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Colorimetric assay for β-lactamase activity using cocktail of penicillin and 4-(2-pyridylazo)resorcinol (PAR)−$2Hg^{2+}$ complex

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ABSTRACT

A colorimetric assay method for measuring β-lactamase activity was developed using a cocktail of penicillin and 4-(2-pyridylazo)resorcinol (PAR)−$2Hg^{2+}$ complex. The penicilloic acid that hydrolyzed form of penicillin by β-lactamase chelates with mercury ion of PAR−$2Hg^{2+}$ complex effectively, conversion of PAR−$2Hg^{2+}$ complexes to free PAR induced the color change from red to yellow. Exact measurement of β-lactamase activity can be performed by the spectrophotometric method, and the facile monitoring of β-lactamase activity was possible in real time. Also, this colorimetric method was applied to determine the Michaelis constant (K_m) of β-lactamase and the IC_{50} of clavulanic acid. Given the facts that its simplicity and high assay speed, this method may be extended to high-throughput screening of β-lactamase inhibitors.

1. Introduction

β-lactam antibiotic blocks cell wall synthesis of bacteria, thus it has been developed as a drug for the clinical treatment of bacterial infections [1–4]. However, some bacteria are resistant to β-lactam antibiotics, such as penicillin and cephalosporin [5–8]. The major cause of increasing bacterial resistance to β-lactam antibiotics is the expression of enzyme β-lactamase in bacteria [9–12]. β-lactamase (EC 3.5.2.6) hydrolyzes β-lactam antibiotics in bacteria that contain the β-lactamase coding gene, thereby inactivating the antibiotics and preventing cell lysis [13,14]. Thus, combining β-lactam antibiotics with β-lactamase inhibitors and modifying the structure of β-lactam antibiotics have been the clinical challenges to combat antibiotic resistance in bacteria. Therefore, development of rapid, sensitive, and simple detection methods for assessing β-lactamase activity, applicable for high-throughput screening of potent β-lactamase inhibitors and new β-lactam antibiotics also gained importance.

In recent years, various methods for assessing β-lactamase activity have been developed. Some methods depend on the hydrolyzed substrate bearing a new carboxylic acid group. For instance, the iodometric assay is based on the reduction of iodine by the hydrolyzed substrate [15–18], and an acidimetric assay uses an acid-base indicator [19,20]. Other methods use specific substrates, such as a chromogenic cephalosporin called nitrocefin, CENTA, or PADAC [21–23], a fluorescent substrate as the reporter for β-lactamase expression [24–28], or a newly synthesized organic substrate facilitating the aggregation of gold nanoparticles [29,30]. However, these methods have some major drawbacks. They are time-consuming, real time monitoring is not possible, and they involve complex synthesis of substrates.

We developed a simple and rapid colorimetric assay method using organic dye-metal complexes, for easier detection of β-lactamase activity. Many organic dye and metal complexes have been used in various chemosensors, based on the ligand exchange between the organic dye and the target molecule or ion [31–40]. When β-lactam antibiotics are hydrolyzed by β-lactamase, the product contains carboxylic acid and an amine functional group, which can chelate metal ion effectively (Scheme 1A). Thus, we designed the β-lactamase assay based on the ligand exchange between an organic dye and the product of hydrolysis of β-lactam antibiotics by the enzyme. Among various dye-metal complexes, PAR−$2Hg^{2+}$ complex was selected for the colorimetric detection of β-lactamase activity. A typical β-lactam antibiotic, penicillin G, was used as a substrate because most of the β-lactamase−producing bacteria are resistant to penicillin G and hydrolyze it readily [5–8]. If penicillin G is hydrolyzed by β-lactamase, the product of the enzymatic reaction, penicilloic acid, can chelate the metal ion. Conversion of PAR−$2Hg^{2+}$ complexes to free PAR changes the color of assay solution from red to yellow (Scheme 1B). Therefore, exact

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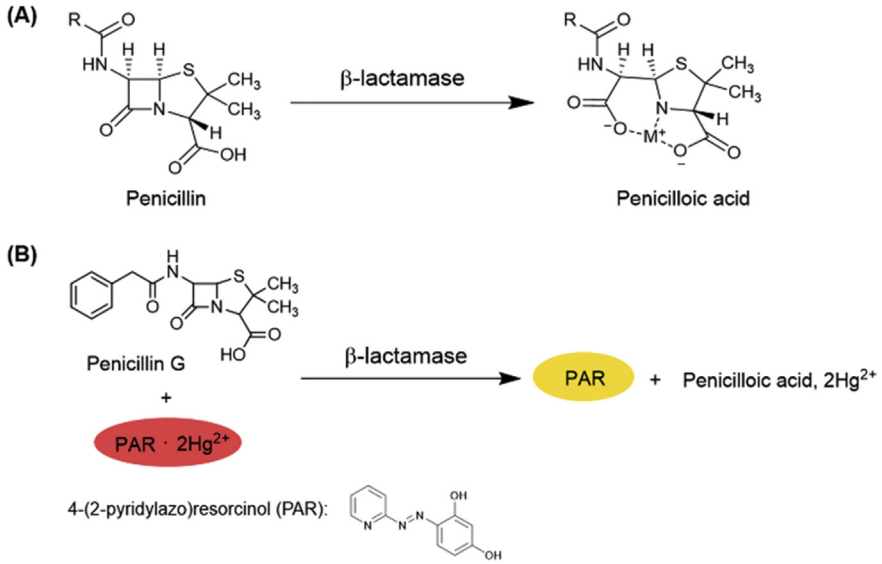

Scheme 1. Schematic illustration of colorimetric β-lactamase assay based on ligand exchange between penicilloic acid and PAR–$2Hg^{2+}$ complex.

measurement of β-lactamase activity can be performed by the spectrophotometric method. This colorimetric assay, using a cocktail of penicillin and PAR–$2Hg^{2+}$ complex, can be also used for the screening potent β-lactamase inhibitors.

2. Experimental section

2.1. Materials and methods

For the enzymatic assay, β-lactamase from *Bacillus cereus* and penicillin G sodium salt were purchased from Sigma Aldrich and penicilloic acid was purchased from Aurum pharmatech. All the metal reagents for organic dye-metal complex, $Hg(NO_3)_2 \cdot H_2O$, $Ca(ClO_4)_2 \cdot 4H_2O$, $Cd(ClO_4)_2 \cdot H_2O$, $Cu(ClO_4)_2 \cdot 6H_2O$, $Pb(ClO_4)_2 \cdot H_2O$, $Mg(ClO_4)_2$, $Mn(ClO_4)_2 \cdot H_2O$, $Ni(ClO_4)_2 \cdot 6H_2O$, $KClO_4$, $NaClO_4$, $Zn(ClO_4)_2 \cdot 6H_2O$, $Fe(ClO_4)_2 \cdot H_2O$, $SrCl_2 \cdot 6H_2O$, $Ba(ClO_4)_2$ $Co(ClO_4)_2 \cdot 6H_2O$ were purchased from Sigma Aldrich. Indigo carmine, thiazole yellow G, alizarin red S, pyrocatechol violet, and glycine red sodium salt from Sigma Aldrich, PAR from TCI, methyl red from Yakuri, and Congo red from Fluka were used as the organic dyes.

All UV/Vis spectra were recorded with SCINCO S-3100 UV/Vis spectrophotometer in 1 cm quartz cells at 25 °C.

2.2. Screening of dye-metal complex

First, 15 different metal stock solutions (50 μM, Fe^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Na^{+}, Pb^{2+}, and Hg^{2+}) were added to each sample containing eight different dyes (10 μM, indigo carmine, PAR, thiazole yellow G, methyl red, alizarin red S, pyrocatechol violet, Congo red, and glycine cresol red sodium salt) in sodium phosphate buffer (pH 7.0, 20 mM). The color of the solution was compared before and after the addition of metal stock solution, and dye-metal complexes that showed significant color change were chosen for the next step.

Then, various amounts of penicilloic acid (10 μM, 50 μM, 500 μM, and 1 mM) were added to the samples containing 13 pairs of dye (10 μM)-metal (50 μM) complexes, chosen from the first step in sodium phosphate buffer (pH 7.0, 20 mM). Dye-metal complexes that showed a significant color change after the addition of penicilloic acid were chosen for the following analysis using UV/Vis spectrophotometer. Final sample volume was 1 mL.

The four selected pairs of dye (10 μM)-metal (50 μM) complexes in sodium phosphate buffer (pH 7.0, 20 mM) were back titrated with different concentration of penicilloic acid (10 μM, 50 μM, 100 μM, 500 μM, and 1 mM). The dye-metal complexes that were sensitive to small amount of penicilloic acid and showed a large shift in the UV/Vis spectrum were chosen for the colorimetric assay of β-lactamase.

2.3. Preparation of PAR–$2Hg^{2+}$ complex

The PAR solution (20 μM) in sodium phosphate buffer (pH 7.0, 20 mM) was titrated with $Hg(NO_3)_2$ (10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 80 μM, 100 μM, 500 μM, and 1 mM). The change in absorbance at 498 nm was measured. Next, the absorbance at 498 nm of the sample containing PAR solution (20 μM) and $Hg(NO_3)_2$ (40 μM) in sodium phosphate buffer (pH 7.0, 20 mM) was measured for 60 min, using a UV/Vis spectrophotometer.

2.4. Model study using penicilloic acid

The sample containing PAR (20 μM) and Hg^{2+} (40 μM) in sodium phosphate buffer (pH 7.0, 20 mM) was incubated for 40 min. Then, the sample solutions were titrated with varying ratios of penicillin and penicilloic acid ([penicilloic acid]/[penicillin] = 0 to 1), and the absorbance at 413 nm and at 498 nm were recorded using a UV/Vis spectrophotometer.

2.5. Detection of β-lactamase activity using PAR–$2Hg^{2+}$ complex

The sample containing PAR (20 μM), Hg^{2+} (40 μM) and penicillin G (100 μM) in sodium phosphate buffer (pH 7.0, 20 mM) was incubated for 40 min. Then, β-lactamase from *Bacillus cereus* (0.02 U/mL) was added to the sample, and the absorbance of the solution mixture was recorded for 20 min using a UV/Vis spectrophotometer.

2.6. Kinetic measurements of enzymatic reaction

The sample containing PAR (20 μM), Hg^{2+} (40 μM), and penicillin G (100 μM) in sodium phosphate buffer (pH 7.0, 20 mM) was

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