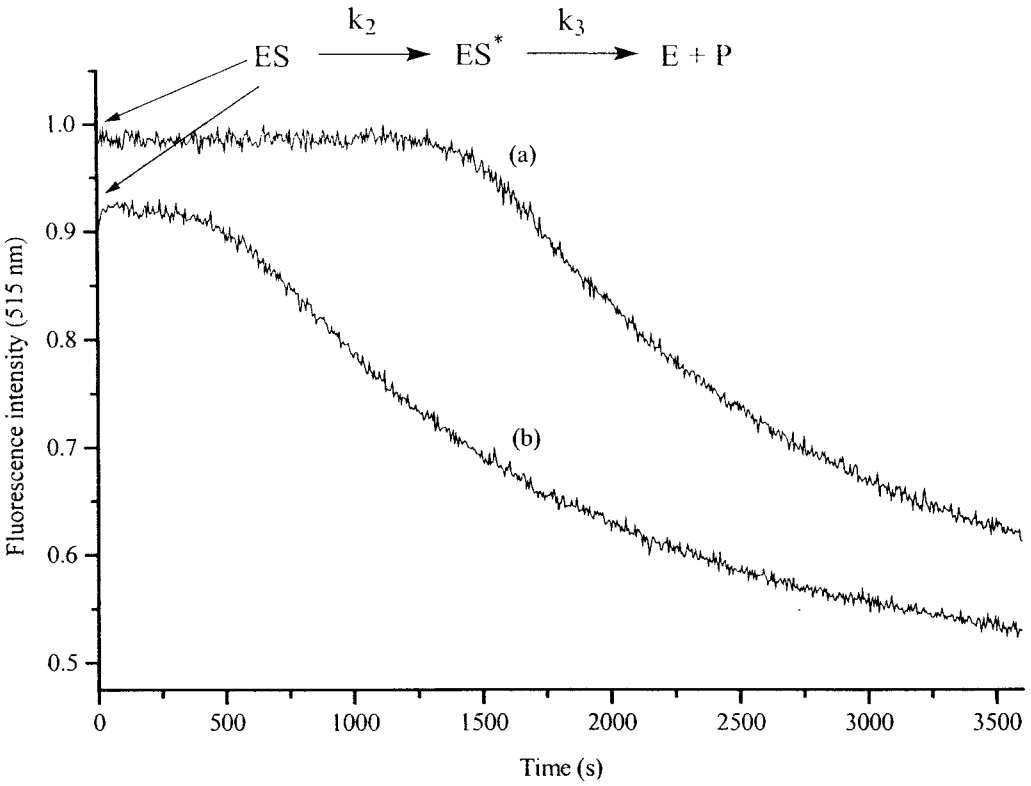
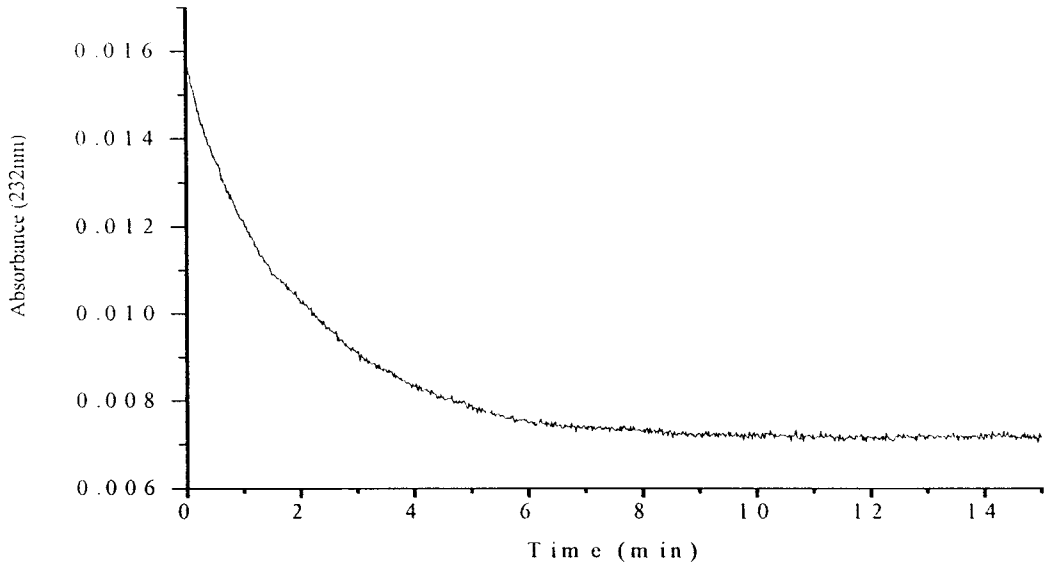


Figure 3.21 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M penicillin G (a) and 1.0 x 10^{-5} M penicillin G (b). Excitation wavelength: 494 nm.

(a)



(b)

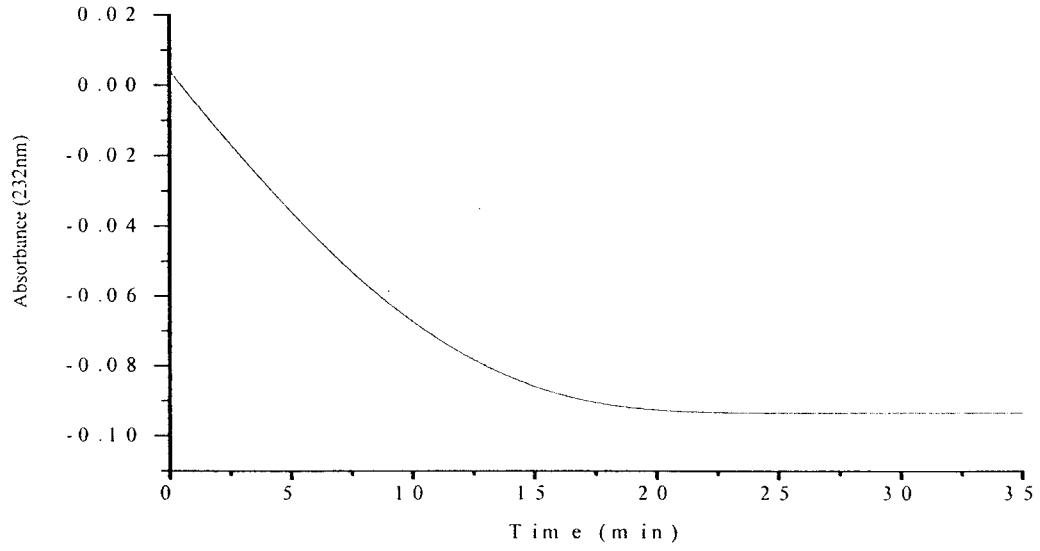


Figure 3.22 Spectrophotometric assays of the E166Cf enzyme in 50 mM phosphate buffer (pH 7.0) with 1.0 x 10^{-5} M penicillin G (a) and 1.0 x 10^{-4} M penicillin G (b) as substrates.

The absence of a declining fluorescence signal for cefuroxime, cefoxitin and moxalactam can be ascribed to the poor hydrolytic activities of the E166Cf enzyme towards these antibiotics (very low k_2 and/or k_3) (Figure 3.23). As a result, the cephalosporin substrates stay in the active sites. To verify this, the activities of the labeled enzyme with cefuroxime, cefoxitin and moxalactam as substrates were monitored by circular dichroism (CD) spectropolarimetry. The principle of this assay is that β-lactam antibiotics, due to the asymmetric property of their fused ring systems, exhibit strong CD signals, but will become CD inactive when they are hydrolyzed by β-lactamase (Figure 3.24) [91]. Therefore, by measuring the CD signals of cefuroxime, cefoxitin and moxalactam (1.0×10^{-4} M) at 258, 264 and 265 nm respectively as a function of time in the presence of E166Cf enzyme (1.2×10^{-7} M), the hydrolytic activities of the labeled enzyme towards these antibiotics can be monitored. To ensure that any changes in the CD signals are due to the enzymatic activity instead of spontaneous β-lactam hydrolysis, negative controls were set up in which no E166Cf enzymes were added to the antibiotic solutions. As shown in Figures 3.25 to 3.27, no significant changes in the CD signals appear after incubating the E166Cf enzymes with the cephalosporin antibiotics for one hour. This indicates that cefuroxime, cefoxitin and moxalactam are resistant to the hydrolytic activity of the labeled enzyme. This is consistent with previous reports that the accommodation

of the 7α-methoxy group on the cephalosporin β-lactam ring into the active site of β-lactamase I causes impairment on both acylation and deacylation steps [92, 93].

Compared with the penicillin antibiotics, the rates of increase in fluorescence signal for the cephalosporin antibiotics are much lower. For example, the fluorescence intensity of the E166Cf enzyme (1.2×10^{-7} M) takes 130 s to reach the peak signal in the presence of 10^{-6} M penicillin G, but under the same conditions 3000 s was required for 10^{-6} M cefuroxime. The considerable drop in the rate of increase in fluorescence signal for the cephalosporin antibiotics may be attributed to their bulkier structures which impede their binding to the labeled enzymes.

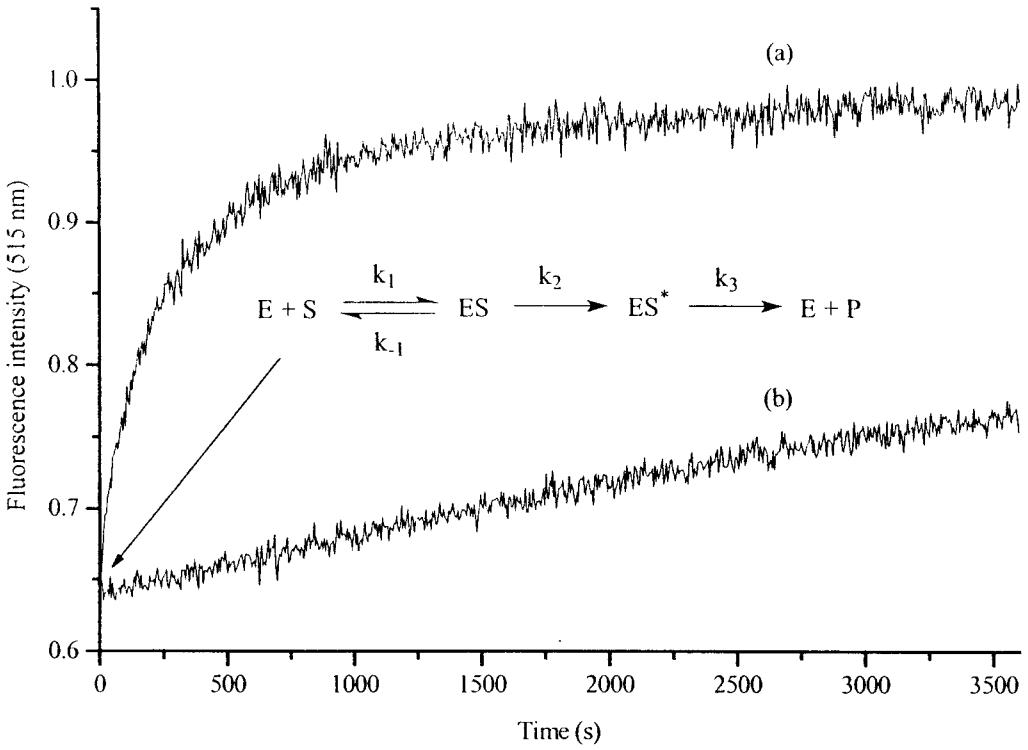


Figure 3.23 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) in the presence of 1.0 x 10^{-4} M cefoxitin (a) and 1.0 x 10^{-6} M cefoxitin (b). Excitation wavelength: 494 nm.

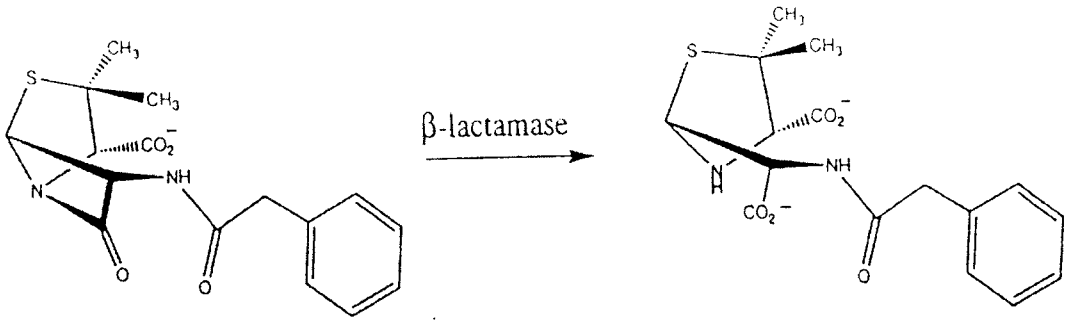
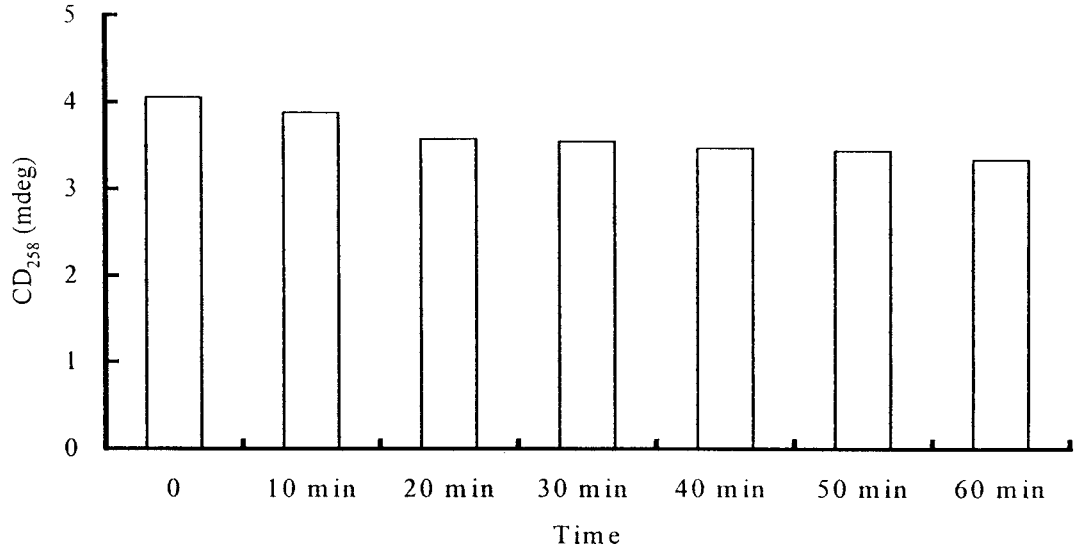


Figure 3.24 Hydrolysis of penicillin G by β-lactamase. The asymmetry of the fused ring system of β-lactam antibiotics is shown schematically [91].

(a)



(b)

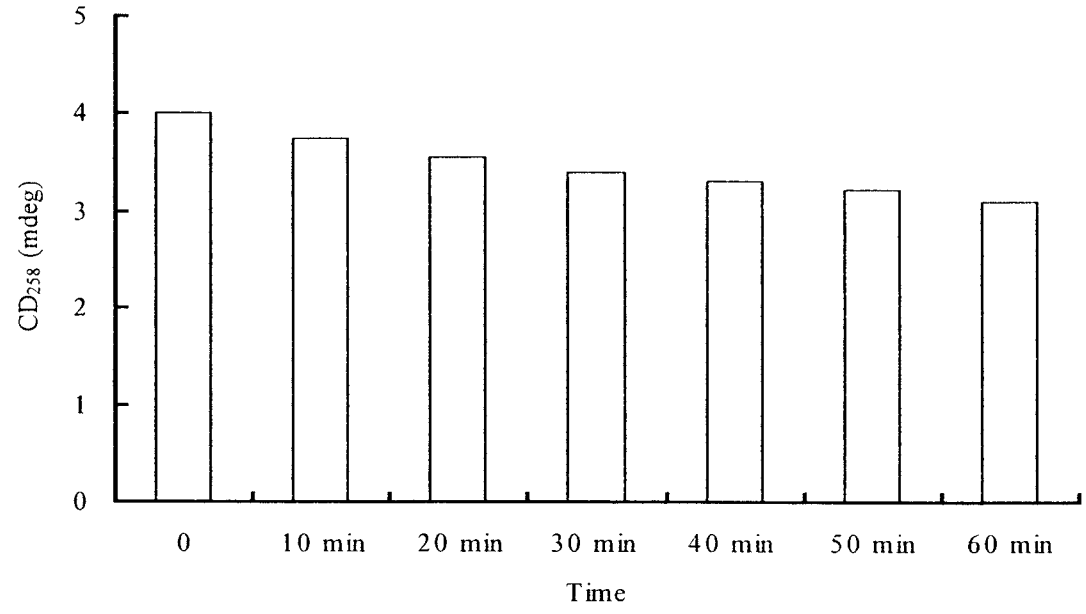
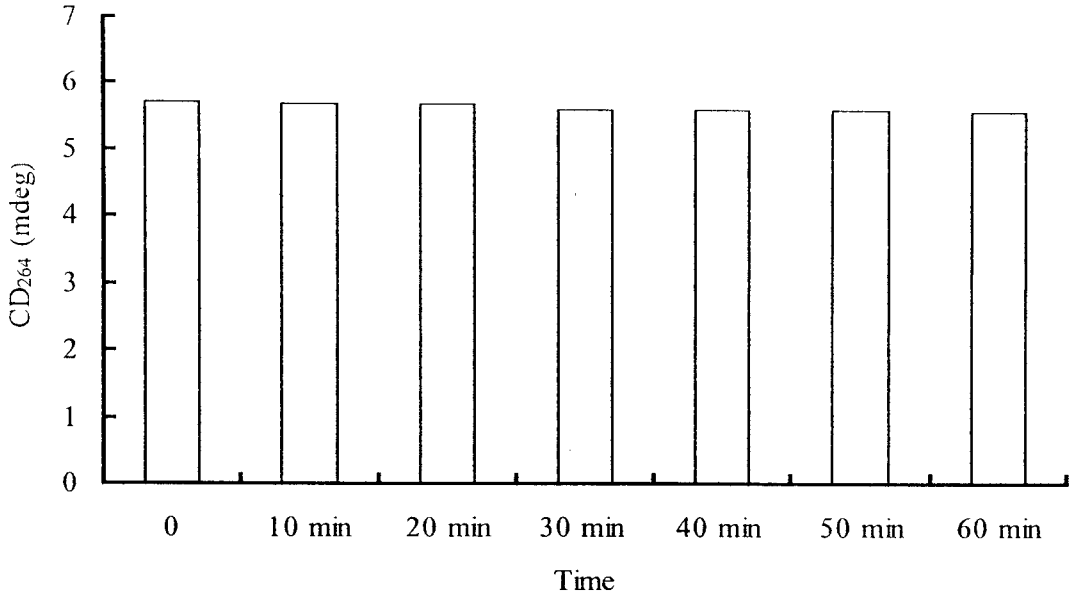


Figure 3.25 Circular dichroism signals (258 nm) of cefuroxime in 50 mM phosphate buffer (pH 7.0) as a function of time in the absence of E166Cf enzyme (a) and in the presence of E166Cf enzyme (1.2×10^{-7} M) (b)

(a)



(b)

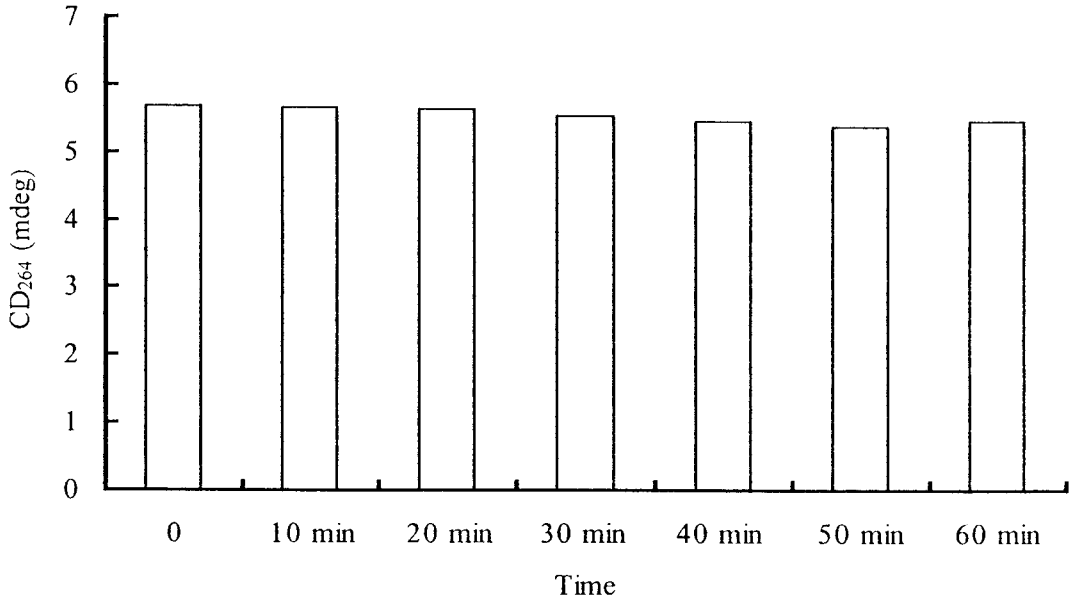
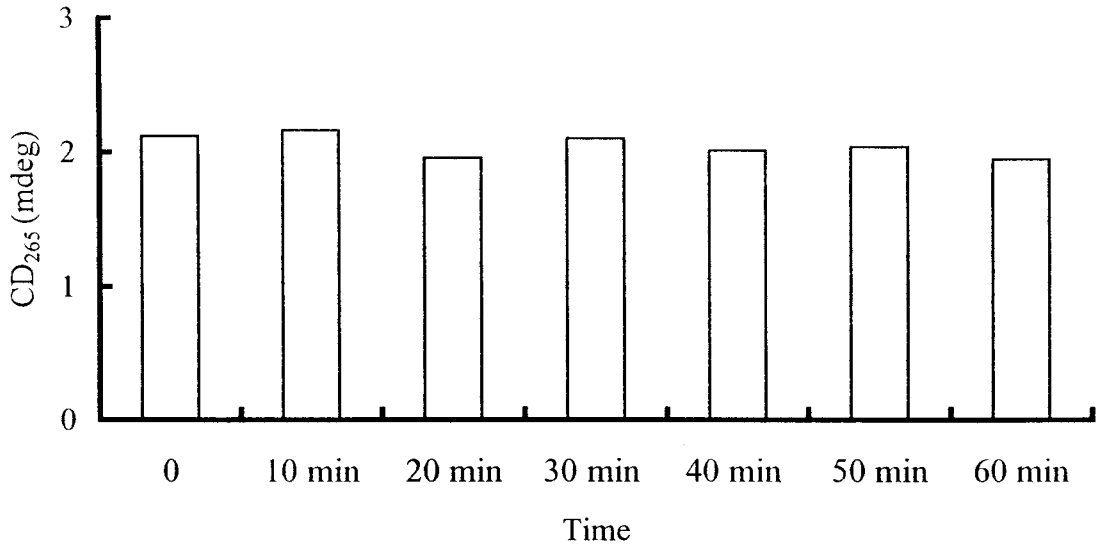


Figure 3.26 Circular dichroism signals (264 nm) of cefoxitin in 50 mM phosphate buffer (pH 7.0) as a function of time in the absence of E166Cf enzyme (a) and in the presence of E166Cf enzyme (1.2×10^{-7} M) (b)

(a)



(b)

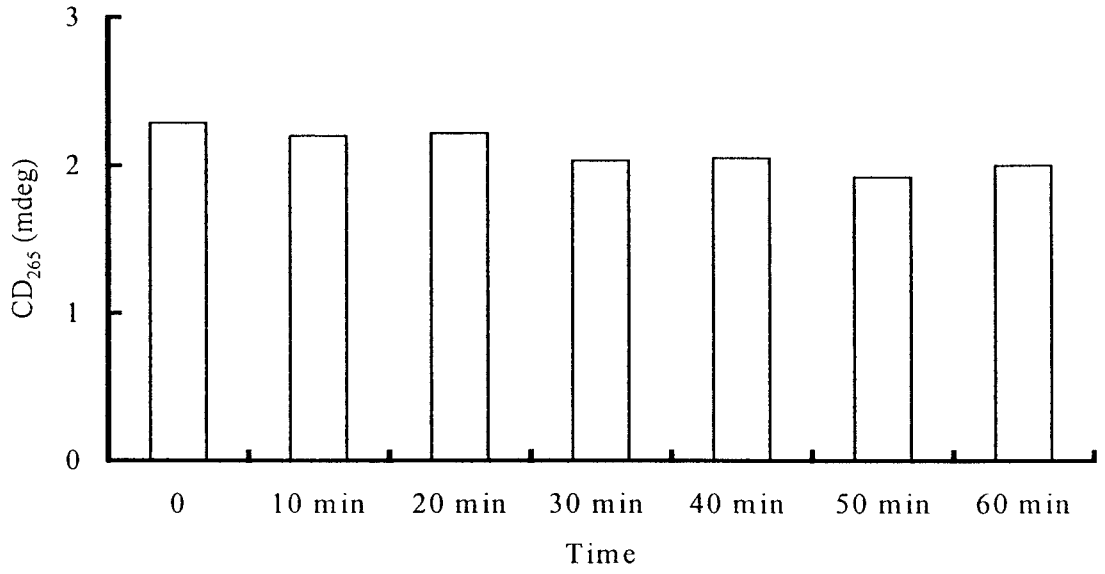


Figure 3.27 Circular dichroism signals (265 nm) of moxalactam in 50 mM phosphate buffer (pH 7.0) as a function of time in the absence of E166Cf enzyme (a) and in the presence of E166Cf enzyme (1.2×10^{-7} M) (b)

3.3.4 Fluorometric studies of the E166Cf enzyme towards sulbactam and clavulanate

Sulbactam and clavulanate are β-lactamase inhibitors which can inactivate β-lactamases by blocking their active sites via the formation of covalent bond [32, 33]. These β-lactamase inhibitors are usually used in combination with other β-lactam antibiotics in antibacterial therapies. The β-lactamase inhibitors serve as sacrificial agents to inactivate bacterial β-lactamases, thus allowing their partners to reach their bacteria targets without being inactivated [34].

The reaction mechanism of sulbactam towards *Escherichia coli* TEM-2 β-lactamase is shown in Figure 3.28 [32]. The initial step involves the formation of acyl-enzyme complex via the acylation of Ser-70 in the active site. This covalently modified complex then dissociates directly to regenerate the enzyme, or undergoes rearrangements to give an enamine ester which eventually leaves the active site via deacylation. In addition to these pathways, the acyl-enzyme complex also reacts to give a cross-linking ester bond in the active site, thus blocking the active site irreversibly.

The reaction mechanism of clavulanate towards class A β-lactamases is quite complicated. Figure 3.29 shows the inhibition mechanism of clavulanate towards *Staphylococcus aureus* PC1 and *Escherichia coli* TEM-2 β-lactamases [32]. The initial step involves the formation of acyl-enzyme complex accompanied by the opening of the oxazole ring. This complex then deacylates directly to regenerate the enzyme or undergoes a series of rearrangements leading to the formation of inert enamine ester. For the latter, the complex undergoes deacylation to give the free enzyme again or reacts to give an ester bond linking Ser-70 and Ser-130 in the active site together. This 'cross-linked' enzyme reacts further to give other covalently modified enzymes.

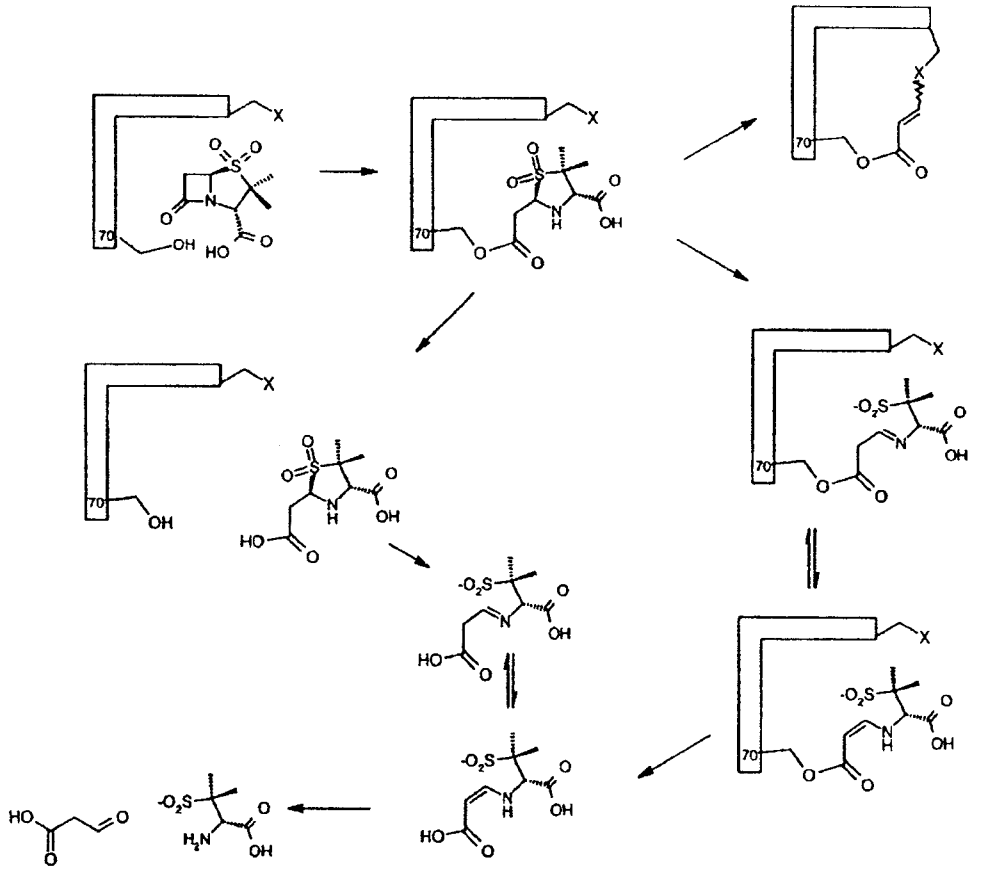


Figure 3.28 Proposed reaction mechanism of sulbactam with *E. coli* TEM-2 β-lactamase. The identity of the nucleophile X is yet unknown but has been postulated to be Lys-273 or Ser-130 [32].

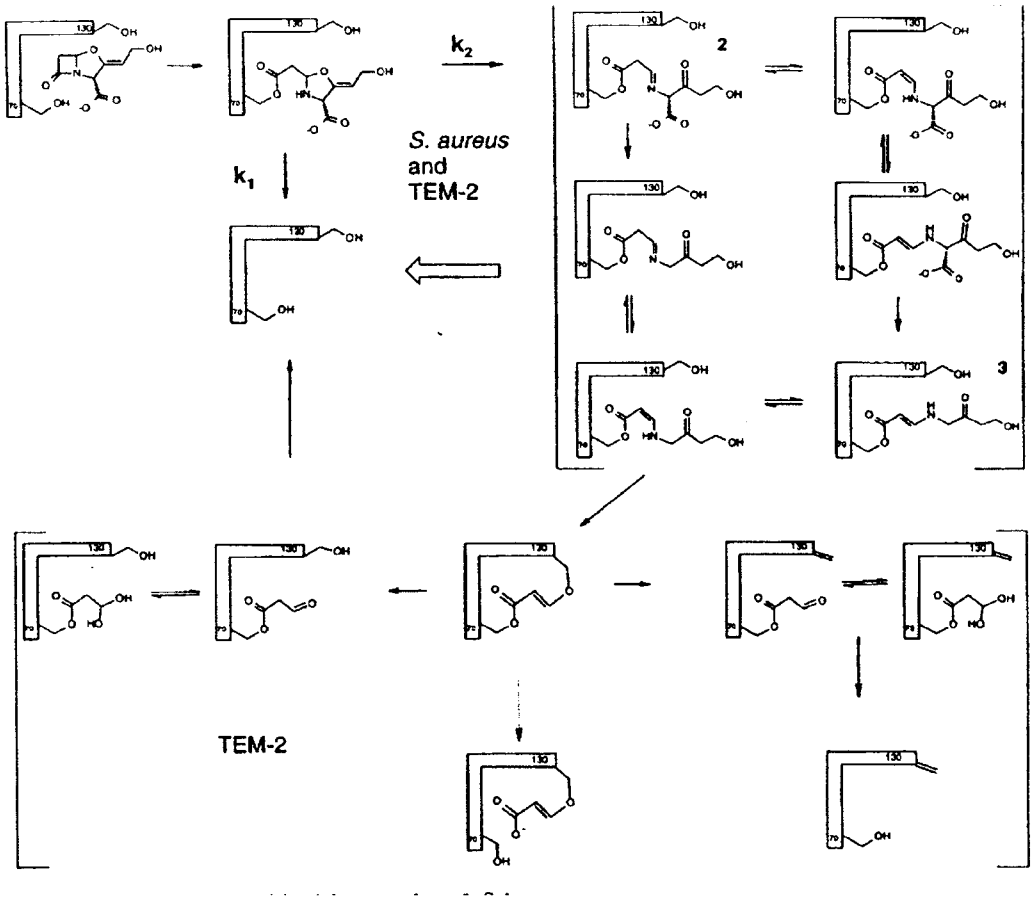
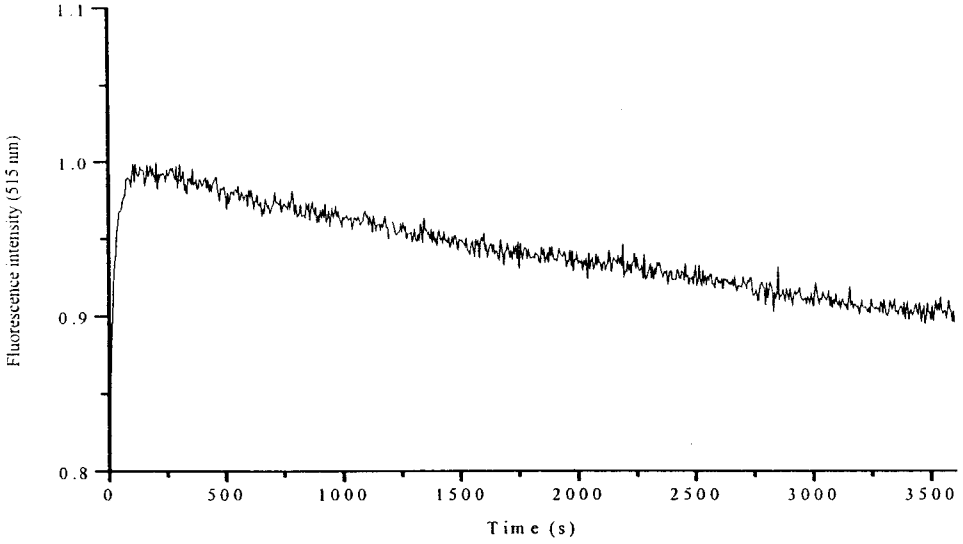


Figure 3.29 Proposed reaction mechanism of clavulanate with *S. aureus* PC1 and *E. coli* TEM-2 β-lactamases [32].

As most class A β-lactamases are susceptible to the inhibitions by sulbactam and clavulanate [33], we reasoned that the E166Cf enzyme could detect these β-lactamase inhibitors. The fluorescence behaviors of the E166Cf enzyme (1.2×10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with sulbactam (1.0×10^{-4} M) and clavulanate (1.0×10^{-3} M) as substrates were investigated by measuring the fluorescence intensities at 515 nm as a function of time. As shown in Figure 3.30, the fluorescence signals obtained with sulbactam and clavulanate are entirely different. For sulbactam, the fluorescence intensity increases rapidly at the initial stage and then declines slowly. For clavulanate, the addition of substrate causes an instantaneous increase in fluorescence signal which declines rapidly within 200 s and then levels off to a plateau.

(a)



(b)

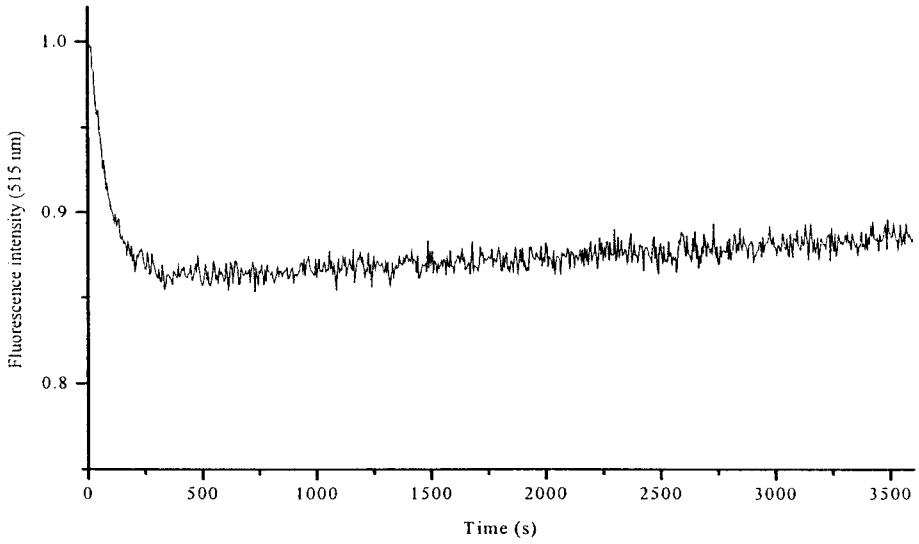


Figure 3.30 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) at 515 nm in 50 mM phosphate buffer (pH 7.0) with 1.0 x 10^{-4} M sulbactam (a) and 1.0 x 10^{-3} M clavulanate (b) as substrates. Excitation wavelength: 494 nm.

The reason for the difference in fluorescence behavior for the E166Cf enzyme in the presence of sulbactam and clavulanate have yet to be confirmed. The fluorescence signals recorded may be rationalized by the inhibition mechanisms shown in Figures 3.28 and 3.29. For sulbactam, the increase in fluorescence signal may be attributed to the formation of covalent ES^* complex. The subsequent decline in fluorescence intensity implies that the covalent ES^* complex may deacylate directly to regenerate the labeled enzyme or undergo rearrangement reactions to give an enamine ester which eventually leaves the active site via deacylation (Figure 3.28). The rapid decline in fluorescence signal for clavulanate may be ascribed to the regeneration of free E166Cf enzyme via the pathways shown in Figure 3.29.

3.4 Concluding remarks

The time-resolved fluorescence measurements demonstrated that the E166Cf enzyme fluoresces more strongly in the presence of penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam. The increase in fluorescence signal might be due to the conformational change in the active site upon binding of a β-lactam substrate. Such a conformational change might cause the fluorescein label to depart from the active site, thus switching on the fluorescence of the fluorescein molecule. As indicated in the time-resolved fluorescence spectra, the E166Cf enzyme is capable of distinguishing 'β-lactamase-unstable' antibiotics (penicillin G, penicillin V and ampicillin) from 'β-lactamase-resistant' antibiotics (cefuroxime, cefoxitin and moxalactam). In the presence of penicillin antibiotics, the fluorescence signal of the labeled enzyme increases and then declines. In contrast, in the presence of cephalosporin antibiotics, the fluorescence intensity increases gradually to give a plateau signal. It should be noted that the E166Cf enzyme can detect β-lactam antibiotics at trace level (10^{-7} M), and would potentially serve as a reagentless tool for the convenient measurement of antibiotics in liquid samples such as milk. Furthermore, the E166Cf can detect sulbactam and clavulanate, and may find its application in the discovery of new β-lactamase inhibitors.

Chapter 4

Application of the fluorescein-labeled β-lactamase I (E166Cf) in screening bacteria for β-lactamases against a panel of β-lactam antibiotics

4.1 Introduction

The effectiveness of β-lactam antibiotics in antibacterial therapies has led to their widespread use in clinical treatments of bacterial infection. However, the clinical importance of β-lactam antibiotics has been challenged by the emergence of bacterial strains that can produce β-lactamases to inactivate β-lactam antibiotics [4-6]. This clinical problem is further worsened by the emergence of new or modified β-lactamases with extended substrate profiles [8] and the widespread ability of pathogenic bacteria to produce β-lactamases [5]. To respond to this clinical problem, the pharmaceutical industry has produced a wide variety of new β-lactam drugs which are more resistant to β-lactamase activities. As a consequence of increasing β-lactam drugs available for clinical treatment, a rapid, sensitive and high-throughput method that can screen bacteria for β-lactamases against a panel of drug candidates is highly desired.

In order to choose an effective antibiotic for a patient confidently, the substrate specificity of the β-lactamase produced by the infecting bacterial species must be known because β-lactamase encoding genes can be transferred among bacterial strains [5]. Thus, a sensitive method for detecting β-lactamase activity against various

antibiotics is highly desired in drug screening. As mentioned in Chapter 1, although a number of novel assays for detection of β-lactamase activity have been developed in recent years (e.g. ECL [45, 46] and fluorescent spot test methods [47-49]), they suffer from disadvantages which limit their applications in drug screening. For example, the fluorescent spot test method can be only applied to the β-lactam antibiotics with an acyl side chain having an α-amino group and an α-phenyl group [47]. Therefore, we are interested in developing the E166Cf enzyme into a novel and easy-to-use biosensor for drug screening.

As indicated in Chapter 3, the E166Cf enzyme can detect both penicillins and cephalosporins at low concentrations. We reasoned that the E166Cf enzyme could also serve as a reagentless tool to screen β-lactamase-producing bacteria against β-lactam antibiotics by monitoring the competition for β-lactam antibiotics between the labeled enzyme and bacterial β-lactamase. When both E166Cf and bacterial β-lactamase enzymes are incubated with β-lactamase-unstable antibiotics, the fluorescence of the E166Cf enzyme will be suppressed because of the greater catalytic efficiency of the bacterial enzyme. In contrast, when bacterial β-lactamases are screened against β-lactamase-resistant antibiotics, the fluorescence of the E166Cf enzyme will be enhanced because of the poor hydrolytic activity of the bacterial

enzyme. In this chapter, we demonstrate that the E166Cf enzyme can be used as a reagentless tool in screening various bacterial β-lactamases (*Bacillus cereus* penPC β-lactamase, *Escherichia coli* TEM-1 β-lactamase, *Bacillus licheniformis* penP β-lactamase and *Bacillus cereus* β-lactamase II) against different β-lactam antibiotics (penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam). These bacterial enzymes are chosen because we are interested in investigating the ability of the E166Cf enzyme to screen β-lactamases from different sources (*B. cereus* penPC, *B. licheniformis* penP and *E. coli* TEM-1 are classified as class A β-lactamase whereas *B. cereus* β-lactamase II belongs to class B β-lactamase [7]) against various β-lactam antibiotics. Moreover, *Bacillus* is a Gram-positive bacterium which secretes β-lactamases into the medium while *E. coli* is a Gram-negative strain which produces β-lactamases intracellularly [4]. The detection of β-lactamases from *E. coli* is more difficult than from *Bacillus*, and therefore we are also interested in investigating the ability of the E166Cf enzyme to detect the β-lactamases produced by *E. coli*.

4.2 Experimental section

4.2.1 Materials

All chemicals were of analytical grade. Penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin, moxalactam and chloramphenicol were purchased from Sigma Chemical Co. Potassium dihydrogenphosphate (KH_2PO_4) and sodium chloride (NaCl) were obtained from Aldrich Chemical Co. Brain heart infusion, nutrient agar, LD broth and yeast extract were purchased from OXOID Ltd. Deionized water was purified by a Milli-Q water purification system (Millipore).

4.2.2 Preparation of *B. cereus* penPC β-lactamase, *B. licheniformis* penP β-lactamase, *E. coli.* TEM-1 β-lactamase and *B. cereus* β-lactamase II

The β-lactamase II, penPC β-lactamase and penP β-lactamase were expressed in *B. subtilis* ϕ105MU331. These enzymes were prepared according to the procedures described previously [73] with slight modifications.

A bacterial strain was streaked on an agar plate containing 5 μg/ml chloramphenicol,

and the plate was incubated at 37 °C for 24 h. A single bacterial colony from the agar plate was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) which was then incubated at 37 °C with shaking at 300 rpm overnight. About 7 ml of overnight inoculum was added to a baffled conical flask containing 100 ml of sterilized BHY medium. The inoculated medium was incubated at 37 °C with shaking at 300 rpm. When the optical density of the bacterial culture at 600 nm reached 3.5 to 4.0, the bacterial culture was heated in a water bath at 51 °C for 5 min. Afterwards, the bacterial culture was incubated at 37 °C with shaking at 300 rpm for further 6 h. The bacterial culture was then harvested and stored at –20 °C. The expressions of penPC β-lactamase, penP β-lactamase and β-lactamase II at each hour after heat shock were monitored by SDS/PAGE assays (Figures 4.1 to 4.3).

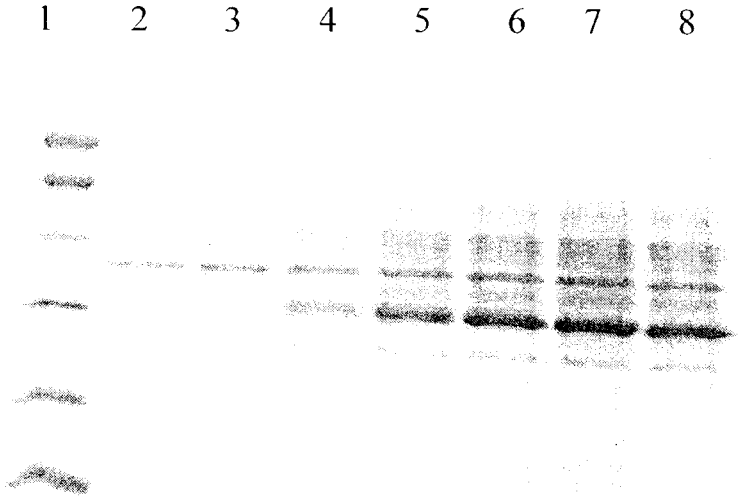


Figure 4.1 SDS/PAGE of the penPC β-lactamase cultures after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

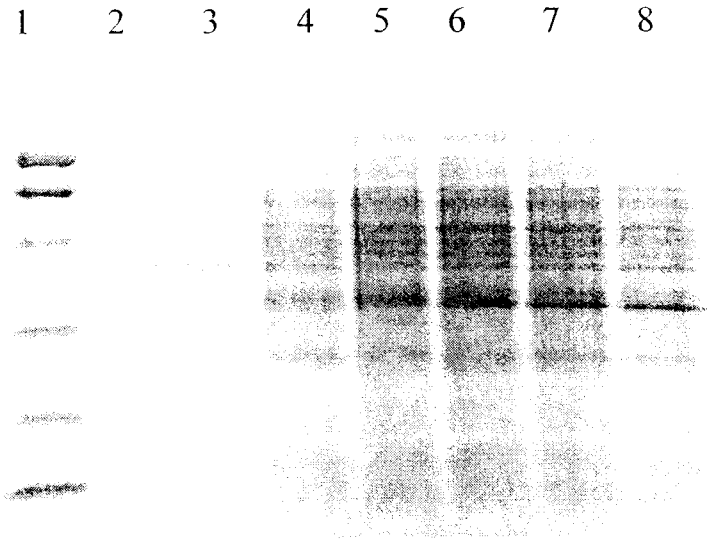


Figure 4.2 SDS/PAGE of the penP β-lactamase cultures after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

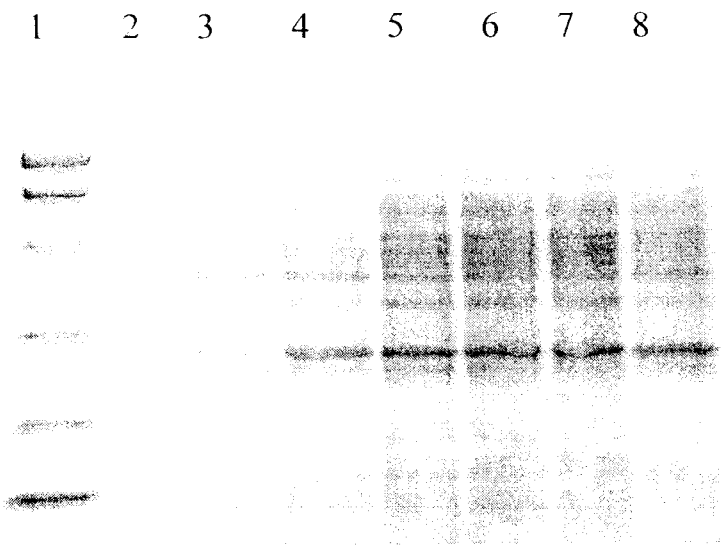


Figure 4.3 SDS/PAGE of the β-lactamase II cultures after heat shock. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: t = 0; lane 3: t = 1 h; lane 4: t = 2 h; lane 5: t = 3 h; lane 6: t = 4 h; lane 7: t = 5 h; lane 8: t = 6 h.

The TEM-1 β-lactamase was expressed in *E. coli.* BL21(DE3). Preparation of the TEM-1 β-lactamase was performed as follows.

A bacterial strain was streaked on an agar plate containing 100 μg/ml ampicillin, and the plate was incubated at 37 °C for 24 h. A single bacterial colony from the plate was inoculated into 100 ml of sterilized LB broth (28 g/l) which was then incubated at 37 °C with shaking at 280 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at –20 °C. The expression of TEM-1 β-lactamase after overnight incubation was monitored by SDS/PAGE assay (Figure 4.4).

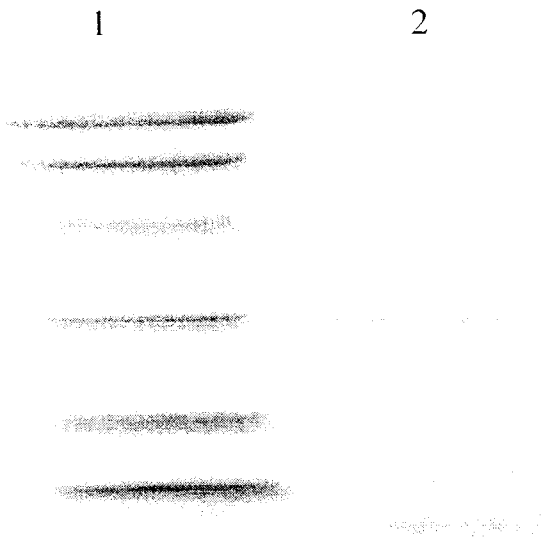


Figure 4.4 SDS/PAGE of the TEM-1 β-lactamase culture after overnight incubation. Lane 1: markers: rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), hen egg white lysozyme (14,400); lane 2: after overnight incubation.

4.2.3 Preparation of *B. subtilis* ϕ105MU331 and *E. coli.* BL21(DE3) cultures

Both *B. subtilis* ϕ105MU331 and *E. coli.* BL21(DE3) cultures (which produce no β-lactamase) were prepared as negative controls for drug screening experiments. Preparation of *B. subtilis* ϕ105MU331 and *E. coli.* BL21(DE3) cultures was performed as follows.

For *B. subtilis* ϕ105MU331, a bacterial strain was streaked on an agar plate containing 5 μg/ml chloramphenicol, and the plate was incubated at 37 °C for 24 h. A single bacterial colony was inoculated into 100 ml of sterilized BHY medium (37 g/l brain heart infusion and 5 g/l yeast extract) which was then incubated at 37 °C with shaking at 300 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at –20 °C.

For *E. coli.* BL21(DE3) , a bacterial strain was streaked on an agar plate containing 100 μg/ml ampicillin, and the plate was incubated at 37 °C for 24 h. A single bacterial colony was inoculated into 100 ml of sterilized LB broth (28 g/l) which was then incubated at 37 °C with shaking at 280 rpm overnight. After overnight incubation, the bacterial culture was harvested and stored at –20 °C.

4.2.4 Physical measurement

Drug screening experiments were performed on a FLUOstar microplate reader (BMG Labtechnologies) equipped with two sample injectors. Excitation and emission filters of 485 and 520 nm respectively were used. Samples including bacterial β-lactamase cultures, bacterial cultures and E166Cf enzymes were placed in a 96-well microtiter plate (Corning Costar) in all measurements. The antibiotic solutions were injected to the samples by the injectors. The injection speed was set at 310 μl/s. All measurements were performed at room temperature.

4.3 Results and discussion

4.3.1 Screening of β-lactamase II against penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam in the presence of the E166Cf enzyme

The fluorescence behaviors of the E166Cf enzyme (1.2×10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0×10^{-4} M) as substrates in the presence of β-lactamase II culture (10 μl) were investigated by measuring the fluorescence intensities at 520 nm as a function of time. Negative controls were also set up in which *B. subtilis* cultures (which produce no β-lactamase II) were added instead of β-lactamase II cultures (Figures 4.5 to 4.10). As shown in the time-resolved fluorescence measurements, the fluorescence intensities of the E166Cf enzyme with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam as substrates increase rapidly in the absence of β-lactamase II. In the presence of β-lactamase II, the fluorescence signal of the labeled enzyme with penicillin G, penicillin V, ampicillin and cefuroxime as substrates declines rapidly. In contrast, no decline in fluorescence signal was observed with cefoxitin and moxalactam.

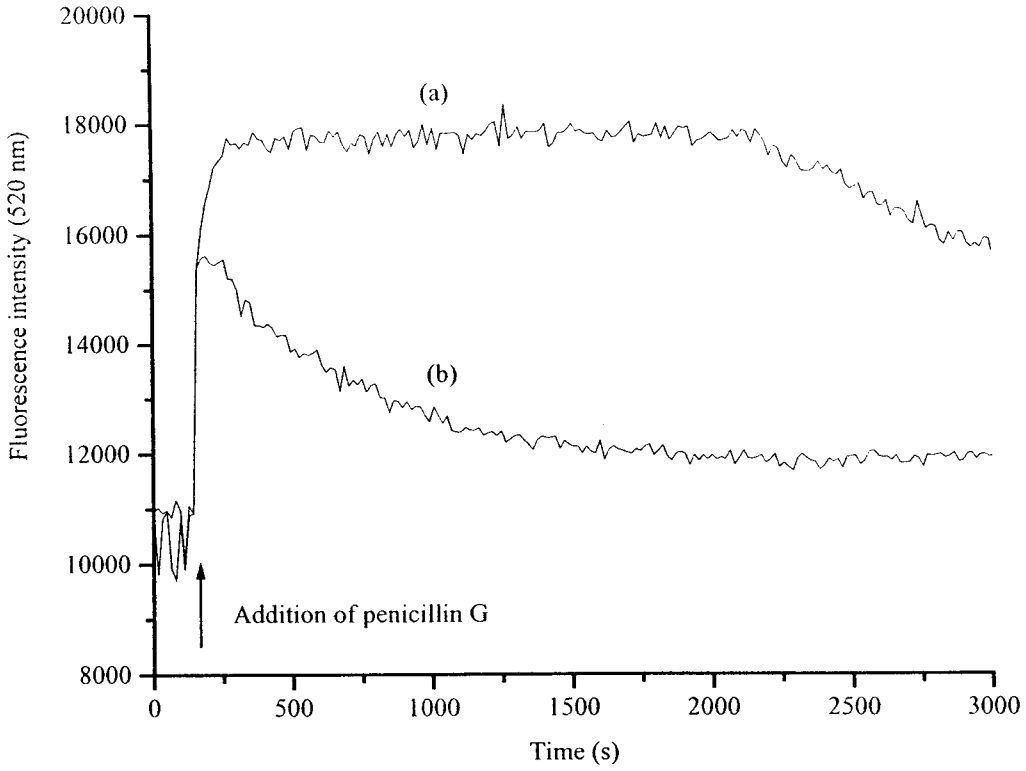


Figure 4.5 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin G (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

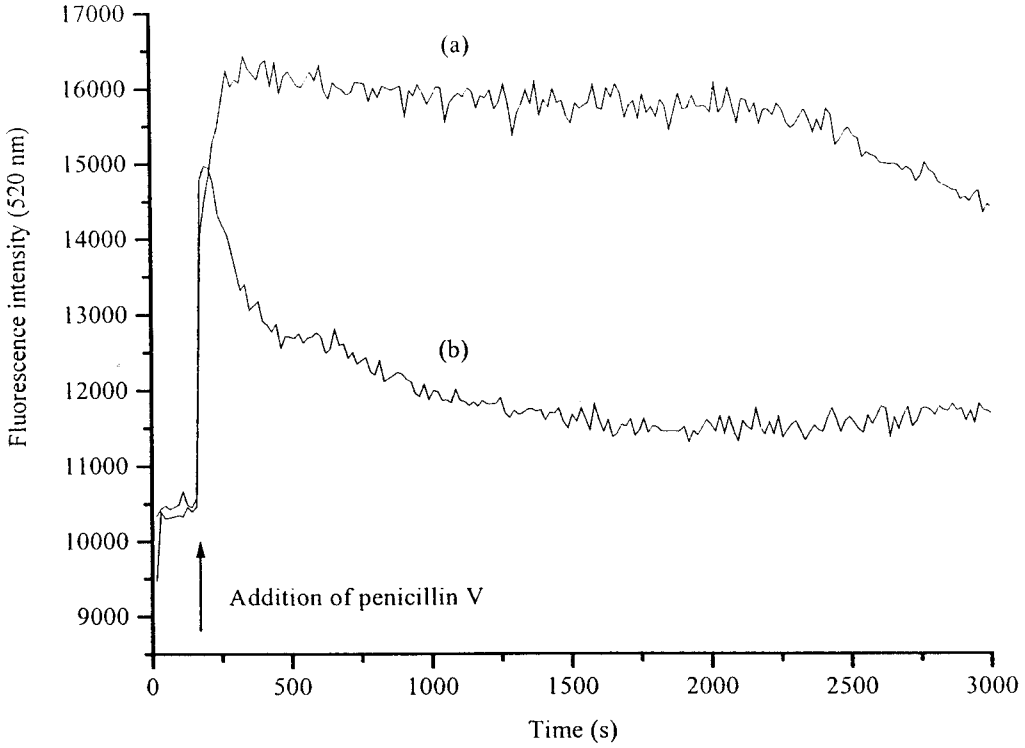


Figure 4.6 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin V (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

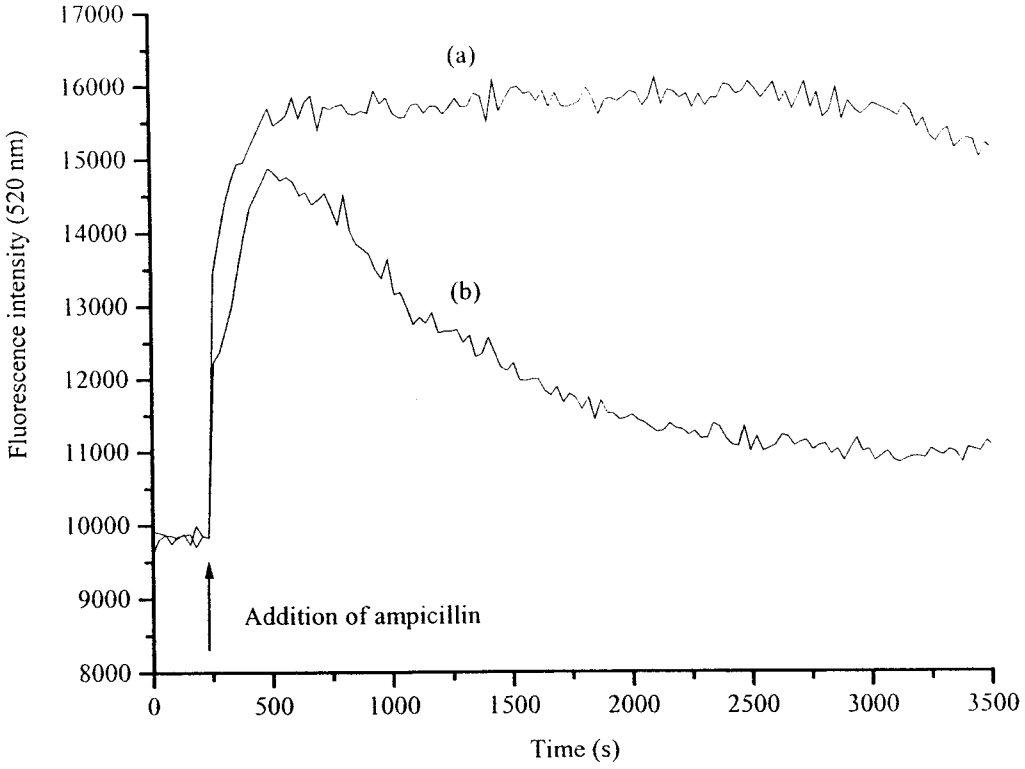


Figure 4.7 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with ampicillin (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

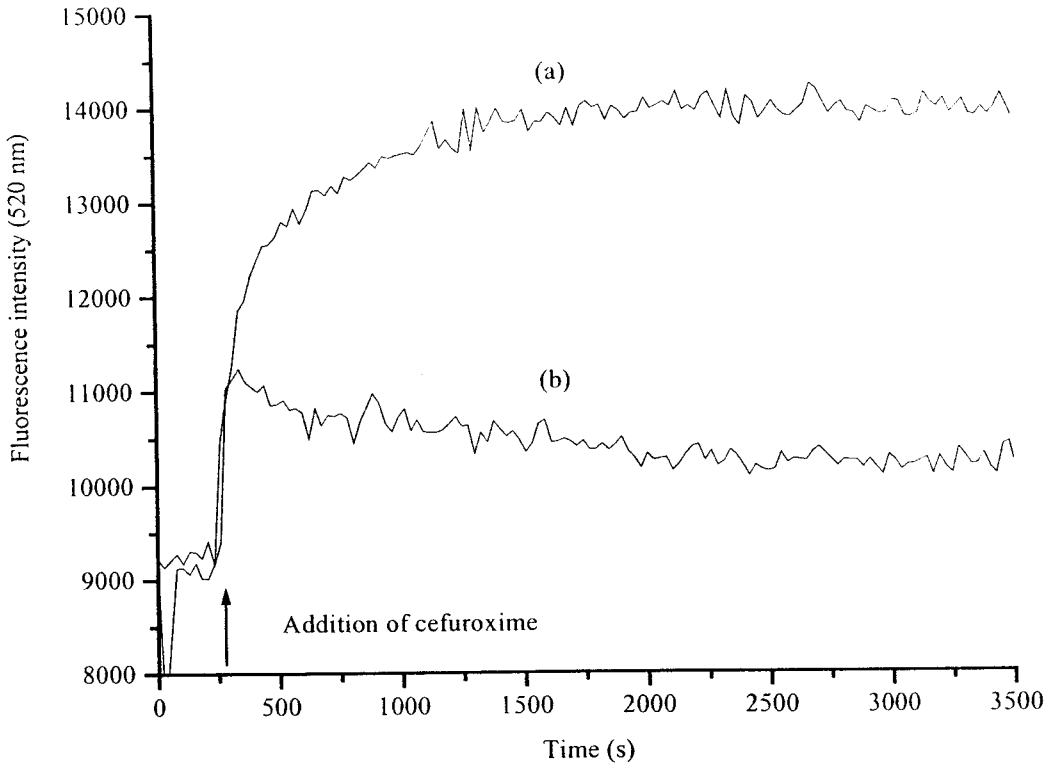


Figure 4.8 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefuroxime (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

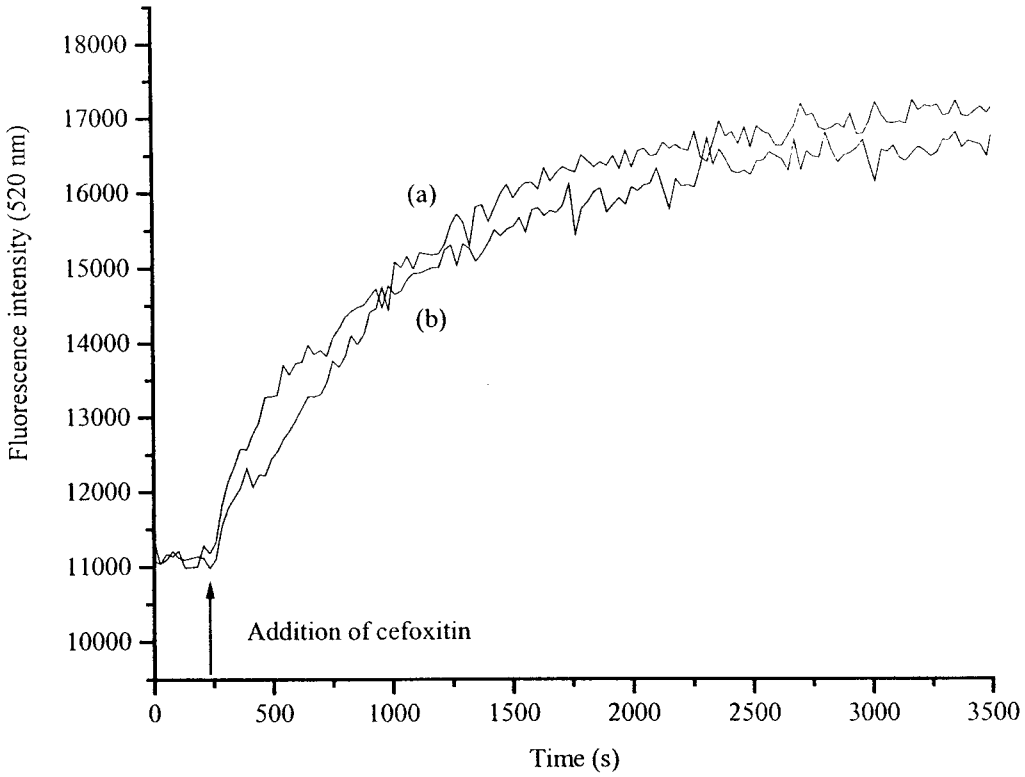


Figure 4.9 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefoxitin (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

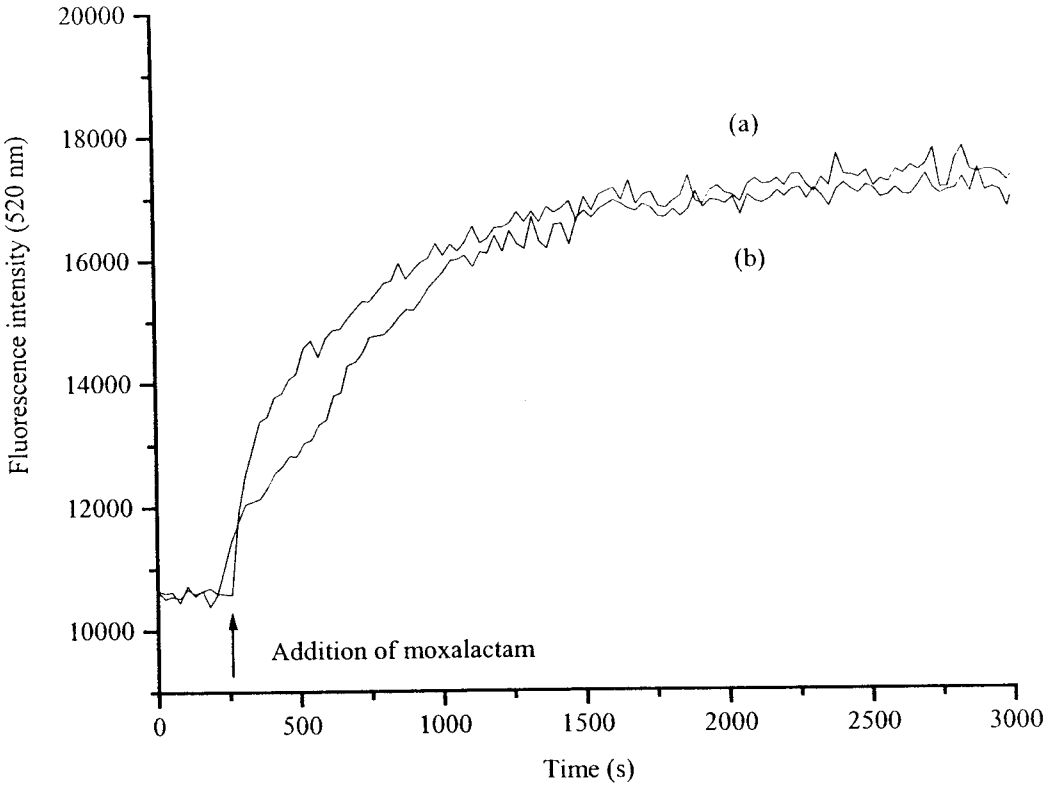


Figure 4.10 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with moxalactam (1.0 x 10^{-4} M) as substrate in the absence of β-lactamase II (a) and in the presence of β-lactamase II (b). Excitation wavelength: 485 nm.

The decline in fluorescence intensity observed with penicillin G, penicillin V, ampicillin and cefuroxime can be attributed to the hydrolytic activities of β-lactamase II towards these antibiotics. As a consequence of substrate competition, the fluorescence of the E166Cf enzyme was suppressed. In contrast, both cefoxitin and moxalactam are resistant to the hydrolytic activities of β-lactamase II, and therefore the labeled enzymes exhibited stronger fluorescence signals with these antibiotics. The results are consistent with previous report that the β-lactamase II from *Bacillus cereus* hydrolyzes penicillin G, ampicillin and cefuroxime more efficiently (larger k_{cat}/K_m) than cefoxitin and moxalactam [18]. Table 4.1 summarizes the kinetic parameters of *B. cereus* β-lactamase II with penicillin G, ampicillin, cefuroxime, cefoxitin and moxalactam as substrates [18].

Table 4.1 Kinetic parameters of *Bacillus cereus* β-lactamase II with penicillin G, ampicillin, cefuroxime, cefoxitin and moxalactam as substrates [18]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Ampicillin	1530 ± 115	1105	7.20×10^5
Penicillin G	1500 ± 200	678	4.50×10^5
Cefuroxime	45 ± 5	35	7.80×10^5
Cefoxitin	2100 ± 465	0.2	9.50×10^1
Moxalactam	644 ± 90	1.2	1.90×10^3

4.3.2 Screening of penPC β-lactamase against penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam in the presence of the E166Cf enzyme

Time-resolved fluorescence measurements of the E166Cf enzyme (1.2×10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0×10^{-4} M) as substrates in the presence of penPC β-lactamase culture (3 μl) were performed. *B. subtilis* cultures (which produce no β-lactamase) were used as negative controls. As shown in Figures 4.11 to 4.16, the fluorescence intensities of the E166Cf enzyme incubated with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam increase significantly in the absence of penPC β-lactamase. In the presence of penPC β-lactamase, the fluorescence intensities of the labeled enzyme with penicillin G, penicillin V and ampicillin as substrates decline. In contrast, no decline in fluorescence signal was observed with cefuroxime, cefoxitin and moxalactam.

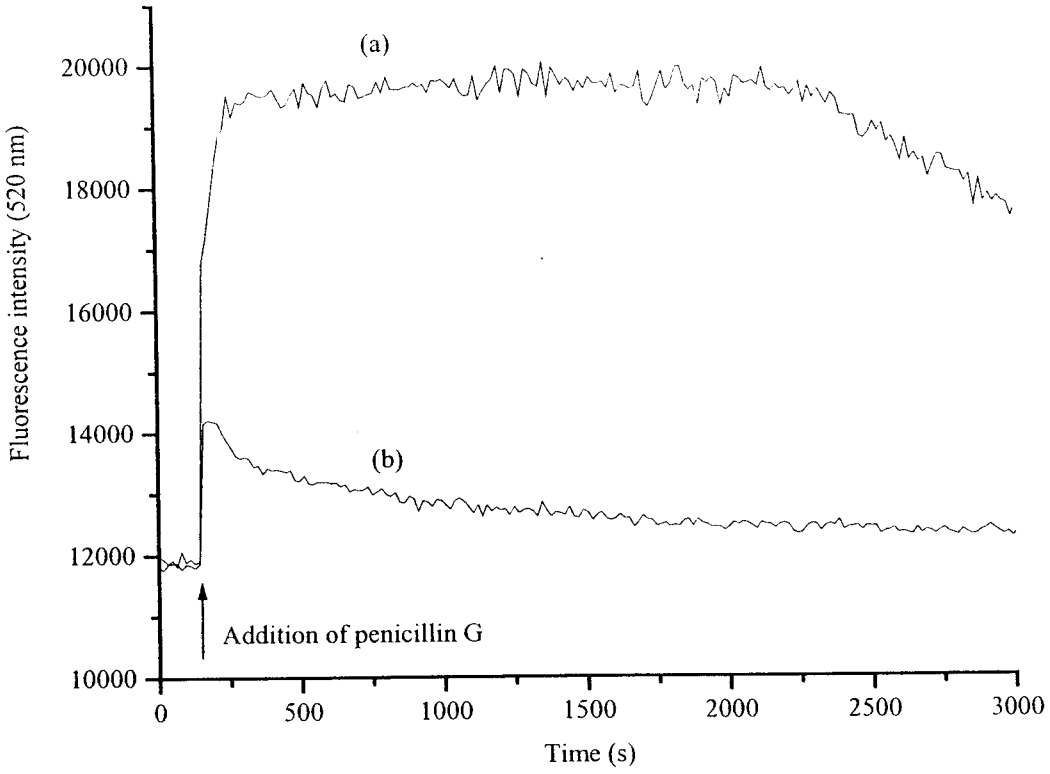


Figure 4.11 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin G (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

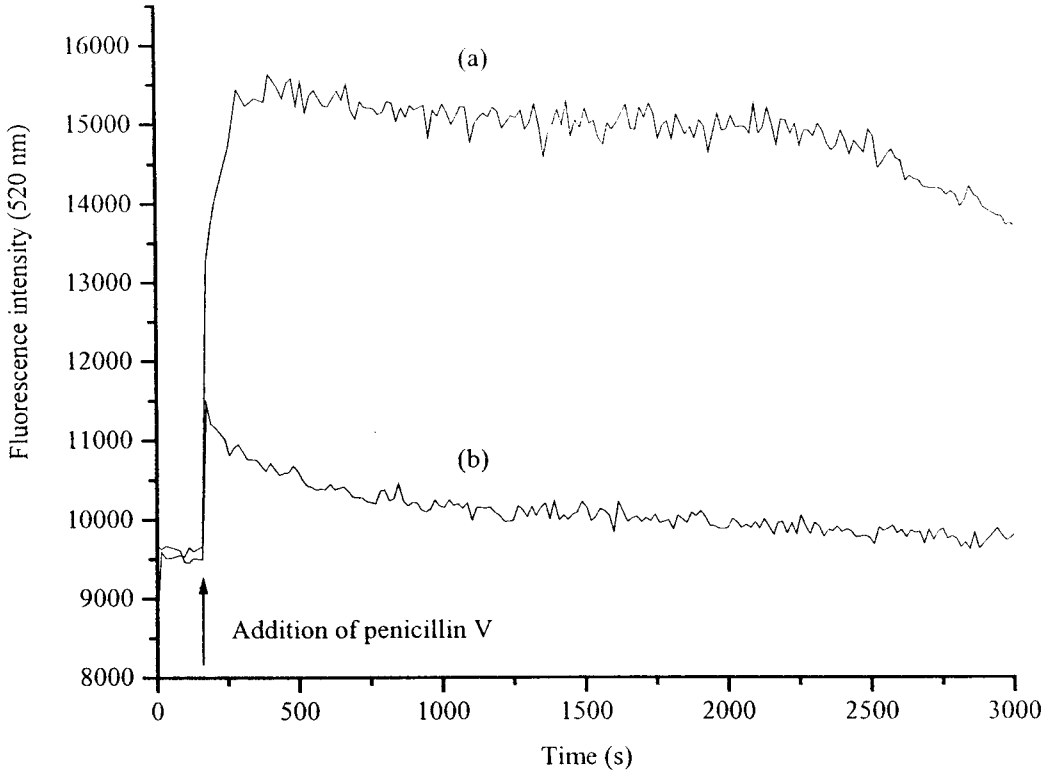


Figure 4.12 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin V (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

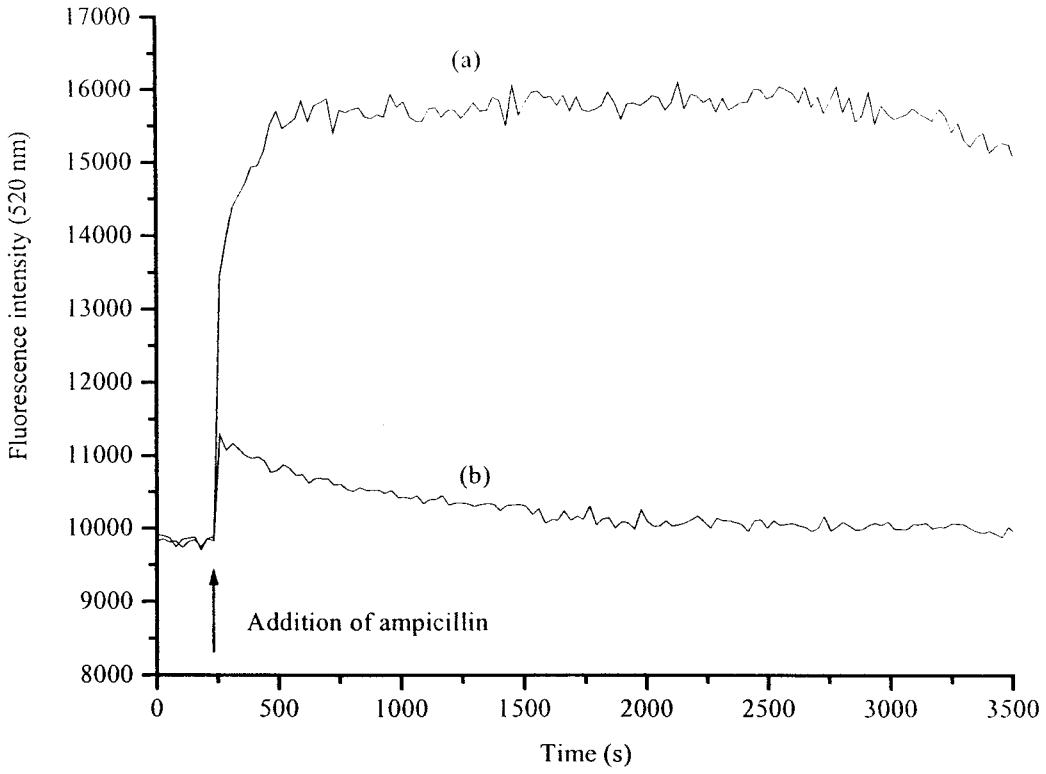


Figure 4.13 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with ampicillin (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

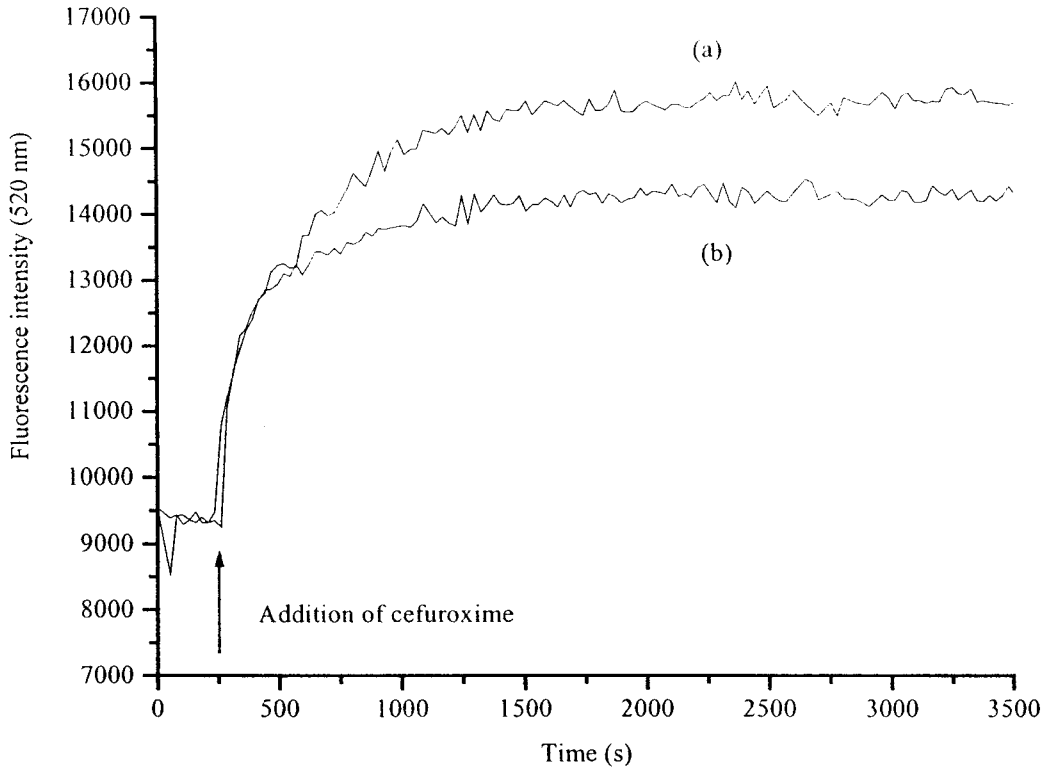


Figure 4.14 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefuroxime (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

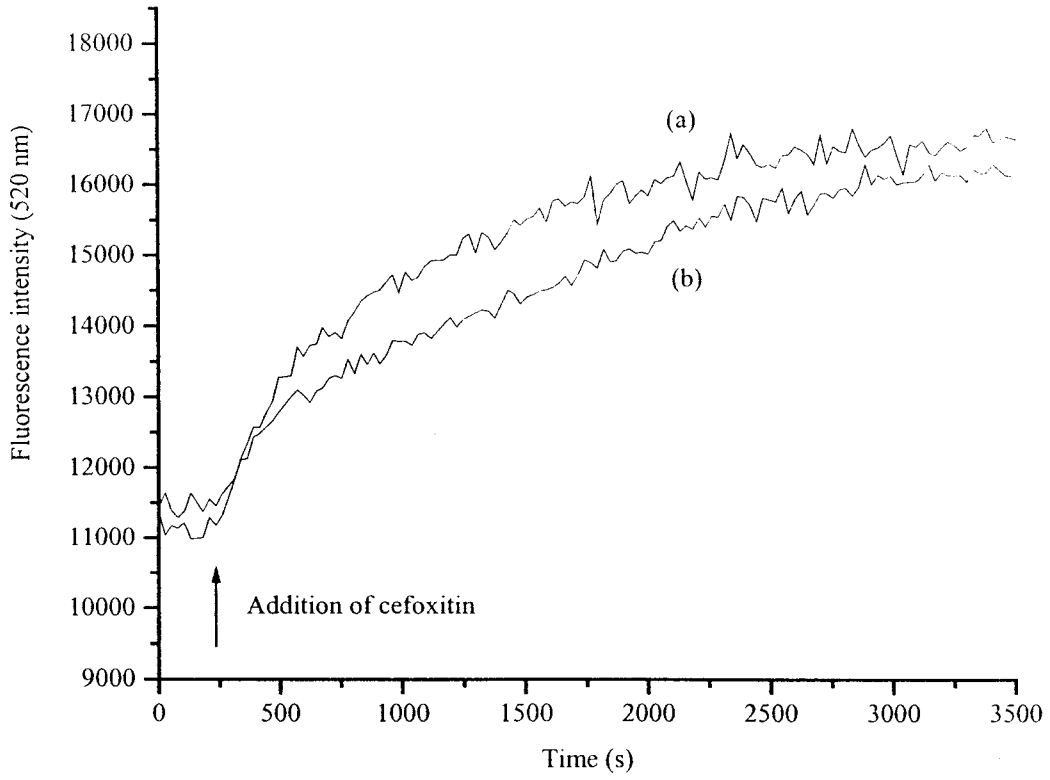


Figure 4.15 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefoxitin (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

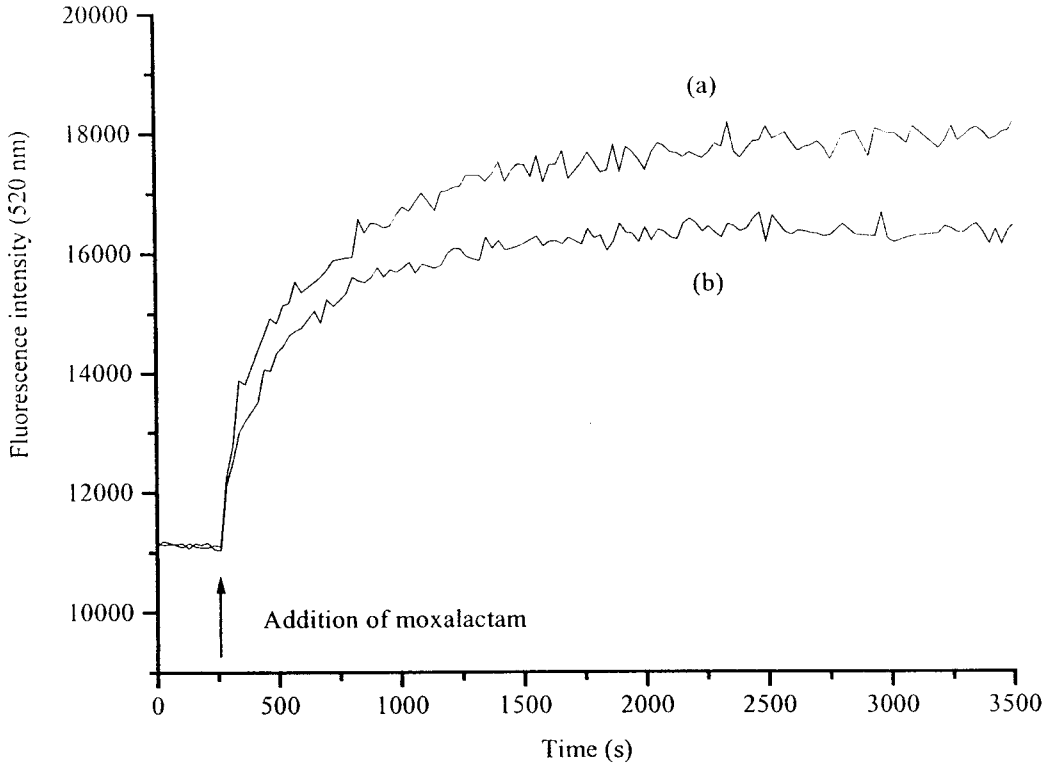


Figure 4.16 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with moxalactam (1.0 x 10^{-4} M) as substrate in the absence of penPC β-lactamase (a) and in the presence of penPC β-lactamase (b). Excitation wavelength: 485 nm.

As indicated in Chapter 2, penPC β-lactamase can effectively hydrolyze penicillin G, penicillin V and ampicillin (large k_{cat}/K_m). Therefore, it is reasonable that the presence of penPC β-lactamase suppressed the fluorescence of the E166Cf enzyme when penicillin G, penicillin V and ampicillin were added. The absence of decline in fluorescence intensity for cefuroxime, cefoxitin and moxalactam can be attributed to the poor hydrolytic activities of penPC β-lactamase towards these antibiotics, as cephalosporin antibiotics are, in general, poor substrates for class A β-lactamases [6-8].

4.3.3 Screening of penP β-lactamase against penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam in the presence of the E166Cf enzyme

The fluorescence behavior of the E166Cf enzyme (1.2×10^{-7} M) in 50 mM phosphate buffer (pH7.0) with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam as (1.0×10^{-4} M) substrates in the presence of penP β-lactamase cultures (6 μl) were monitored by measuring the fluorescence intensities at 520 nm as a function of time. Negative controls were set up in which *B. subtilis* cultures (which produce no β-lactamase) were added instead of penP β-lactamase cultures. As shown in Figures 4.17 to 4.22, the fluorescence intensities of the E166Cf enzyme with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam as substrates in the absence of penP β-lactamase increase remarkably. In the presence of penP β-lactamase, the fluorescence signals of the labeled enzyme with penicillin G, penicillin V, ampicillin and cefuroxime as substrates decline. In contrast, the fluorescence intensities of the labeled enzyme increase with cefoxitin and moxalactam as substrates.

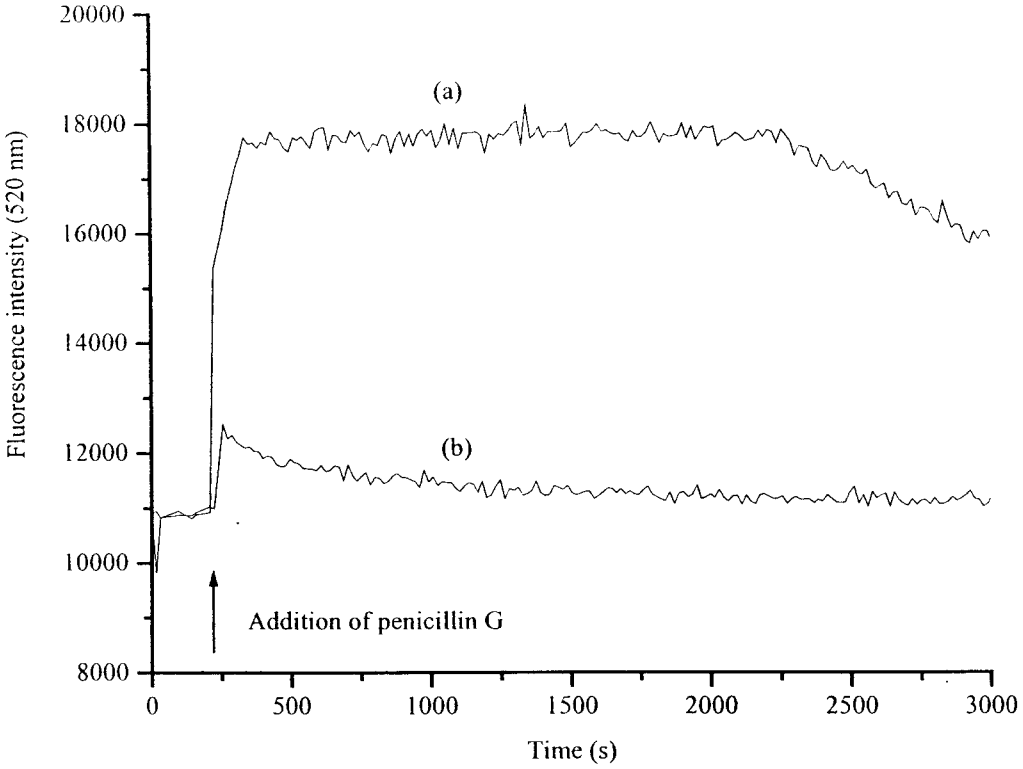


Figure 4.17 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin G (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

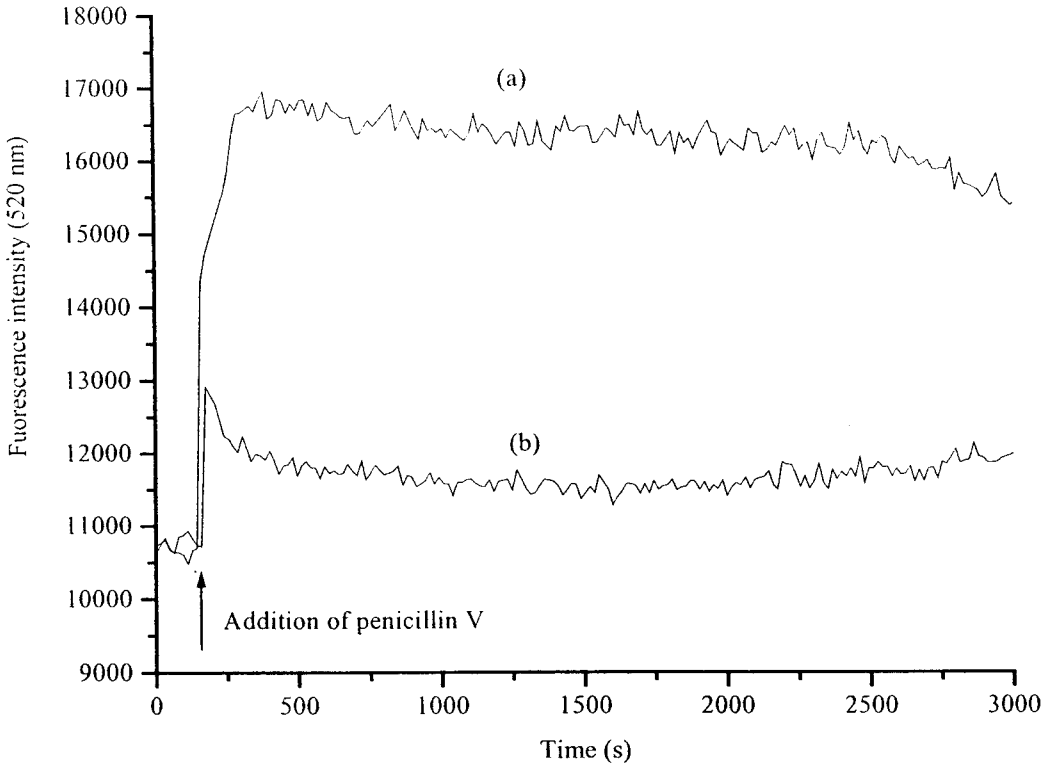


Figure 4.18 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with penicillin V (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

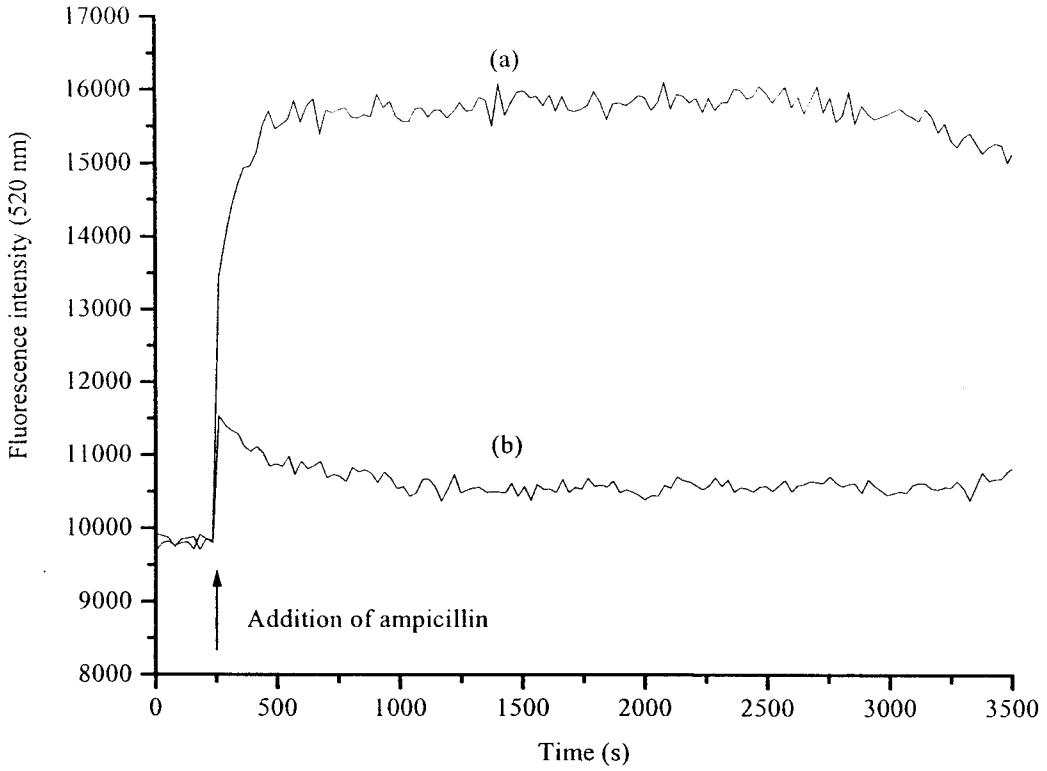


Figure 4.19 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with ampicillin (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

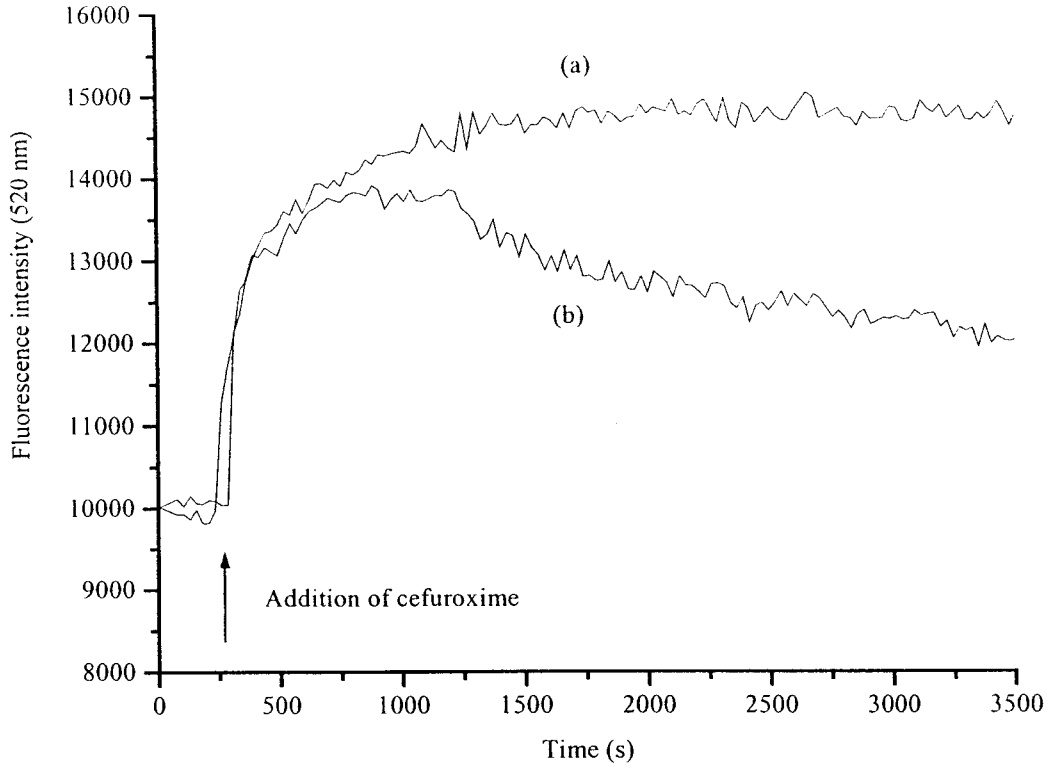


Figure 4.20 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefuroxime (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

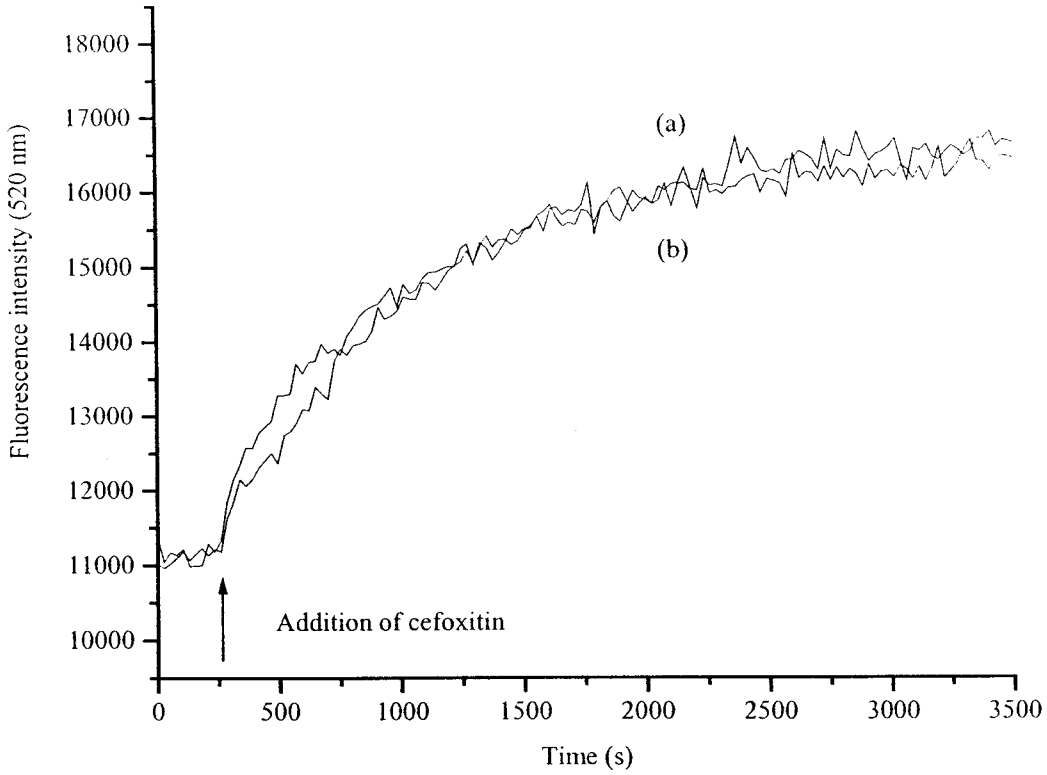


Figure 4.21 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with cefoxitin (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

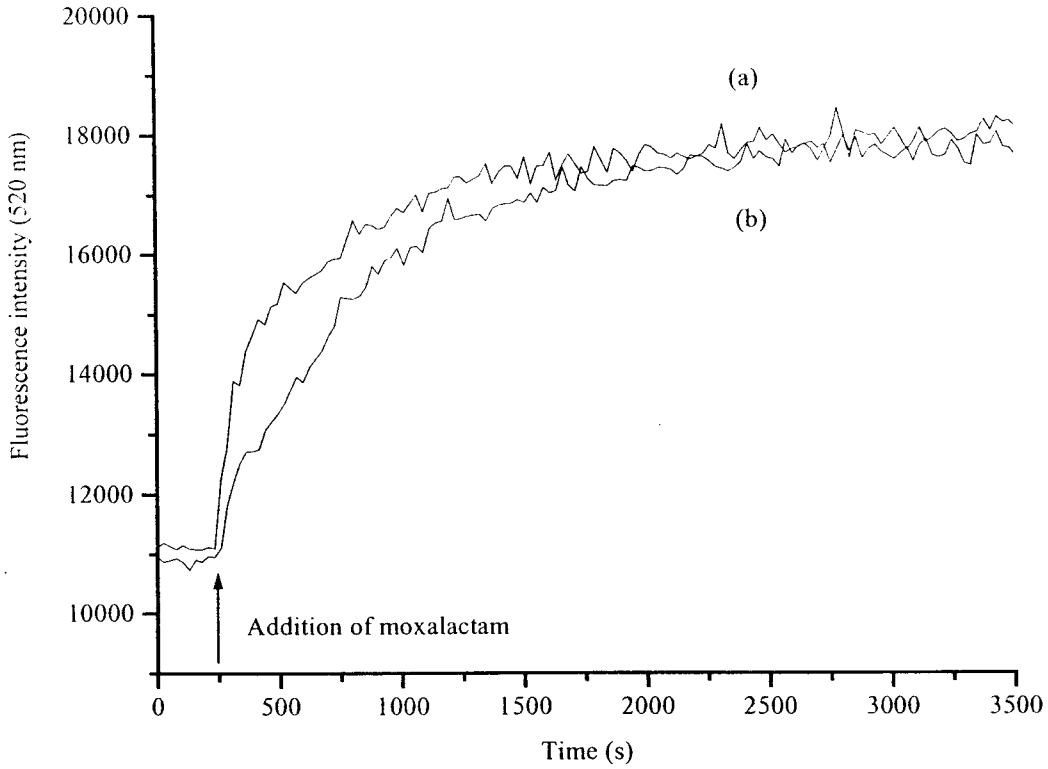


Figure 4.22 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) in 50 mM phosphate buffer (pH 7.0) with moxalactam (1.0 x 10^{-4} M) as substrate in the absence of penP β-lactamase (a) and in the presence of penP β-lactamase (b). Excitation wavelength: 485 nm.

That a decline in fluorescence intensity of the E166Cf enzyme was not observed with cefoxitin and moxalactam as substrates implies that these antibiotics are resistant to the hydrolytic activity of penP β-lactamase. As a result, the labeled enzymes face less competition with the bacterial enzymes for the antibiotic substrates. The decline in fluorescence intensity observed with penicillin G, penicillin V, ampicillin and cefuroxime can be attributed to the hydrolytic activities of penP β-lactamase towards these antibiotics. Table 4.2 summarizes the kinetic parameters of *B. licheniformis* penP β-lactamase with penicillin G, ampicillin, cefoxitin and moxalactam as substrates [7, 94].

Table 4.2 Kinetic parameters of *B. licheniformis* penP β-lactamase with penicillin G, ampicillin, cefoxitin and moxalactam as substrates [7, 94]

	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Penicillin G	2200	2.9×10^4
Ampicillin	1500	1.1×10^4
Cefoxitin	$(0.13 \pm 0.01) \times 10^{-3}$	20 ± 5
Moxalactam	$(0.9 \pm 0.1) \times 10^{-3}$	3 ± 0.4

4.3.4 Screening of TEM-1 β-lactamase against penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam in the presence of the E166Cf enzyme

It should be noticed that the screening of bacteria against antibiotics in real samples are performed in complex media such as bacterial cultures and crude biological fluids. To demonstrate the ability of the E166Cf enzyme to screen bacterial β-lactamases against various antibiotics in a complex medium, time-resolved fluorescence measurements were performed by adding the E166Cf enzymes and antibiotics directly to TEM-1 β-lactamase cultures. Figures 4.23 to 4.28 show the time-resolved fluorescence measurements of the E166Cf enzyme (1.2×10^{-7} M) with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam (1.0×10^{-4} M) as substrates in the presence of TEM-1 β-lactamase cultures (294 μl) and *E. coli.* cultures (which produce no β-lactamase). As indicated in Figures 4.23 to 4.28, an increase in fluorescence intensity of the E166Cf enzyme with penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam as substrates in the absence of TEM-1 β-lactamase was observed. In the presence of TEM-1 β-lactamase, the fluorescence intensity of the labeled enzyme with penicillin G, penicillin V and ampicillin as substrates declines, whereas an increase fluorescence signal was observed for cefuroxime, cefoxitin and moxalactam.

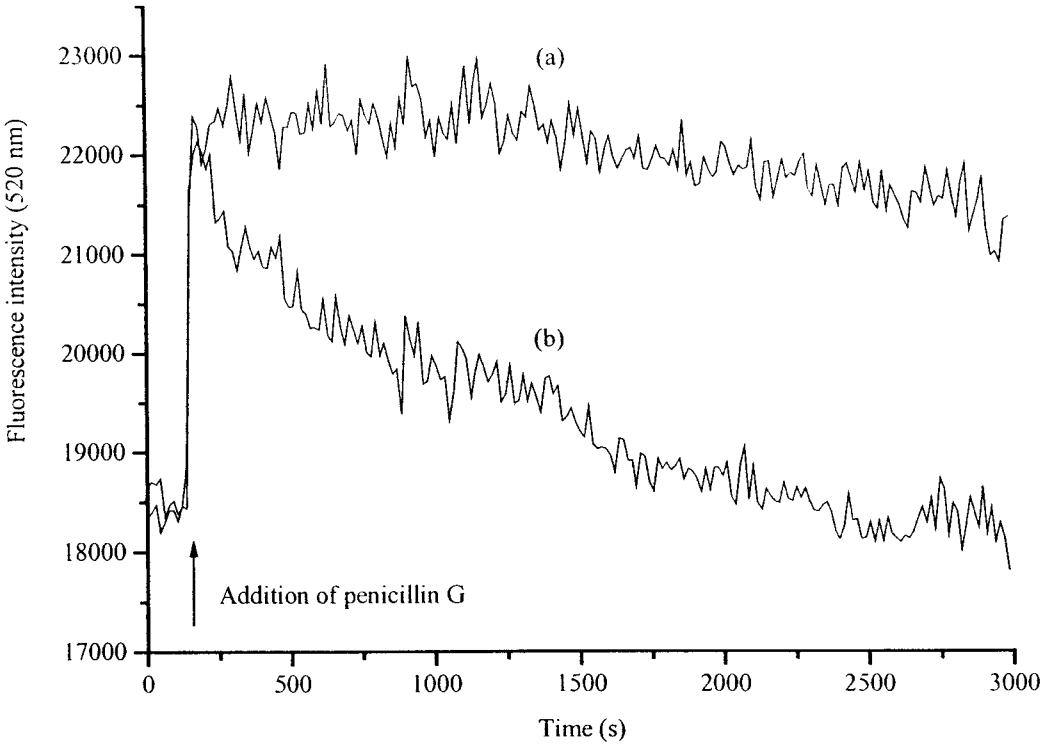


Figure 4.23 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with penicillin G (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

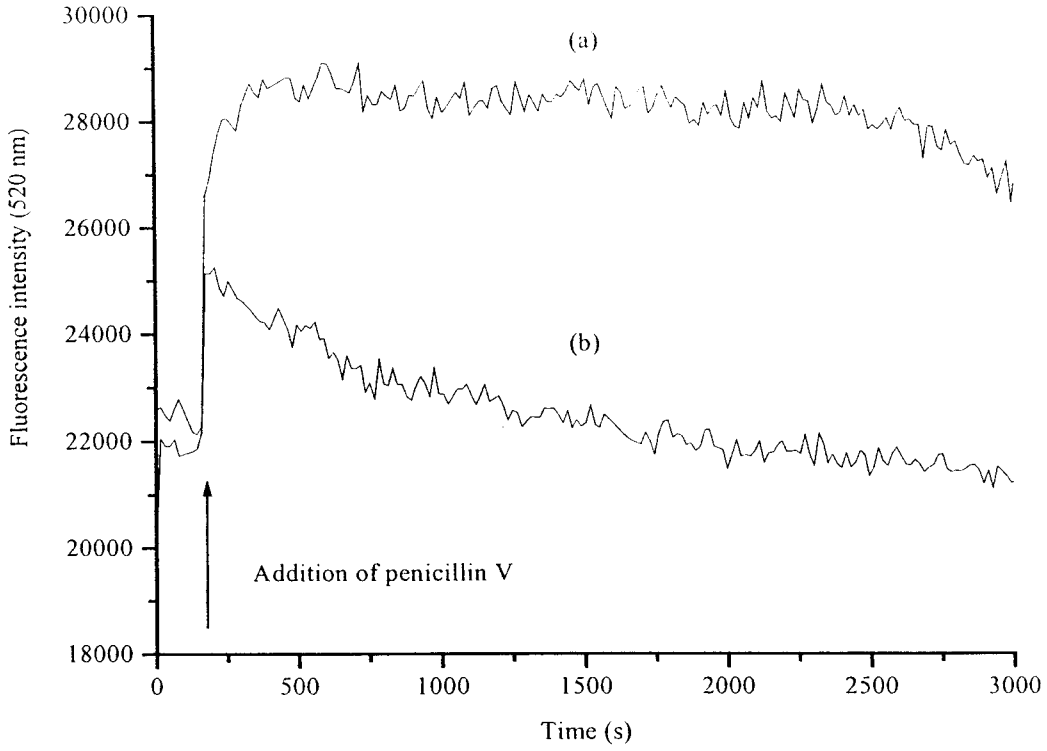


Figure 4.24 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with penicillin V (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

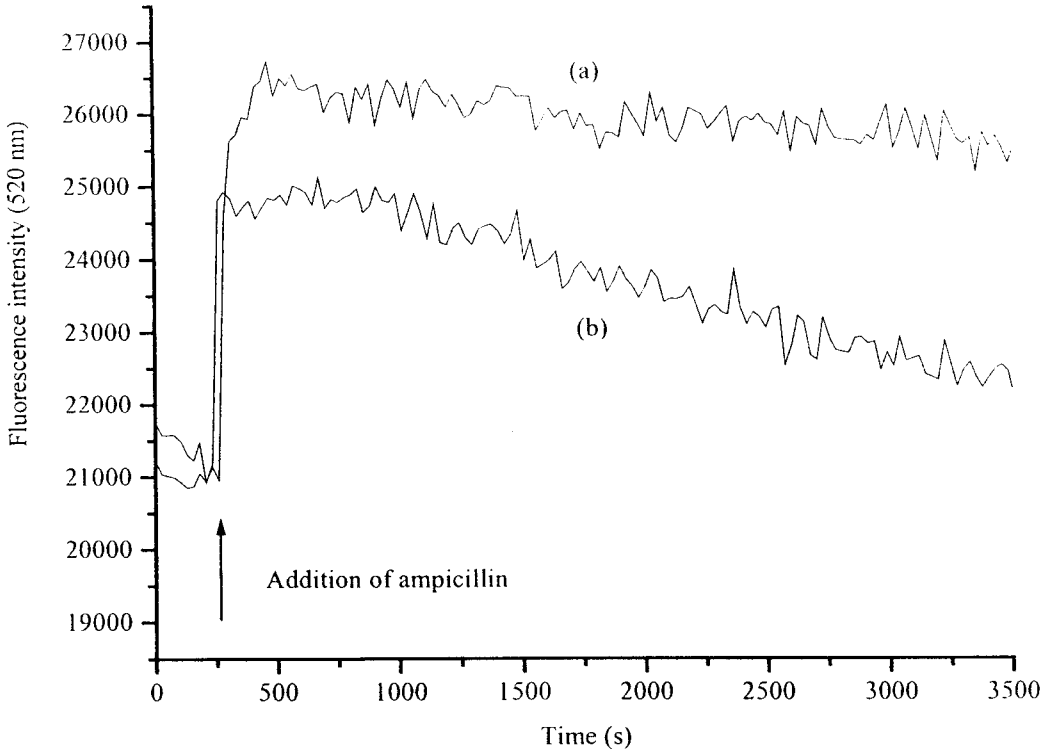


Figure 4.25 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with ampicillin (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

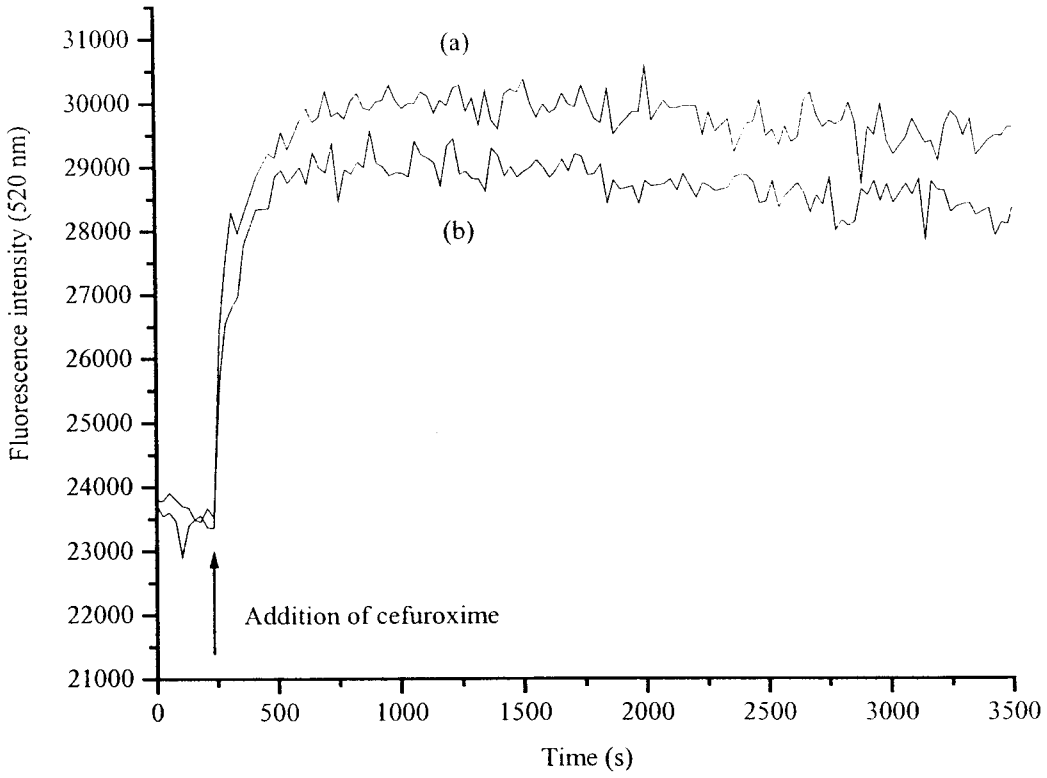


Figure 4.26 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with cefuroxime (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

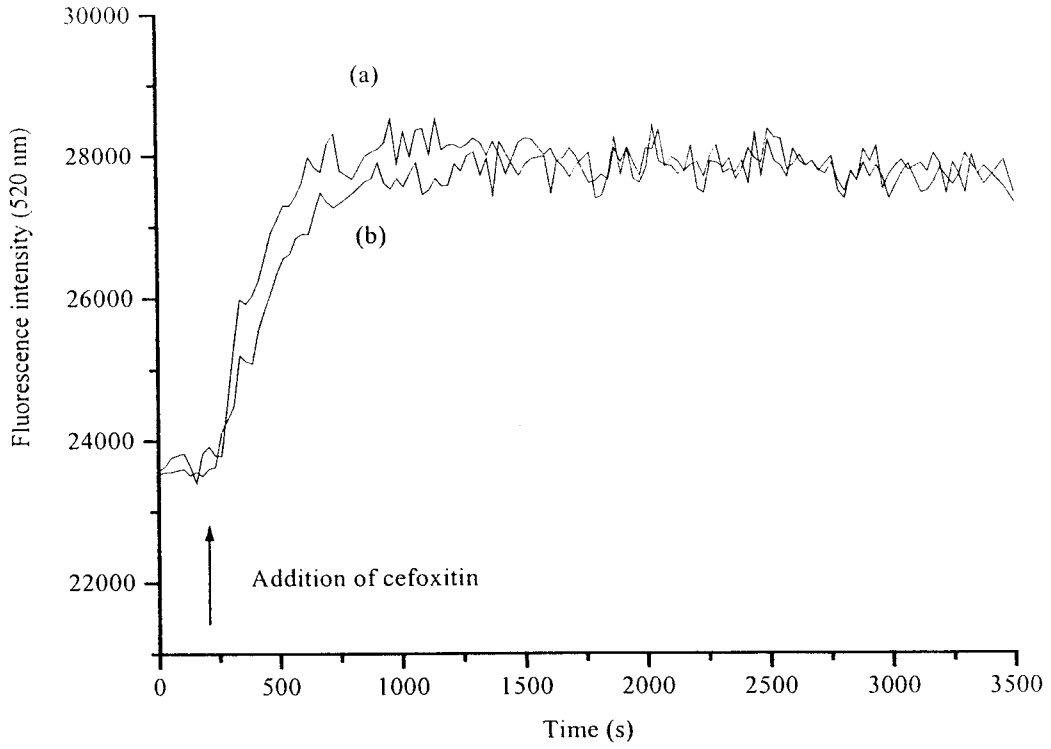


Figure 4.27 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with cefoxitin (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

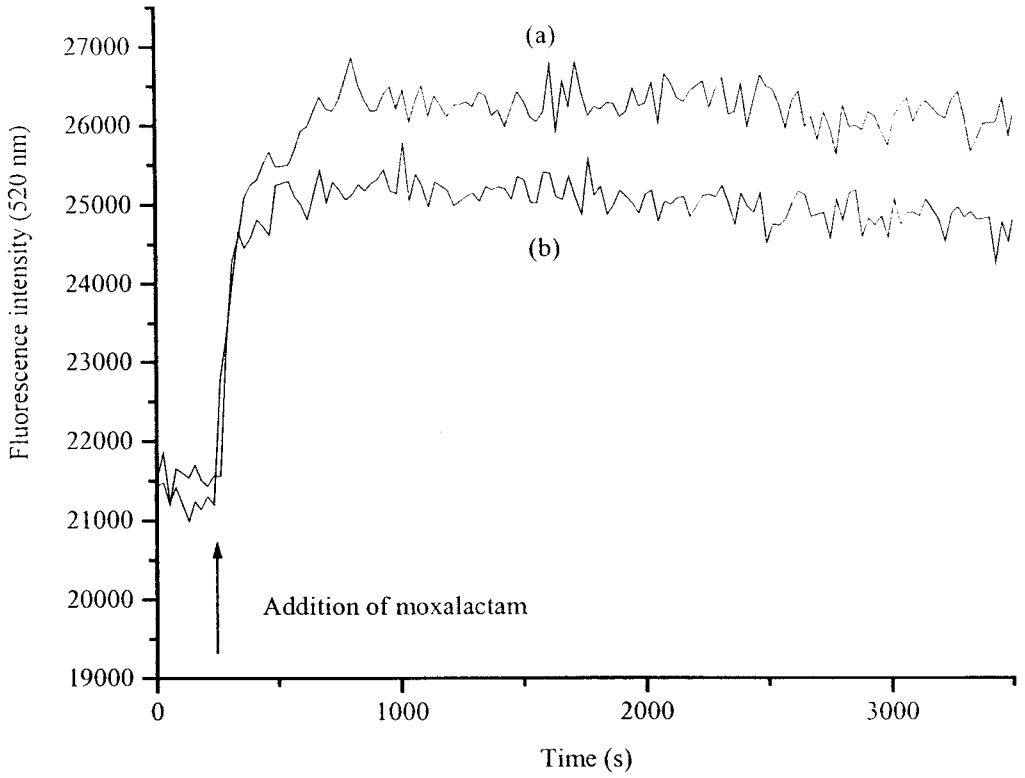


Figure 4.28 Time-resolved fluorescence measurements of the E166Cf enzyme (1.2 x 10^{-7} M) with moxalactam (1.0 x 10^{-4} M) as substrate in the absence of TEM-1 β-lactamase (a) and in the presence of TEM-1 β-lactamase (b). Excitation wavelength: 485 nm.

These results indicate that the E166Cf enzyme is capable of screening bacterial β-lactamase against β-lactam antibiotics in a complex medium. The time-resolved fluorescence measurements revealed that cefuroxime, cefoxitin and moxalactam are resistant to the hydrolytic activities of TEM-1 β-lactamase because no decline in fluorescence signal was observed. In contrast, penicillin G, penicillin V and ampicillin are readily hydrolyzed by TEM-1 β-lactamase. The kinetic parameters of *E. coli* TEM-1 β-lactamase with penicillin, ampicillin and cefoxitin as substrates have been reported [8, 95]. Table 4.3 summarizes the kinetic parameters of *E. coli* TEM-1 β-lactamase with penicillin G, ampicillin, cefoxitin and cefuroxime as substrates [8, 95].

Table 4.3 Kinetic parameters of *E. coli* TEM-1 β-lactamase with penicillin G, ampicillin and cefoxitin as substrates [8, 95]

	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Penicillin G	8.4×10^4
Ampicillin	3.3×10^4
Cefoxitin	0.006
Cefuroxime	6

4.4 Concluding remarks

The time-resolved fluorescence measurements of the E166Cf enzyme in the presence of bacterial β-lactamases against various β-lactam drugs demonstrated the ability of the E166Cf enzyme to serve as a reagentless tool in screening bacterial β-lactamases against β-lactam antibiotics. For example, cefoxitin and moxalactam were found to be resistant to the hydrolytic activities of β-lactamase II, penPC β-lactamase, penP β-lactamase and TEM-1 β-lactamase, whereas penicillin G, penicillin V and ampicillin were found to be ineffective in inactivating these bacterial enzymes. It should be noted that the use of E166Cf enzyme in drug screening offers a number of advantages. Firstly, as indicated in the time-resolved fluorescence measurements, the E166Cf enzyme can search for potent β-lactam antibiotics for bacterial β-lactamases in less than 15 min. Thus, it is time saving to use the E166Cf enzyme in drug screening. Secondly, by using the E166Cf enzyme as a screening tool, it is convenient to screen bacterial β-lactamases against various β-lactam antibiotics because no sample pre-treatment is required. Thirdly, the E166Cf enzyme is capable of screening bacterial β-lactamases against antibiotics even in a complex medium such as bacterial culture. Furthermore, the E166Cf enzyme can be used in high-throughput drug screenings. For example, both the E166Cf enzymes and infecting bacterial samples

(which produce β-lactamases) can be placed into different wells of a microtiter plate (e.g. the standard 96-well microtiter plate) and then incubated with various β-lactam antibiotics. The fluorescence signals from different wells can be monitored simultaneously by a microplate reader. Thus, this method allows the physicians to search for potent antibiotics for the patients quickly. All these advantages should allow the E166Cf enzyme to be used routinely in drug screening in the future.

Chapter 5

Conclusions

In this project, we have prepared and characterized a fluorescein-labeled β-lactamase I (E166Cf) that can detect β-lactam antibiotics and β-lactamase inhibitors, and can screen bacteria for β-lactamases against a panel of antibiotics. The E166Cf enzyme was constructed by labeling the thiol-reactive fluorescein-5-maleimide with the E166C mutant whose Glu-166 on the Ω-loop was replaced with a cysteine residue by site-directed mutagenesis. As the wild-type β-lactamase I contains no cysteine residue [69], this single-point mutation allows the attachment of the thiol-reactive fluorophore to a specific site on the enzyme. Circular dichroism spectropolarimetry showed that the secondary structure of the E166Cf enzyme is conserved after the labeling reaction. Spectrophotometric assays revealed that the catalytic efficiencies of the E166Cf enzyme towards penicillin G, penicillin V and ampicillin are comparable to those of the E166C mutant. Hence the hydrolytic activity of the E166Cf enzyme is conserved even in the presence of the fluorescein label. The Glu-166 residue is close to the active site, and its location on the flexible Ω-loop [70, 71] allows the fluorophore to move when the antibiotic binds to the enzyme's active site.

Time-resolved fluorescence measurements on the E166Cf enzyme indicated that it fluoresces more strongly in the presence of penicillin G, penicillin V, ampicillin, cefuroxime, cefoxitin and moxalactam. The increase in fluorescence intensity is

attributed to the conformational change in the active site upon binding of a β-lactam antibiotic. Such a conformational change causes the fluorescein label to depart from the active site, thus switching on the fluorescence of the fluorescein molecule. The E166Cf enzyme is capable of distinguishing 'β-lactamase-unstable' antibiotics (penicillin G, penicillin V and ampicillin) from 'β-lactamase-resistant' antibiotics (cefuroxime, cefoxitin and moxalactam). In the presence of penicillin antibiotics, the fluorescence intensity of the E166Cf enzyme increases and then declines. In contrast, in the presence of cephalosporin antibiotics, the fluorescence intensity increases gradually to give a plateau signal. The E166Cf enzyme can detect β-lactam antibiotics down to 10^{-7} M, and therefore has the potential to be developed into a reagentless tool for the convenient measurement of antibiotics in liquid samples such as milk. Furthermore, the E166Cf enzyme can detect β-lactamase inhibitors such as sulbactam and clavulanate, and therefore may find its future applications in the discovery of new β-lactamase inhibitors.

We have demonstrated the ability of the E166Cf enzyme to serve as a high-throughput tool in screening bacterial β-lactamases against various β-lactam antibiotics by monitoring the fluorescence change of the labeled enzyme in the presence of bacterial culture and antibiotics. For example, cefoxitin and moxalactam were found to be

resistant to the hydrolytic activities of β-lactamase II, penPC β-lactamase, penP β-lactamase and TEM-1 β-lactamase, whereas penicillin G, penicillin V and ampicillin were found to be ineffective in inactivating these bacterial enzymes.

Although the E166Cf enzyme can function effectively in screening bacteria for β-lactamases against a panel of antibiotics, there is room for further improvement if it is going to be used as a biosensor for detecting antibiotics in food samples and as a tool in the discovery of new β-lactamase inhibitors. Firstly, the fluorescence signal of the E166Cf enzyme towards 'β-lactamase-unstable' antibiotics (e.g. penicillin G, penicillin V and ampicillin' varies with time. Thus, it is difficult to determine the antibiotic concentration in an unknown sample accurately just based on the increase in fluorescence signal. Secondly, the fluorescence intensity of the E166Cf enzyme towards cephalosporin antibiotics increases too slowly due to poor binding, and this results in an exceedingly long detection time for these antibiotics. Thirdly, the detection limit of the E166Cf enzyme for most β-lactam antibiotics in liquid samples is ~10^{-7} M, which is above the allowed maximum limit of 10^{-8} M β-lactam antibiotics in milk as regulated by the European Union [52].

We propose that the following approaches to overcome the above disadvantages of the E166Cf enzyme. Firstly, the E166Cf enzyme can be further modified in such a way that only substrate binding takes place in the enzyme's active site but no hydrolytic action occurs (k_2 and $k_3 = 0$). This can be done by site-directed mutagenesis so that the essential amino acids (e.g. Ser-70) required for the hydrolytic action are removed. The resulting labeled enzyme will become a 'pure binding protein' ($E + S \rightleftharpoons ES$) and therefore the fluorescence intensity should remain constant after the binding of antibiotics. Secondly, a new fluorescein-labeled β-lactamase can be constructed in which the class C β-lactamase is used as a recognizing element. Unlike class A β-lactamases, class C β-lactamases can bind to cephalosporin antibiotics and hydrolyze them to inactive carboxylic acids effectively [7]. Thus, the detection time for cephalosporin antibiotics can be greatly shortened when this labeled enzyme is used as a sensor. Thirdly, improvement in instrumentation might also improve the detection limit of the labeled enzyme. For example, by immobilizing the labeled enzyme on a suitable surface, light scattering by colloid particles in the sample can be reduced and the detection limit can be raised.

References

References

1. Page, M. I., "Structure-Activity Relationships: Chemical", In Page, M. I., ed., *The Chemistry of β-Lactams*, Blackie Academic & Professional, London, pp. 79-100 (1992).

2. Ghuysen, J. M., "Molecular Structures of Penicillin-Binding Proteins and β-Lactamases", *Trends in Microbiology*, Vol. 2, pp. 372-380. (1994).

3. Frere, J. M. and Joris, B., "Penicillin-Sensitive Enzymes in Peptidoglycan Biosynthesis", *CRC Critical Reviews in Microbiology*, Vol. 11, pp. 299-396 (1985).

4. Jacoby, G. A., "Extrachromosomal Resistance in Gram-Negative Organisms: the Evolution of β-Lactamase", *Trends in Microbiology*, Vol. 2, pp. 357-360. (1994).

5. Davies, J., "Inactivation of Antibiotics and the Dissemination of Resistance Genes", *Science*, Vol. 264, pp. 375-382. (1994).

6. Frere, J. M., "Beta-Lactamases and Bacterial Resistance to Antibiotics", *Molecular Microbiology*, Vol. 16, pp. 385-395. (1995).

7. Matagne, A., Dubus, A., Galleni, M. and Frere, J. M., "The β-Lactamase Cycle: a Tale of Selective Pressure and Bacterial Ingenuity", *Natural Product Reports*, Vol. 16, pp. 1-19. (1999).

8. Matagne, A., Lamotte-Brasseur, J. and Frere, J. M., "Catalytic Properties of Class A β-Lactamases: Efficiency and Diversity", *Biochemical Journal*, Vol. 330, pp. 581-598. (1998).

9. Bush, K., Jacoby, G. A. and Medeiros, A. A., "A Functional Classification Scheme for β-Lactamases and its Correlation with Molecular Structure", *Antimicrobial Agents and Chemotherapy*, Vol. 39, pp. 1211-1233. (1995).

10. Huovinen, P., Huovinen, S. and Jacoby, G. A., "Sequence of Pse-2 Beta-Lactamase", *Antimicrobial Agents and Chemotherapy*, Vol. 32, pp. 134-136 (1988).

11. Joris, B., Ledent, P., Dideberg, O., Fonze, E., Lamottebrasseur, J., Kelly, J. A., Ghuysen, J. M. and Frere, J. M., "Comparison of the Sequences of Class-A β-Lactamases and of the Secondary Structure Elements of Penicillin-Recognizing Proteins", *Antimicrobial Agents and Chemotherapy*, Vol. 35, pp. 2294-2301 (1991).

12. Sabath, L. D. and Abraham, E. P., "Zinc as a Cofactor for Cephalosporinase from *Bacillus cereus* 569", *Biochemical Journal*, Vol. 98, pp. 11C-13C. (1966).

13. Kuwabara, S. and Abraham, E. P., "Some Properties of Two Extracellular β-Lactamases from *Bacillus cereus* 569/H", *Biochemical Journal*, Vol. 103, pp. 27C-30C. (1967).

14. Carfi, A., Duee, E., Paul-Soto, R., Galleni, M., Frere, J. M. and Dideberg, O., "X-Ray Structure of the Zn^{II} β-Lactamase from *Bacteroides fragilis* in an Orthorhombic Crystal Form", *Acta Crystallographica*, Vol. D54, pp. 45-57. (1998).

15. Concha, N. O., Rasmussen, B. A., Bush, K. and Herzberg, O., "Crystal Structure of the Wide-Spectrum Binuclear Zinc β-Lactamase from *Bacteroides fragilis*", *Structure*, Vol. 4, pp. 823-836. (1996).

16. Carfi, A., Pares, S., Duee, E., Galleni, M., Duez, C., Frere, J. M. and Dideberg, O., "The 3-D structure of a Zinc Metallo-β-Lactamase from *Bacillus cereus* Reveals a New Type of Protein Fold", *European Molecular Biology Organization Journal*, Vol. 14, pp. 4914-4921. (1995).

17. Yang, Y. J., Wu, P. J. and Livermore, D. M., "Biochemical Characterization of a β-Lactamase that Hydrolyzes Penems and Carbapenems from Two *Serratia marcescens* Isolates", *Antimicrobial Agents and Chemotherapy*, Vol. 34, pp. 755-758. (1990).

18. Felici, A., Amicosante, G., Oratore, A., Strom, R., Ledent, P., Joris, B., Fanuel, L. and Frere, J. M., "An Overview of the Kinetic Parameters of Class B β-Lactamases", *Biochemical Journal*, Vol. 291, pp. 151-155. (1993).

19. Felici, A. and Amicosante, G., "Kinetic Analysis of Extension of Substrate Specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* Metallo-β-Lactamases", *Antimicrobial Agents and Chemotherapy*, Vol. 39, pp. 192-199. (1995).

20. Felici, A., Perilli, M., Segatore, B., Franceschini, N., Setacci, D., Oratore, A., Stefani, S., Galleni, M. and Amicosante, G., "Interactions of Biapenem with Active-Site Serine and Metallo-β-Lactamases", *Antimicrobial Agents and Chemotherapy*, Vol. 39, pp. 1300-1305. (1995).

21. Yang, Y. and Bush, K., "Biochemical Characterization of the Carbapenem-Hydrolyzing β-Lactamase AsbM1 from *Aeromonas sobria* AER 14M: A Member of a Novel Subgroup of Metallo-β-Lactamases", *Federation of European Microbiological Societies Microbiology Letters*, Vol. 137, pp. 193-200. (1996).

22. Fisher, J., Belasco, J. G., Khosla, S. and Knowles, J. R., "β-Lactamase Proceeds via an Acyl-Enzyme Intermediate. Interaction of the *Escherichia coli* RTEM Enzyme with Cefoxitin", *Biochemistry*, Vol. 19, pp. 2895-2901. (1980).

23. Knox, J. R. and Moews, P. C., "β-Lactamase of *Bacillus licheniformis* 749/C. Refinement at 2 A Resolution and Analysis of Hydration", *Journal of Molecular Biology*, Vol. 220, pp. 435-455. (1991).

24. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. and Frere, J. M., "β-Lactamase of *Bacillus licheniformis* 749/C at 2 A Resolution", *Proteins: Structure, Function, and Genetics*, Vol. 7, pp. 156-171 (1990).

25. Dideberg, O., Charlier, P., Wery, J. P., Dehottay, P., Dusart, J., Erpicum, T., Frere, J. M. and Ghuysen, J. M., "The Crystal Structure of the β-Lactamase of *Streptomyces albus* G at 0.3 nm Resolution", *Biochemical Journal*, Vol. 245, pp. 911-913. (1987).

26. Herzberg, O. and Moult, J., "Bacterial Resistance to β-Lactam Antibiotics: Crystal Structure of β-Lactamase from *Staphylococcus aureus* PC1 at 2.5 A Resolution", *Science*, Vol. 236, pp. 694-701. (1987).

27. Jelsch, C., Mourey, L., Masson, J. M. and Samama, J. P., "Crystal Structure of *Escherichia coli* TEM1 β-Lactamase at 1.8 A Resolution", *Proteins: Structure, Function, and Genetics*, Vol. 16, pp. 364-383. (1993).

28. Herzberg, O., "Refined Crystal Structure of β-Lactamase from *Staphylococcus aureus* PC1 at 2.0 A Resolution", *Journal of Molecular Biology*, Vol. 217, pp. 701-719. (1991).

29. Baguley, C., "The Structure and Activity of β-Lactamase I from *Bacillus cereus*", *D. Phil. Thesis*, University of Oxford (1990).

30. Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frere, J. M. and Ghuysen, J. M., "Mechanism of Acyl Transfer by the Class A Serine β-Lactamase of *Streptomyces albus* G", *Biochemical Journal*, Vol. 279, pp. 213-221. (1991).

31. Lamotte-Brasseur, J., Jacob-Dubuisson, F., Dive, G., Frere, J. M. and Ghuysen, J. M., "*Streptomyces albus* G Serine β-Lactamase. Probing of the Catalytic Mechanism via Molecular Modelling of Mutant Enzymes", *Biochemical Journal*, Vol. 282, pp. 189-195. (1992).

32. Page, M. G., "β-Lactamase Inhibitors", *Drug Resistance Updates*, Vol. 3, pp. 109-125. (2000).

33. Yang, Y., Rasmussen, B. A. and Shlaes, D. M., "Class A β-Lactamase - Enzyme-Inhibitor Interactions and Resistance", *Pharmacology & Therapeutics*, Vol. 83, pp. 141-151 (1999).

34. Zhang, Y. L. and Li, J. T., "The In Vitro Activity of Sulbactam Combined with Third Generation Cephalosporins against Third Generation Cephalosporin-Resistant Bacteria", *International Journal of Antimicrobial Agents*, Vol. 17, pp. 143-146 (2001).

35. McGhie, D., Clarke, P. D., Johnson, T. and Hutchison, J. G., "Detection of β–Lactamase Activity of *Haemophilus influenzae*", *J Clinical Pathology*, Vol. 30, pp. 585-587. (1977).

36. Masuda, G., "Studies on Bactericidal Activities of β-Lactam Antibiotics on Agar Plates: the Correlation with the Antibacterial Activities Determined by the Conventional Methods", *Journal of Antibiotics*, Vol. 29, pp. 1237-1240. (1976).

37. Dale, J. W. and Smith, J. T., "The Purification and Properties of the β-Lactamase Specified by the Resistance Factor R-1818 in *Escherichia coli* and *Proteus mirabilis*", *Biochemical Journal*, Vol. 123, pp. 493-500. (1971).

38. Catlin, B. W., "Iodometric Detection of *Haemophilus influenzae* β-Lactamase: Rapid Presumptive Test for Ampicillin Resistance", *Antimicrobial Agents and Chemotherapy*, Vol. 7, pp. 265-270. (1975).

39. Jorgensen, J. H., Lee, J. C. and Alexander, G. A., "Rapid Penicillinase Paper Strip Test for Detection of β-Lactamase-Producing *Haemophilus influenzae* and *Neisseria gonorrhoeae*", *Antimicrobial Agents and Chemotherapy*, Vol. 11, pp. 1087-1088. (1977).

40. Rosen, I. G., Jacobson, J. and Rudderman, R., "Rapid Capillary Tube Method for Detecting Penicillin Resistance in *Staphylococcus aureus*", *Applied Microbiology*, Vol. 23, pp. 649-650. (1972).

41. Duma, R. J. and Kinz, L. J., "Simple Test for Identifying Penicillinase-Producing *Staphylococci*", *Applied Microbiology*, Vol. 16, pp. 1261-1262. (1968).

42. Escamilla, J., "Susceptibility of *Haemophilus influenza* to Ampicillin as Determined by Use of a Modified, One-minute β-Lactamase Test", *Antimicrobial Agents and Chemotherapy*, Vol. 9, pp. 196-198. (1976).

43. Basu, M., Sanyal, A. K. and Banerjee, A. B., "A New Method of Colorimetric Assay of β-Lactamase Suitable for Estimation of β-Lactamase Inhibition in Crude Microbial Culture Filtrates", *Folia Microbiology*, Vol. 47, pp. 32-36 (2002).

44. Waley, S. G., "A Spectrophotometric Assay of β-Lactamase Action on Penicillins", *Biochemical Journal*, Vol. 139, pp. 789-790. (1974).

45. Liang, P., Sanchez, R. I. and Martin, M. T., "Electrochemiluminescence-Based Detection of β-Lactam Antibiotics and β-Lactamases", *Analytical Chemistry*, Vol. 68, pp. 2426-2431. (1996).

46. Priano, G., Ruzal, S. M. and Battaglini, F., "An Electrochemiluminescent Method for the Detection of β-Lactamases in Microorganisms", *Analytical Biochemistry*, Vol. 290, pp. 379-382. (2001).

47. Chen, K. C., Chen, L. and Lin, J. Y., "Fluorescent Spot Test Method for Specific Detection of β-Lactamases", *Analytical Biochemistry*, Vol. 219, pp. 53-60. (1994).

48. Chen, K. C., Knapp, J. S. and Holmes, K. K., "Rapid, Inexpensive Method for Specific Detection of Microbial β-Lactamases by Detection of Fluorescent End Products", *Journal of Clinical Microbiology*, Vol. 19, pp. 818-825. (1984).

49. Chen, K. C. and Holmes, K. K., "Enhancement of Fluorescence Development of End Products by Use of a Fluorescence Developer Solution in a Rapid and Sensitive Fluorescent Spot Test for Specific Detection of Microbial β-Lactamases", *Journal of Clinical Microbiology*, Vol. 23, pp. 539-544. (1986).

50. Lawung, R., Danielsson, B., Prachayasittikul, V. and Bulow, L., "Calorimetric Analysis of Cephalosporins using an Immobilized TEM-1 β-Lactamase on Ni^{2+} Chelating Sepharose Fast Flow", *Analytical Biochemistry*, Vol. 296, pp. 57-62. (2001).

51. Chambers, S. J., Wyatt, G. M. and Morgan, M. R., "Investigation of the Interactions Between β-Lactams and a Metallo-β-Lactamase from *Bacillus cereus* Using a Monoclonal Antibody", *Analytical Biochemistry*, Vol. 288, pp. 149-155. (2001).

52. Commission Regulation (EC), *Official Journal of the European Communities*, No. 508, pp. L60/16-L60/52 (1999).

53. Kulp, T. J., Camins, I., Angel, S. M., Munkholm, C. and Walt, D. R., "Polymer Immobilized Enzyme Optrodes for the Detection of Penicillin", *Analytical Chemistry*, Vol. 59, pp. 2849-2853 (1987).

54. Riediker, S. and Stadler, R. H., "Simultaneous Determination of Five β-Lactam Antibiotics in Bovine Milk using Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry", *Analytical Chemistry*, Vol. 73, pp. 1614-1621 (2001).

55. Chen, S., Yan, G., Schwartz, M. A., Perrin, J. H. and Schulman, S. G., "Penicillin-Enhanced Chemiluminescence of the Luminol-H_2O_2-Co^{2+} System", *Journal of Pharmaceutical Sciences*, Vol. 80, pp. 1017-1019 (1991).

56. Sun, J. S., Schulman, S. G. and Perrin, J. H., "Chemiluminescence of β-Lactam Antibiotics Following Oxidation by Potassium Superoxide", *Analytica Chimica Acta*, Vol. 338, pp. 1-2 (1997).

57. Huth, S. P., Warholic, P. S., Devou, J. M., Chaney, L. K. and Clark, G. H., "Parallux™ β-Lactam: A Capillary-Based Fluorescent Immunoassay for the Determination of Penicillin-G, Ampicillin, Amoxicillin, Cloxacillin, Cephapirin, and Ceftiofur in Bovine Milk", *Journal of the Association of Official Analytical Chemists International*, Vol. 85, pp. 355-364 (2002).

58. Buick, R. K., Greer, N. M. and Elliott, C. T., "A Microtitre Plate Assay for the Detection of Antibiotics in Porcine Urine", *Analyst*, Vol. 125, pp. 395-396 (2000).

59. Gilardi, G., Zhou, L. Q., Hibbert, L. and Cass, A. E., "Engineering the Maltose Binding Protein for Reagentless Fluorescence Sensing", *Analytical Chemistry*, Vol. 66, pp. 3840-3847. (1994).

60. Post, P. L., Trybus, K. M. and Taylor, D. L., "A Genetically Engineered, Protein-Based Optical Biosensor of Myosin II Regulatory Light Chain Phosphorylation", *Journal of Biological Chemistry*, Vol. 269, pp. 12880-12887. (1994).

61. Schauer-Vukasinovic, V., Cullen, L. and Daunert, S., "Rational Design of a Calcium Sensing System Based on Induced Conformational Changes of Calmodulin", *Journal the American Chemical Society*, Vol. 119, pp. 11102-11103 (1997).

62. Morii, T., Sugimoto, K., Makino, K., Otsuka, M., Imoto, K. and Mori, Y., "A New Fluorescent Biosensor for Inositol Trisphosphate", *Journal of the American Chemical Society*, Vol. 124, pp. 1138-1139. (2002).

63. Marvin, J. S. and Hellinga, H. W., "Engineering Biosensors by Introducing Fluorescent Allosteric Signal Transducers: Construction of a Novel Glucose Sensor", *Journal of the American Chemical Society*, Vol. 120, pp. 7-11 (1998).

64. Brune, M., Hunter, J. L., Corrie, J. E. and Webb, M. R., "Direct, Real-Time Measurement of Rapid Inorganic Phosphate Release Using a Novel Fluorescent Probe and Its Application to Actomyosin Subfragment 1 ATPase", *Biochemistry*, Vol. 33, pp. 8262-8271. (1994).

65. Lundgren, J. S., Salins, L. L., Kaneva, I. and Daunert, S., "A Dynamical Investigation of Acrylodan-Labeled Mutant Phosphate Binding Protein", *Analytical Chemistry*, Vol. 71, pp. 589-595. (1999).

66. Tolosa, L., Gryczynski, I., Eichhorn, L. R., Dattelbaum, J. D., Castellano, F. N., Rao, G. and Lakowicz, J. R., "Glucose Sensor for Low-Cost Lifetime-Based Sensing Using a Genetically Engineered Protein", *Analytical Biochemistry*, Vol. 267, pp. 114-120. (1999).

67. Walkup, G. K. and Imperiali, B., "Fluorescent Chemosensors for Divalent Zinc Based on Zinc Finger Domains. Enhanced Oxidative Stability, Metal Binding Affinity, and Structural and Functional Characterization", *Journal of the American Chemical Society*, Vol. 119, pp. 3443-3450 (1997).

68. Walkup, G. K. and Imperiali, B., "Design and Evaluation of a Peptidyl Fluorescent Chemosensor for Divalent Zinc", *Journal of the American Chemical Society*, Vol. 118, pp. 3053-3054 (1996).

69. Madgwick, P. J. and Waley, S. G., "β-Lactamase I from *Bacillus cereus*-Structure and Site-Directed Mutagenesis", *Biochemical Journal*, Vol. 248, pp. 657-662 (1987).

70. Castillo, R., Silla, E. and Tunon, I., "Role of Protein Flexibility in Enzymatic Catalysis: Quantum Mechanical-Molecular Mechanical Study of the Deacylation Reaction in Class A β-Lactamases", *Journal of the American Chemical Society*, Vol. 124, pp. 1809-1816. (2002).

71. Vijayakumar, S., Ravishanker, G., Pratt, R. F. and Beveridge, D. L., "Molecular Dynamics Simulation of a Class A β-Lactamase: Structural and Mechanistic Implications", *Journal of the American Chemical Society*, Vol. 117, pp. 1722-1730 (1995).

72. Reddy, P. Y., Kondo, S., Fujita, S. and Toru, T., "Efficient Synthesis of Fluorophore-Linked Maleimide Derivatives", *Synthesis-Stuttgart*, Vol. pp. 999-1002 (1998).

73. Leung, Y. C., "Properties of Mutant Derivatives of β-Lactamase I from *Bacillus cereus*", *D. Phil. Thesis*, University of Oxford (1994).

74. Schmidt, U., Kenklies, J., Rudolph, R. and Bohm, G., "Site-Specific Fluorescence Labelling of Recombinant Polyomavirus-Like Particles", *Biological Chemistry*, Vol. 380, pp. 397-401 (1999).

75. Bradford, M. M., "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. (1976).

76. Mann, M. and Wilm, M., "Electrospray Mass Spectrometry for Protein Characterization", *Trends in Biochemical Sciences*, Vol. 20, pp. 219-224. (1995).

77. Drake, A. F., "Circular Dichroism", In Jones, C., Mulloy, B. and Thomas, A. H., eds., *Methods in Molecular Biology, Vol. 22: Microscopy, Optical Spectroscopy and Macroscopic Techniques*, Humana Press, Totowa, pp. 219-243 (1994).

78. Stanislawski, J., Enzyme Kinetics, Trinity Software, Hampshire, USA (1991).

79. Doi, N. and Yanagawa, H., "Design of Generic Biosensors Based on Green Fluorescent Proteins with Allosteric Sites by Directed Evolution", *Federation of European Biochemical Societies Letters*, Vol. 453, pp. 305-307. (1999).

80. http://www.rcsb.org/pdb/

81. Jones, T. A., Zou, J. Y., Cowan, S. W. and Kjeldgaard, M., "Improved Methods for Building Protein Models in Electron-Density Maps and the Location of Errors in These Models", *Acta Crystallographica*, Vol. A47, pp. 110-119 (1991).

82. van Aalten, D. M. F., Bywater, R., Findlay, J. B. C., Hendlich, M., Hooft, R. W. W. and Vriend, G., "PRODRG, A Program for Generating Molecular Topologies and Unique Molecular Descriptors from Coordinates of Small Molecules", *Journal of Computer-Aided Molecular Design*, Vol. 10, pp. 255-262 (1996).

83. Rice, L. M. and Brunger, A. T., "Torsion Angle Dynamics-Reduced Variable Conformational Sampling Enhances Crystallographic Structure Refinement", *Proteins: Structure, Function, and Genetics*, Vol. 19, pp. 277-290 (1994).

84. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. and Warren, G. L., "Crystallography & NMR system: A New Software Suite for Macromolecular Structure Determination", *Acta Crystallographica*, Vol. D54, pp. 905-921 (1998).

85. Hubbard, S. J. and Thornton, J. M., NACCESS, Computer Program, Department of Biochemistry and Molecular Biology, University College London (1993).

86. Esnouf, R. M., "An Extensively Modified Version of MolScript that Includes Greatly Enhanced Coloring Capabilities", *Journal of Molecular Graphics & Modelling*, Vol. 15, pp. 132-136 (1997).

87. Merritt, E. A. and Bacon, D. J., "Raster3D: Photorealistic Molecular Graphics", In Charles, W. C., Jr. and Robert, M. S., eds., *Methods in Enzymology, Vol. 277: Macromolecular Crystallography*, Academic Press, San Diego, pp. 505-524 (1997).

88. Nicholls, A., Sharp, K. A. and Honig, B., "Protein Folding and Association-Insights from the Interfacial and Thermodynamic Properties of Hydrocarbons", *Proteins: Structure, Function, and Genetics*, Vol. 11, pp. 281-296 (1991).

89. Martin, M. M. and Lindqvist, L., "The pH-Dependence of Fluorescein Fluorescence", *Journal of Luminescence*, Vol. 10, pp. 381-390 (1975).

90. Klonis, N. and Sawyer, W. H., "Spectral Properties of the Prototropic Forms of Fluorescein in Aqueous Solution", *Journal of Fluorescence*, Vol. 6, pp. 1996 (1996).

91. Long, D. M., "Enzymatic Assay of β-Lactamase using Circular Dichroism Spectropolarimetry", *Analytical Biochemistry*, Vol. 247, pp. 389-393. (1997).

92. Fonze, E., Vanhove, M., Dive, G., Sauvage, E., Frere, J. M. and Charlier, P., "Crystal Structures of the *Bacillus licheniformis* BS3 Class A β-Lactamase and of the Acyl-Enzyme Adduct Formed with Cefoxitin", *Biochemistry*, Vol. 41, pp. 1877-1885. (2002).

93. Matagne, A., Lamotte-Brasseur, J., Dive, G., Knox, J. R. and Frere, J. M., "Interactions Between Active-Site-Serine β-Lactamases and Compounds Bearing a Methoxy Side Chain on the α-Face of the β-Lactam Ring: Kinetic and Molecular Modelling Studies", *Biochemical Journal*, Vol. 293, pp. 607-611. (1993).

94. Jamin, M., Damblon, C., Bauduin-Misselyn, A. M., Durant, F., Roberts, G. C., Charlier, P., Llabres, G. and Frere, J. M., "Direct N.M.R. Evidence for Substrate-Induced Conformational Changes in a β-Lactamase", *Biochemical Journal*, Vol. 301, pp. 199-203. (1994).

95. Raquet, X., Lamotte-Brasseur, J., Fonze, E., Goussard, S., Courvalin, P. and Frere, J. M., "TEM β-Lactamase Mutants Hydrolysing Third-Generation Cephalosporins. A Kinetic and Molecular Modelling Analysis", *Journal of Molecular Biology*, Vol. 244, pp. 625-639. (1994).