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Catalytically impaired fluorescent Class C β-lactamase enables rapid and sensitive cephalosporin detection by stabilizing fluorescence signals: Implications for biosensor design

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Biosensors have found applications in many sectors including the food industry, where cephalosporin detection has played an important role in reducing the incidence of cephalosporin contamination, ensuring food safety, and reducing the spread of antibiotic resistance. Taking advantage of the specific interaction between β-lactamase and its cephalosporin substrates/inhibitors, we previously constructed a biosensor based on a fluorescein-labeled class C β-lactamase mutant ,V211Cf, for specific and reagentless detection of cephalosporins and class C β-lactamase inhibitors (Anal. Chem. 2011, 83, 1996–2004). Upon the addition of substrate/ inhibitor (i.e. the biosensor's analyte), the analyte induced a change in the local environment of the fluorescein molecule that was covalently tethered to a site close to the enzyme's active site (the 211 position), triggering a fluorescence enhancement of V211Cf. To improve the performance of V211Cf for better cephalosporin detection of the biosensor, we have developed Y150S/V211Cf, a derivative of V211Cf constructed by introducing the Y150S mutation to suppress the hydrolytic activity of V211Cf thereby improving the stability of the fluorescence signal. From our results, Y150S/V211Cf not only demonstrated improved fluorescence signal sustainability over V211Cf, but also showed a rapid response towards cephalothin (a first generation cephalosporin). These features make it feasible to of use Y150S/V211Cf for the rapid and specific detection of cephalosporins, and illustrate the possibilities for rational biosensor design of catalytically impaired fluorescent enzymes for rapid and sensitive analyte detection purposes.

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Abbreviations: BHI, Brain heart infusion; **BHY**, medium composed of 37 g/L BHI and 5 g/L yeast extract; **f**, fluorescein; ***k***, rate constant; k_{cat}, catalytic rate constant; K_m, Michaelis constant

Keywords: β-lactamase · Cephalosporin · Fluorescein · Fluorescent biosensor · Food industry

1 Introduction

Cephalosporins are a major class of β-lactam antibiotics used to treat bacterial infections [1–3]. They have been widely used as food supplements in livestock production [4], and are currently one of the types of antibiotic contamination commonly found in poultry and dairy products. Over-use and misuse of cephalosporins intensify the contamination problem. Recent concerns have been raised regarding the problems associated with cephalosporin contamination, which include the spreading of bacterial resistance [5], antibiotic allergies in hypersensitive individuals [6], and the inhibition of starter cultures in the pro-

Table 1. Methods for cephalosporin detection

Methods	Principal	Characteristics	References
Microbial inhibition assay (e.g. Delvotest SP and Charm AIM-96)	Inhibition of microbial growth by β-lactam antibiotics	– Broad spectrum detection – Simple – Requires incubation for microbial growth – Time per test ranges from 2–4 h – Low cost per test	[7–9]
Rapid Test – immunoassay (e.g. Parallux™) and receptor assay (e.g., Charm Test™ and Fluorophors BetaScreen E.U. test)	Interaction between antibody/receptor protein and cephalosporins	– Specific detection – Sensitive – Semi-quantitative – Easy to perform – Time per test ranges from 5–10 min	[10–13]
High performance liquid chromatography (HPLC)	Identification and quantification of cephalosporins based on their partition coefficient	– Sensitive – Quantitative – Requires sample preparation step – Requires sophisticated instrumentation	[14]
Mass spectrometry	Identification and quantification of cephalosporins based on mass-to-charge ratio	– Sensitive – Quantitatve – Requires sample preparation step – Requires sophisticated instrumentation	[15]
Capillary zone electrophoresis	Migration behavior of cephalosporins in electrophoretic gel	– Low sensitivity – Quantitative – Automated	[16]
Surface plasmon resonance-based biosensor	Resonance upon binding of β-lactam antibiotics with β-lactam receptor protein	– Sensitive – Requires sophisticated instrumentation	[17, 18]
Electrochemiluminescence	Electrochemiluminescence generated by β-lactam antibiotics in chemical reaction	– Sensitive – Simple	[19]
Spectrofluorometry	Formation of fluorescent products by cephalosporins with other chemical substances	– Sensitive	[20]
Potentiometry and voltammetry	Electro-oxidation of cephalosporins	– Sensitive	[21, 22]
β-lactamase based fluorescent biosensor (E166Cf, V211Cf and Y150S/V211Cf)	Fluorescence signal triggered by interaction between fluorescent β-lactamase mutant and cephalosporins	– Specific and simple detection – Sensitive and rapid – Semi-quantitative – Real-time detection – Allows high throughput screening	[23, 34] This study

duction of dairy products. To solve these problems, rapid and quantitative detection of cephalosporin residues in food has become particularly important for quality control and to reduce the incidence of contamination.

At this time a variety of approaches have been developed for cephalosporin detection (Table 1). Microbial inhibition tests and rapid tests are currently the standard methods for detecting β-lactam antibiotics [7–13]. Microbiological tests are simple, able to detect a broad spectrum of antibiotics, and require lengthy incubations to allow microbial growth, thereby having the drawbacks of lacking specificity and being time-consuming. On the contrary, rapid tests are sensitive methods based on antibodies or receptor proteins that are specific for β-lactam antibiotics. They are superior to microbial inhibition assays in terms of sensitivity and test duration time, however many of them only provide semi-quantitative measurements. Additional methods for the determination of cephalosporin levels have also been reported [14–22]. However, these methods are usually laborious, tedious, and require complicated sample preparation steps, thereby limiting their application to the routine examination of β-lactam antibiotic levels.

Recently, we have developed a fluorescent enzyme biosensor (E166Cf) from a class A β-lactamase from *Bacillus cereus*, which is an enzyme able to hydrolyze the

β-lactam ring of β-lactam antibiotics [23]. The replacement of a catalytically important residue (Glu166) with a cysteine by site-directed mutagenesis resulted in the severe impairment of the β-lactamase activity of this enzyme (the E166C mutant) losing most of its ability to hydrolyze β-lactam antibiotics while retaining its ability to recognize and bind to its β-lactam substrates. A fluorescein molecule (f) was then site-specifically tethered to the single cysteine residue of the E166C mutant through the formation of a covalent bond between the fluorescein-5-maleimide and the SH-group of cysteine at position 166, giving rise to E166Cf. As the fluorescein, located at the flexible **Ω**-loop of the enzyme, is in close proximity to the active site, substrate binding induces a change in the fluorescein's local environment. In response to such a change in the local environment, flexibility of the loop motif allows movement of the fluorescein from its original hydrophobic environment to a more solvent exposed environment, triggering an increase in the fluorescence intensity of E166Cf. The substrate driven change in fluorescence is the basis for fluorescent biosensing mechanism of E166Cf which can be utilized for the detection of β-lactam antibiotics [24-26].

For the rapid and specific detection of cephalosporins, we focused on constructing a new β-lactamase-based biosensor using a class C β-lactamase. We postulated that a class C β-lactamase would be a suitable candidate to act as a biosensor scaffold for detecting cephalosporins based on the following rationales. First, class C β-lactamases possess high structural and functional similarities and a common mechanism for the hydrolysis of β-lactam substrates (Eq. 1) with their evolutionarily related class A counterparts [27–29]. Thus, it is envisioned that a similar approach in E166Cf fabrication can be used for the construction of a class C-based biosensor. Second, class C β-lactamases, so-called cephalosporinases, demonstrate a substrate preference for cephalosporins and often display more efficient cephalosporin binding and hydrolysis than that of class A enzymes. Class C β-lactamases are more favorable for accommodating cephalosporins that are bulky in size [30]. This implies that a class C-based biosensor would probably lead to a more selective and specific detection for cephalosporins. Third, in terms of kinetic properties, most class C β-lactamases exhibit a low K_m and low k_{cat} for hydrolyzing β-lactam antibiotics [31–33]. These kinetic properties make class C enzymes attractive as biosensors for their substrates because low K_m corresponds to a higher affinity of the biosensor for the antibiotic analytes whereas low k_{cat} implies a lower rate in the destruction of the analytes, leading to a more stable and detectable fluorescence signal.

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

Where E is a β-lactamase enzyme, S is a β-lactam antibiotic substrate, ES is a non-covalent enzyme-substrate complex, ES* is a covalent acyl enzyme-substrate complex, and P is the product.

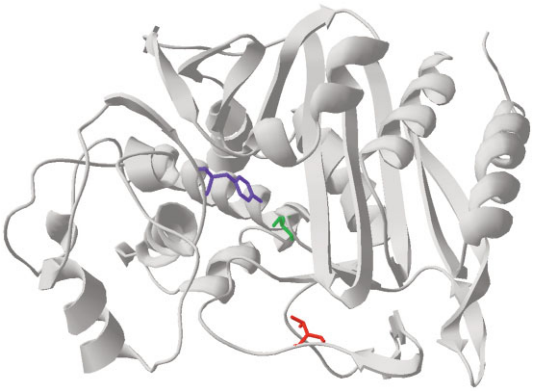

Figure 1. Structural architecture of the P99 AmpC β-lactamase. Three conserved residues in the active site pocket, including Ser64, Tyr150, and Val211, are highlighted. Ser64 (Green) is the catalytic centre responsible for acylating the β-lactam substrates; Tyr150 (Blue) is a residue playing a pivotal role in the deacylation step of the hydrolytic action; and Val211 (Red), located on a long flexible Ω-loop with its side chain protruding toward the active site, is a residue in the R1 cleft binding site that is related to substrate recognition.

Our recently constructed class C β-lactamase-based V211Cf biosensor has validated our postulations [34]. A non-catalytic residue, Val211 on the **Ω**-loop of the P99 AmpC β-lactamase from *Enterobacteria cloacae* P99 (Figure 1) was first substituted with a cysteine, followed by the incorporation of a fluorescein at the same position. The resulting V211Cf showed fluorescence enhancement in the presence of β-lactam antibiotics. Molecular modeling data illustrated that the fluorescein molecule gained higher solvent accessibility upon β-lactam substrate binding, revealing that the fluorescence signal increase is probably due to greater exposure of the fluorescein to the solvent. Moreover, mass spectrometric analysis revealed that the fluorescence signal was attributed to the formation of the enzyme-substrate adduct (ES*). As V211Cf has preserved the catalytic properties of the wild-type enzyme, V211Cf is behaving like a "natural drug target", which can be very useful for screening class C β-lactamase specific inhibitors. However, as a tool for cephalosporin detection, V211Cf with catalytic efficiency similar to wild-type β-lactamase has the drawback of poor sustainability of the fluorescence signals when attempting to detect the rapidly-hydrolyzed substrates.

To improve the stability of the fluorescence signals, we postulated that if we mutate a residue on the enzyme to lower the catalytic activity and keep the binding capability, we could use the class C β-lactamase as the biosensor scaffold for stable and specific cephalosporin detection. Tyr150, conserved in class C β-lactamases, plays the indispensible role in the final step of β-lactamase's hydrolytic action. The deacylation catalysed by Tyr150 involves the breakdown of the acyl-enzyme and the sub-

sequent release of the hydrolyzed product (see Eq. 1) from the enzyme's active site [35]. In the present study, we mutated Tyr150 into serine to impair the catalytic ability of V211Cf. Here, we report the preparation and characterization of Y150S/V211Cf. The performance of Y150S/V211Cf in detecting cephalothin (a rapidly-hydrolyzed substrate of class C β-lactamase) was studied and compared with those of E166Cf and V211Cf so as to demonstrate the feasibility of using Y150S/V211Cf as a platform for cephalosporin detection.

2 Materials and methods

2.1 Chemicals

Cephalothin, chloramphenicol, sodium chloride, Tris, HCl, potassium dihydrogenphosphate, ammonium acetate, and hen-egg white lysozyme were purchased from Sigma (St. Louis, MO). Ampicillin was purchased from USB (Cleveland, OH). Fluorescein-5-maleimide was purchased from Molecular Probes Inc. (Eugene, O.R.). Brain heart infusion (BHI) and yeast extract were obtained from Oxoid Ltd (Nepean, Ontario, Canada). The chemical structures of fluorescein-5-maleimide and cephalothin used in this study are shown in Supporting information, Fig. S1.

2.2 Site-directed mutagenesis

The Y150S/V211C mutant of the P99 β-lactamase was constructed by mutagenesis using a QuikChange® Site-directed Mutagenesis Kit (Strategene, La Jolla, CA), according to the manufacturer's protocol. Plasmid pSG1113/M containing the P99 β-lactamase V211C mutated gene [34] was used as a template for the construction of the mutant. The Y150S/V211C mutant was constructed by using the mutagenic primer pair: 5' – GCA CAA CGC GTC TTA GCG CCA ACG CCA GC – 3' (sense) and 5' – CGT GTT GCG CAG AAT CGC GGT TGC GGT CG – 3' (anti-sense). DNA sequencing was performed to confirm the introduction of the desired mutation into the gene.

2.3 Expression of β-lactamases

The *B. cereus* β-lactamase I E166C mutant and the *E. cloacae* P99 β-lactamase mutants (V211C and Y150S/V211C) were expressed in *Bacillus subtilis* strain IA304 (ϕ105MU331) as extracellular and N-terminally $(His)_6$-tagged intracellular proteins, respectively [23,34]. The bacterial strain harboring the expression construct was streaked onto an agar plate supplemented with 5 μg/mL chloramphenicol and allowed to grow at 37°C for 24 h. A few single bacterial colonies from the agar plate were inoculated into 100 mL of sterile BHY medium (37 g/L BHI and 5 g/L yeast extract), which was then incubated at 37°C with shaking at 300 rpm for 11–12 h. This overnight culture was transferred to a conical flask containing sterile BHY medium at a dilution factor of 1:20. The freshly inoculated media was cultivated at 37°C with shaking at 300 rpm. When the OD_{600} value reached 3.0–3.5, protein expression was triggered by heat-shocking the bacterial culture at 50°C for 5 min. Afterwards, the bacterial culture was subjected to a further 5 h incubation at 37°C with shaking at 300 rpm. For extracellular expression of the *B. cereus* β-lactamase I E166C mutant, culture supernatant containing the expressed E166C protein was collected after centrifugation at 9,000 rpm, at 4°C for 20 min and immediately subjected to purification. For intracellular expression of P99 β-lactamase mutants, bacterial cells were collected by centrifugation at 9,000 rpm and 4°C for 20 min and stored at –80°C until purification.

2.4 Purification of β-lactamase mutants

To purify the E166C mutant, the culture supernatant was first mixed with 40 g celite for 20 min at 4°C. After discarding the supernatant, the celite was washed with deionized water so as to remove the non-specific unbound proteins. After that, celite-bound β-lactamase was eluted by mixing the celite with 300 ml of protein elution buffer (100 mM Tris-HCl, 2 M NaCl and 100 mM trisodium citrate, pH 7.0). The protein solution was then concentrated to 10 ml at 4 °C using an Amicon concentrator equipped with a YM-1 membrane (Millipore, MWCO = 1000). The concentrated protein solution was exchanged with 20 mM ammonium bicarbonate, freeze-dried, and then stored at -20°C.

To purify V211C and Y150S/V211Cf mutants, the cell pellet was re-suspended in solubilization buffer (50 mM Tris-HCl, 0.1 M NaCl, pH 8.0), incubated with 75 μg/mL lysozyme for 1 h at 30°C, and then sonicated to lyse the cells. The bacterial lysate was collected by centrifugation at 10,000 rpm and 4°C for 1 h and loaded onto a Ni^{2+}-charged 5 mL HiTrap chelating column (Amersham-Pharmacia Biotech Inc.) pre-equilibrated with start buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.4). The column was then washed with 6 column volumes of the same buffer, and the $(His)_6$-tagged enzyme was eluted by a linear imidazole gradient of 0–0.2 M. Fractions containing the enzyme were pooled and then dialyzed against 20 mM potassium phosphate buffer (pH 7.4) at 4°C. For long-term storage, the purified protein solution was buffer-exchanged with 20 mM ammonium bicarbonate, freeze-dried, and stored at –20°C.

2.5 Fluorescein labeling of β-lactamase mutants

To prepare E166Cf, lyophilized E166C mutant protein was dissolved in 6 M guanidinium hydrochloride. The protein solution was incubated at room temperature for 30 min to allow the protein to unfold. Afterwards, a 10 fold molar

excess of fluorescein-5-maleimide in dimethylformamide was added to the protein solution and the pH of the mixture was adjusted to 7.5 with 0.2 M sodium hydroxide. The labeling reaction was carried out by stirring the mixture at room temperature for 3 h in the dark. After that, removal of excess dye was achieved by buffer exchange with 50 mM potassium phosphate by dialysis or ultracentrifugation using an Amicon Ultra-15 (NMWL = 10,000) centrifugal filter device. The labeled mutant was stored at –80°C.

For preparation of V211Cf and Y150S/V211Cf, lyophilized protein was first dissolved in 50 mM potassium phosphate (pH 7.0). Labeling of mutants with fluorescein was performed by adding a 10 fold molar excess of fluorescein-5-maleimide to the protein solution, followed by shaking at 300 rpm in the dark for 30 min. Afterwards, excess dye was removed by dialysis with 50 mM potassium phosphate or by ultracentrifugation using an Amicon Ultra-15 (NMWL = 10,000) centrifugal filter device. The labeled mutant was then stored at –80°C.

2.6 Enzyme kinetic studies

Hydrolysis of cephalothin was monitored by following the absorbance change at 263 nm, resulting from the breakdown of the β-lactam ring, using a Perkin Elmer Lambda Bio 20 UV/VIS spectrometer. All kinetic measurements were performed in 50 mM potassium phosphate buffer (pH 7.0) at 20°C in triplicate. Kinetic parameters (K_m and k_{cat}) were determined by fitting the initial rate of cephalothin hydrolysis to the Michaelis-Menten equation using non-linear regression analysis.

2.7 Fluorescence measurements

A 5 μL aliquot of 10 μM fluorescein-labeled mutant (in 50 mM phosphate buffer, pH 7) was first mixed with 490 μL of 50 mM phosphate buffer (pH 7.0) in a quartz cuvette with a 1 cm path length. A series of 1, 10, 100, 1000, and 10000 μM cephalothin were prepared as the stock solutions (in 50 mM phosphate buffer, pH 7.0). A 5 μL portion of the cephalothin stock solution was then added to the enzyme solution to give a reaction mixture containing the desired antibiotic concentration (0.1, 1, 10, 100 and 1000 μM) and 0.1 μM fluorescein-labeled mutant. The reaction mixture was mixed immediately, and the fluorescence signal was recorded using a Perkin-Elmer LS50B spectrofluorimeter (Beaconsfield, Buckinghamshire, England). Both excitation and emission slit widths were set at 5 nm. For E166Cf, excitation and emission wavelengths were set at 494 nm and 515 nm, respectively. For V211Cf and Y150S/V211Cf, excitation and emission wavelengths were 498 nm and 517 nm, respectively. Stopped-flow fluorescence measurement of V211Cf was performed with an Applied Photophysics SX.18MV-R stopped-flow spectrophotometer (Leatherhead, UK). Both excitation and emission slit widths were 5 nm. The emission was observed through a 515 nm cutoff filter. All fluorescence measurements were performed at 20°C.

3 Results

3.1 Fabrication and characterization of the Y150S/V211Cf biosensor

Our previously constructed class A β-lactamase-based biosensor, E166Cf, exhibited a relatively slow response towards the cephalosporins, resulting in exceedingly long time required for the detection of these antibiotics [23]. We reasoned that the slow response was due to the poor affinity of class A β-lactamase (a poor cephalosporinase) for cephalosporins. As a result, we postulated that a class C β-lactamase, which is a cephalosporin-preferred hydrolase, could be tailor-made into an efficient cephalosporin biosensor that could be used for the rapid and specific detection of cephalosporins. Recently, we reported the fabrication of the class C enzyme-based sensor, V211Cf, that can detect β-lactam antibiotics and class C β-lactamase specific inhibitors. While this implied that a similar strategy for constructing the class A β-lactamase-based biosensor could be applied to turn a class C β-lactamase into a biosensor, V211Cf ,with similar catalytic capability as its parental wild-type enzyme, actually gave a short-lived fluorescence signal due to the rapidly hydrolyzed β-lactam antibiotics [34]. This property meant that V211Cf was not useful for detecting first and second generation cephalosporins, which are the preferred substrates of class C enzymes. To overcome this shortcoming, we attempted to develop a catalytically impaired derivative of V211Cf with a reduced rate of the hydrolytic action.

Our previous data illustrated that the fluorescence signal given by V211Cf in the presence of β-lactam antibiotics was solely attributed to the formation of acyl-enzyme complex (ES*) [34]. β-lactamase's hydrolytic action is a three step mechanism which is composed of substrate binding, acylation, and deacylation (Eq. 1). Substrate binding and acylation affect the rate of ES* formation, while deacylation contributes to the breakdown of ES*. In order to improve the performance of V211Cf, we hypothesized that the new version of the biosensor should be impaired in deacylation (to reduce the degradation of β-lactam analytes) with its acylation rate not affected (to retain the substrate binding ability of β-lactamase). Therefore, we decided to mutate Tyr150, a residue important for deacylation, into a serine residue [35]. We predicted that such a mutation would lead to a drastic decrease in β-lactamase activity without affecting the substrate binding step of the mechanism.

To fabricate the Y150S/V211Cf biosensor, two mutations were introduced into the wild-type P99 *E. cloacae* β-lactamase via site-directed mutagenesis. The first

mutation was the introduction of a cysteine residue to replace Val211 and allow fluorophore attachment, and the second mutation was the replacement of Tyr150 with a serine residue for suppressing the hydrolytic activity of β-lactamase. This double mutant was then over-expressed, purified, and labeled with a fluorescein molecule via a thiol reaction between the maleimide group of fluorescein-5-maleimide and the sulfhydryl group of the cysteine residue of the protein. The labeling efficiency of Y150S/V211Cf with fluorescein was determined by SDS-PAGE analysis and electrospray ionization mass spectrometry (ESI-MS). It was estimated that more than 90% of Y150S/V211C was successfully labeled with fluorescein (Supporting information, Figs. 2–4).

To evaluate the effects of site-directed mutagenesis and fluorophore coupling on the catalytic function of the β-lactamase, hydrolytic activity of the wild-type P99 β-lactamase and its mutants (V211C, Y150S/V211C and Y150S/V211Cf) towards cephalothin (a first generation cephalosporin) was studied by spectrophotometry (Table 2). K_m is the Michealis-Menten constant that reflects the substrate binding affinity of the enzyme whereas k_{cat} is the turnover number that indicates how fast the substrate is being consumed by the enzyme. The catalytic efficiency k_{cat}/K_m generally depicts the catalytic ability of the enzyme. According to our kinetic results, the V211C mutant demonstrated similar K_m and k_{cat} to the wild-type, illustrating that the V211C mutation did not have much effect on the binding and catalytic properties of P99 β-lactamase. In contrast, the Y150S/V211C mutant showed a similar K_m to the wild-type and V211C while it possessed a much lower k_{cat} than the wild-type (a decrease of ~1800 fold) and V211C mutant (~800 fold decrease). This indicated that the Y150S mutation greatly reduced the catalytic ability of the P99 β-lactamase and had no significant effect on the substrate binding ability of the enzyme. The significant effect of the Y150S mutation on the deacylation rate (reflected by the low k_{cat}) is consistent with the indispensable role of Tyr150 in the deacylation mechanism reported by Oefner et al. [36]. In addition, the drastic reduction in β-lactamase activity of Y150S/V211C implied that the incorporation of a single point mutation Y150S in the V211C mutant was sufficient to suppress the hydrolytic activity of the β-lactamase. Furthermore, it was shown that Y150S/V211Cf had an approximately 3 fold higher in K_m than Y150S/V211C. The increase in K_m value after fluorescein labeling might be due to the introduction of steric hindrance at the active site by the attached fluorescein. On the contrary, Y150S/V211Cf showed a similar k_{cat} to the unlabeled Y150S/V211C protein, illustrating that labeling the active site with a fluorescein at position 211 did not affect the catalytic function of Y150S/V211C. Taken together, Y150S/V211Cf retained a similar substrate binding capability as the wild-type P99 β-lactamase and was severely impaired in hydrolyzing the cephalosporin substrate.

Table 2. Kinetic parameters for the hydrolysis of cephalothin by the wild-type P99 β-lactamase and the mutants. Initial rates of hydrolysis of 6–8 different concentrations of cephalothin were measured (n = 3 for each concentration) and fitted to Michaelis-Menten equation for determination of K_m and k_{cat}.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Wild-type[a)]	9.06 ± 0.69	117.60 ± 2.30	12.97 ± 1.05
V211C[a]	25.56 ± 1.24	53.98 ± 0.99	2.11 ± 0.11
V211Cf[a]	64.49 ± 6.14	47.34 ± 1.90	0.73 ± 0.08
Y150S/V211C	15.31 ± 1.38	0.064 ± 0.002	0.004 ± 0.0004
Y150S/V211Cf	51.72 ± 6.23	0.073 ± 0.002	0.001 ± 0.0001

a) Kinetic results were cited from our previous published data [34].

3.2 Performance of Y150S/V211Cf in cephalosporin detection

To provide insight into the feasibility of Y150S/V211Cf for cephalosporin detection, its performance in detecting cephalothin, which is very susceptible to β-lactamase hydrolysis, was assessed and compared with the performance of the class A β-lactamase E166Cf biosensor and the class C β-lactamase V211Cf biosensor (Figs. 2 and 3). Various parameters of the biosensors are summarized in Table 3 and discussed in sections 3.2.1–3.2.3.

3.2.1 Fluorescence signal pattern and intensity

As revealed by the fluorescence profiles, the engineered fluorescent enzymes typically exhibited signals with an increase and leveling-off of fluorescence intensity in response to cephalothin addition. Within the 30 min measurement window, fluorescence signals for V211Cf returned to the basal level due to the hydrolysis of cephalothin whereas for catalytically deficient Y150S/C211Cf and E166Cf, fluorescence signals did not diminish. Regarding the fluorescence signal intensity, class C-based biosensors generally showed signals with a smaller magnitude (a smaller change in fluorescence intensity) than E166Cf. For the saturating cephalothin concentration (1×10^{-3} M), the percentage changes in the fluorescence intensity of V211Cf and Y150S/V211Cf were 37% and 28% respectively, approximately 2 fold lower than that of E166Cf (which was 70%).

3.2.2 Response time

As V211Cf retained most of the parental wild-type's hydrolytic activity, it exhibited the shortest response time among the three fluorescein-conjugated β-lactamases by showing a plateau signal 1000 fold faster than E166Cf and Y150S/V211Cf. However, the signal disappeared rapidly as the substrate was hydrolyzed. Among the two catalytically impaired β-lactamases, E166Cf and Y150S/V211Cf, the class C enzyme-based Y150S/V211Cf displayed a response time shorter by 2 fold than the class A β-lacta-

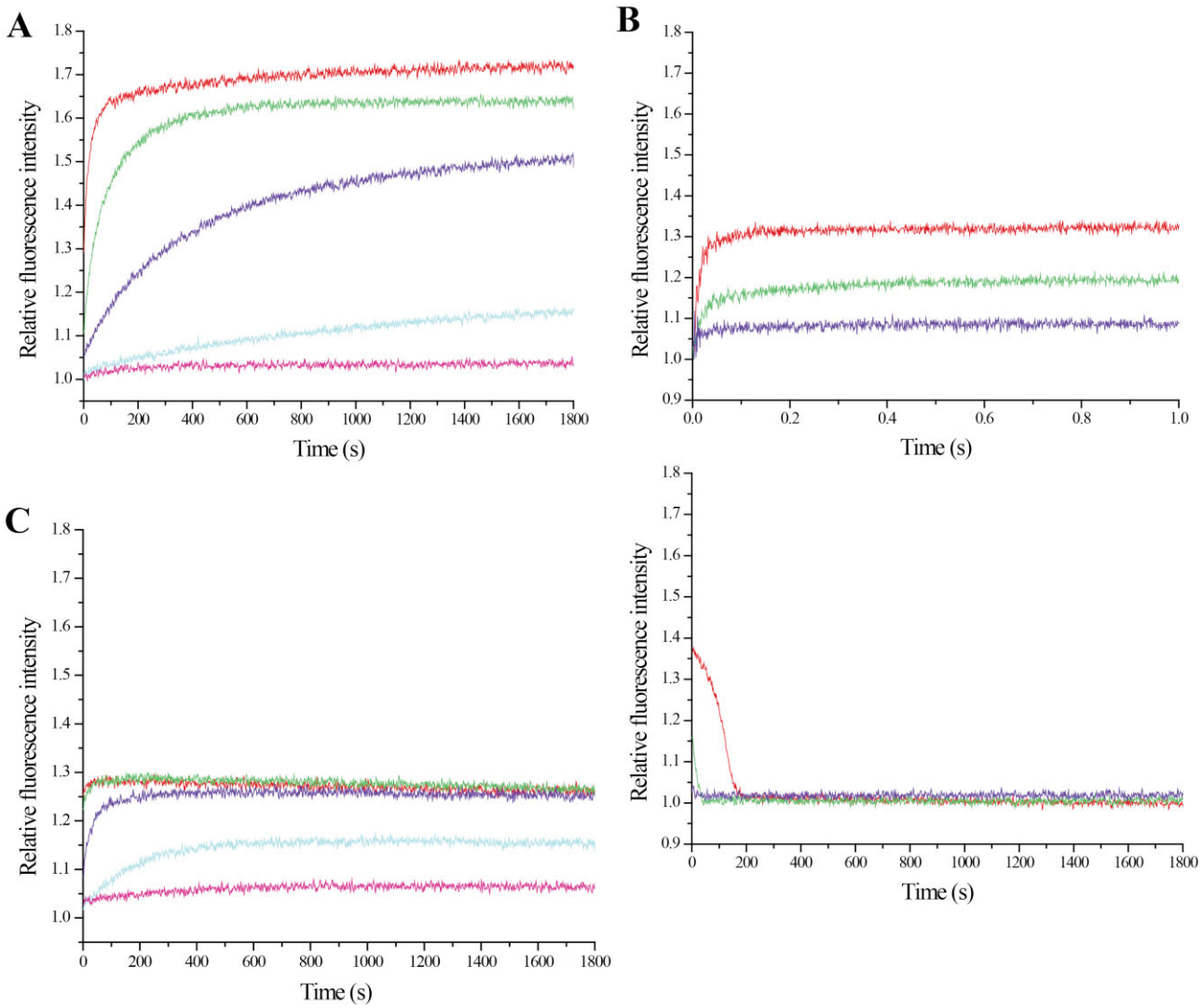

Figure 2. Steady-state fluorescence measurements of 0.1 μM of (**A**) E166Cf, (**B**) V211Cf, and (**C**) Y150S/V211Cf in 50 mM potassium phosphate with various concentrations of cephalothin: 1.0×10^{-7} M (magenta); 1.0×10^{-6} M (cyan); 1.0×10^{-5} M (blue); 1×10^{-4} M (green); 1.0×10^{-3} M (red). In (**B**), the upper panel shows the data collected in the first one second by the stopped-flow fluorescence method. Excitation and emission wavelengths were 498 nm and 517 nm for V211Cf and Y150S/V211Cf respectively; and 494 nm and 515 nm for E166Cf. For stopped-flow fluorescence measurement of V211Cf, the emission was monitored through a 515 nm cutoff filter. Both excitation and emission slit widths were 5 nm. All fluorescence measurements were conducted at 20°C.

mase-derived E166Cf. Taken together, these findings suggested that the fluorescein-labeled class C β-lactamases were superior to the class A β-lactamase-based E166Cf in the parameter of detection time, validating our speculation that a cephalosporinase-based sensor would provide a more rapid response for cephalosporin detection than the fluorescent class A penicillinase.

3.2.3 Lowest detection level

V211Cf exhibited the lowest detection level of 10^{-5} M for cephalothin, which was 100 fold less sensitive than the hydrolytic defective E166Cf and Y150S/V211Cf (10^{-7} M). It can be reasoned that V211Cf rapidly hydrolyzed the substrate, thereby failing to give detectable fluorescence signal towards trace amount of cephalothin. Therefore, Y150S/V211Cf can detect lower levels of cephalothin than V211Cf, offering better cephalosporin detection compared to the V211Cf biosensor.

3.3 Introduction of the Y150S mutation to the V211Cf biosensor improved the stability of the fluorescence signal

With suppressed hydrolytic activity, the class A E166Cf and the class C Y150S/V211Cf β-lactamases generally demonstrated a stable signal over the 30 min time-course

Table 3. Biosensor characteristics of E166Cf, V211Cf and Y150S/V211Cf in detecting cephalothin

	Response time[a](s)	Recovery time[b](s)	Maximum percentage change in fluorescence intensity[c](%)	Lowest detectable level (M)
E166Cf	100	–[d]	70	10^{-7}
V211Cf	0.05	210	37	10^{-5}
Y150S/V211Cf	50	–[d]	28	10^{-7}

a) Response time is defined as the time required by the biosensor to give a signal reaching a plateau level after adding 1.0×10^{-3} M of cephalothin.
b) Recovery time is defined as the time required by the biosensor to return the fluorescence signal to its original basal level after adding 1.0×10^{-3} M of cephalothin.
c) Maximum percentage change in fluorescence intensity is assessed at the concentration of 1.0×10^{-3} M cephalothin.
d) –, not applicable.

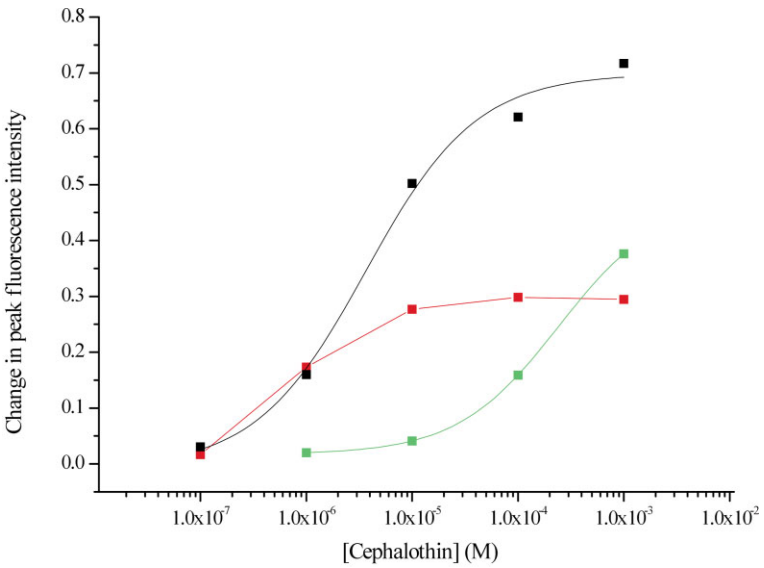

Figure 3. Plots of change in peak fluorescence intensity as a function of concentration of cephalothin for E166Cf (in black line), V211Cf (in green line) and Y150S/V211Cf (in red line).

during which fluorescence was monitored whereas V211Cf, with nearly full hydrolytic activity, showed a rapid hydrolysis of cephalothin, resulting in unstable transient signals towards this good cephalosporin substrate. Over the 30 min measurement period, recovery of baseline fluorescence signal was only observed in V211Cf when cephalothin was used as the substrate. This illustrated that the introduction of the Y150S mutation to the V211Cf biosensor improved the stability of the fluorescence signal.

4 Discussion

For decades cephalosporins have been regarded as one of the most important antimicrobial agents in human medicine due to their high effectiveness and safety. Apart from treating bacterial infections in humans, they have often been applied to livestock as growth promoters by farmers and food producers, leading to cephalosporin contamination in food. Currently, improper use or abuse of cephalosporins has accelerated the problems of widespread of bacterial resistance and antibiotic allergies that have resulted from cephalosporin contamination. To reduce the incidence of cephalosporin contamination of food and the environment, the ability to detect cephalosporins is an important tool for use in poultry and food industries.

With the ultimate goal of developing an efficient system for cephalosporin detection, we developed a new β-lactamase-based fluorescent biosensor, Y150S/V211Cf, to achieve this purpose. Biosensors derived from natural binding proteins have provided useful platforms for reagent-independent biosensing applications due to the non-destructive nature of the binding of these proteins to their native ligands [37]. The recent development of E166Cf, by "active-site-labeling" technology in our laboratory, has opened a new opportunity to have a catalytically defective hydrolase modified into a molecular sensor for the direct, reagentless detection of its hydrolytically-susceptible substrates [31,32]. We proved that enzymes can be turned into "binding proteins' and through site-directed mutagenesis and site-specific fluorophore labeling in order to generate novel biosensors for sensitive and rapid detection. This result motivated us to make use of a class C β-lactamase which can efficiently destroy β-lactam antibiotics (particularly cephalosporins) for cephalosporin detection.

Our first version of the fluorescent class C β-lactamase, V211Cf was a fluorescent hydrolytic enzyme capable of probing the local environment of the active site. A change in fluorescence intensity resulted from the binding of β-lactam substrates or inhibitors (analytes) into the active site pocket of V211Cf, thus indicating the presence of analytes in the test samples. While V211Cf, as a biosensor, demonstrated advantageous features of reagentless, high selectivity, high specificity, and amenability to high-throughput formats for detecting β-lactam substrates or inhibitors, it has the shortcoming of poor signal stability (short-lasting signal) when detecting its preferred β-lactam substrates (cephalosporins) because in most cases it can rapidly hydrolyze its preferred substrates, thereby only providing transient signals that may be difficult to observe or measure (Fig. 2B). As a consequence, in the present study, Y150S/V211Cf, an engineered fluorescent enzyme with suppressed activity, was designed in a rational way for optimizing the performance of V211Cf by rapidly generating a long-lasting signal for detecting the substrate analytes. Our results show that Y150S/V211Cf was able to report the presence of cephalosporins by showing an increase in fluorescence intensity. With a severe impairment in catalytic activity resulting from the substitution of Tyr150 with a serine, Y150S/V211Cf showed improved fluorescence signal stability over V211Cf due to the reduced rate of deacylation (k_3 of Eq. 1) catalysed by the enzyme. Interestingly, the rate of acylation (k_2 of Eq. 1) was not drastically affected, suggesting that Tyr150 is more important for deacylation than for acylation. In addition, when compared with our E166Cf for detecting cephalothin, Y150S/V211Cf revealed a much faster response than the class A β-lactamase-based E166Cf, probably due to faster binding of substrate and subsequent acylation (Eq. 1).

Our newly developed Y150S/V211Cf biosensor provides a rapid method for the detection of trace amounts of cephalosporins. It offers specific detection of cephalosporins because of its natural binding preference for cephahosporins, and because of its impaired hydrolytic capability it generates a stable fluorescence signal to allow semi-quantitative measurements. In addition, it can allow direct and real-time cephalosporin detection. Moreover, unlike analytical methods such as HPLC and mass spectrometry, the Y150S/V211Cf biosensor does not require tedious sample preparation steps prior to detection. Furthermore, as suggested in our previous study of fluorescent-labeled β-lactamase biosensors [29, 34], Y150S/V211Cf is amenable to microplate format, thus enabling high throughput detection of cephalosporins. Taken together, our results illustrate the potential of Y150S/V211Cf as a versatile tool for simple, rapid, and specific cephalosporin detection. Furthermore, both class A and class C β-lactamases are highly efficient enzymes in their wild-type forms [38, 39]. Here we further demonstrated that these enzymes can be engineered, by simple site-directed mutagenesis, to become "binding proteins" able to bind their substrates and retain them in the active site for long periods of time. The innovative "active-site-labeling" strategy adopted in the construction of Y150S/V211Cf is an example of the rational biosensor design of catalytically impaired fluorescent enzymes for rapid and sensitive analyte detection purposes. Further improvement of the sensitivity of the biosensor will be made by modifying the β-lactamase mutant with other fluorophores in future work.

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