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Enantioselective resolution of γ-lactam utilizing a novel (+)-γ-lactamase from *Bacillus thuringiensis*

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ABSTRACT

(-)-γ-Lactam ((-)-2-azabicyclo[2.2.1]hept-5-en-3-one) is a significant chiral synthon that can be used to synthesize a large number of carbocyclic nucleosides such as antiviral drugs abacavir and carbovir. As a new emerging group of enantioselective biocatalysts, (+)-γ-lactamase has drawn more and more attention because of its application for kinetic resolution of racemic γ-lactam to obtain optically pure (-)-γ-lactam. In this paper, a novel (+)-γ-lactamase (designated as SYJ322B5) derived from *Bacillus thuringiensis* was identified by genome mining method. The gene was cloned, functionally expressed in *Escherichia coli* system and the protein was purified to homogeneity. Subsequently, biochemical characterization of SYJ322B5 showed that the optimal temperature and pH were 50 °C and 7.0, respectively. Enzyme assay showed that the recombinant enzyme could enantioselectively catalyze the bioresolution of racemic γ-lactam with a high enantiomeric excess (ee) of 99.8% and enantiomeric ratio (E) > 200. More importantly, SYJ322B5 exhibited the highest enzyme activity among all reported (+)-γ-lactamases. Bioinformatics analysis and molecular docking studies showed that the enzyme was not a conventional amidase superfamily member but belonged to the isochorismatase-like hydrolases superfamily. It possesses a conserved Asp9-Lys81-Cys114 catalytic triad like other members in which the cysteine is considered to perform nucleophilic attack in the enzymatic catalysis process. Overall, this novel (+)-γ-lactamase may become a potential tool for the production of (-)-γ-lactam.

1. Introduction

The study of γ-lactam started with the observation of the increasing bacterial resistance toward traditional β-lactam [1]. (-)-2-Azabicyclo [2.2.1] hept-5-en-3-one ((-)-γ-lactam) is a versatile precursor in the pharmaceutical field. It can be used for the synthesis of a series of antiviral drugs such as abacavir and peramivir [2–4]. Abacavir is a carbocyclic 2′-deoxyguanosine nucleoside analogue [5] and widely used as a reverse transcriptase inhibitor in the treatment of AIDS and herpes virus infection [6,7]. Over the past few decades, (-)-γ-lactam has received increasing attention due to its role in the industrial synthesis of carbocyclic nucleotides drugs [8]. Traditionally, (-)-γ-lactam was synthesized by chemical methods [9]. Velazquez and Olivo first studied the asymmetric synthesis of (-)-γ-lactam [10]. However, the method required expensive chemical materials and it proved to be quite time-consuming. As an alternative, the enzymatic synthesis of (-)-γ-lactam has been investigated by many researchers [11–17].

(+)-γ-Lactamase is a class of enzymes which are capable of cleaving the amide bond of (+)-γ-lactam enantiomer from the racemic γ-lactam (Fig. 1) [18]. To date, several (+)-γ-lactamases-containing strains have been found [4] and from which only eight (+)-γ-lactamases have been identified and purified [3,8,13,19,20,22]. According to previous literatures [7,22], (+)-γ-lactamases can be classified into three different types based on the sequence and structural information. Type I (+)-γ-lactamases belong to the amidase family and normally contain about 500 amino acid residues. The representatives of type I (+)-γ-lactamases are those from *Sulfolobus solfataricus* [21] *and Bradyrhizobium japonicum* USDA 6 [8]. Type II (+)-γ-lactamases belong to acetamidase/formamidase family and consist of approximate 400 amino acids. Its representatives are the (+)-γ-lactamases produced by *Comamonas acidovorans* [11] and *Aeropyrum pernix* [7]. According to the protein structures, type I (+)-γ-lactamases can be divided into two regions, the core region and an unusual isolated N-terminal domain. The α-helical N-terminal domain consists of 2–53 amino acid residues and forms two α-helices and this domain is isolated from the core architecture. Whereas, type II (+)-γ-lactamases barely share this feature. Type III (+)-γ-lactamases are not the conventional amidase superfamily homologs. They belong to isochorismatase-like hydrolase (IHL) superfamily. The best and first representative is Mh33H4-5540 from *Microbacterium hydrocarbonoxydans* [23]. It stands for a new emerging group

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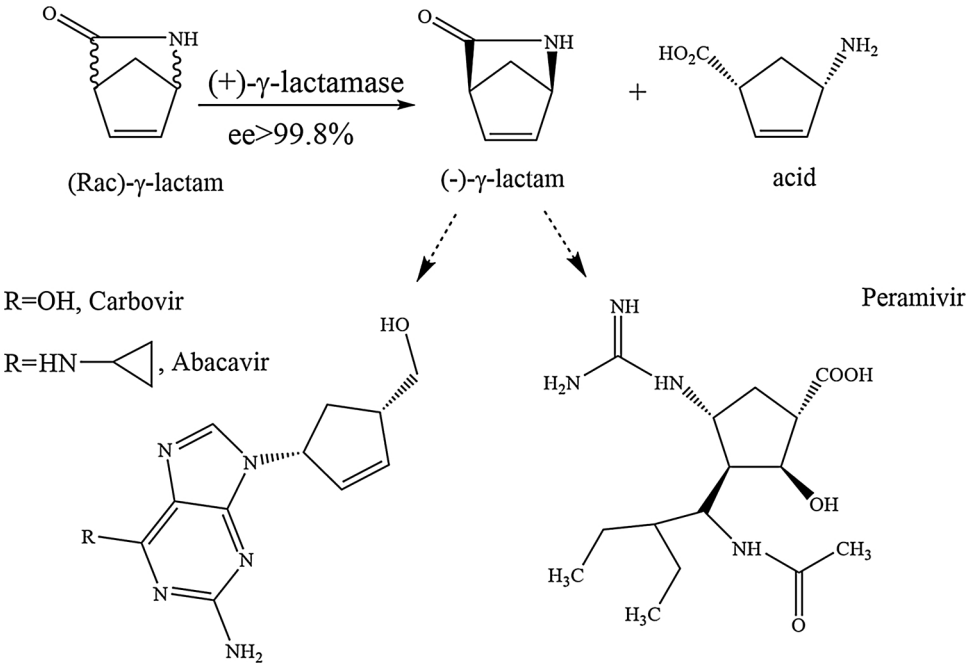

Fig. 1. The resolution of racemic γ-lactam by (+)-γ-lactamase and the application of (-)-γ-lactam.

of (+)-γ-lactamases.

In this work, a new (+)-γ-lactamase named SYJ322B5 was identified from *Bacillus thuringiensis*. SYJ322B5 belongs to the isochorismatase-like hydrolase superfamily, the same as Mh33H4-5540. SYJ322B5 was successfully expressed in *E. coli* expression system as a 6 His C-terminal tagged fusion protein and then purified to homogeneity. The activity of the enzyme was detected by high performance liquid chromatography (HPLC). Results showed SYJ322B5 can enantioselectively catalyze the bioresolution of racemic γ-lactam with a high enantiomeric excess of 99.8% and enantiomeric ratio (E) > 200. These findings indicate that this enzyme has a great practical potential for industrial production of (-)-γ-lactam.

2. Materials and methods

2.1. Chemicals, bacterial strains, plasmids, and molecular biology tools

Chemical reagents (analytical standard grade), unless stated otherwise, were purchased from Beijing Beihua Fine Chemicals Co., Ltd. (Beijing, China). *Bacillus thuringiensis* was isolated from the soil by our laboratory several years ago. The whole genome sequence can be downloaded from NCBI data bank with the accession number of NZ_CP009335.1. *B. thuringiensis* strain was cultured in lysogeny broth (LB) medium at 37 °C. *E. coli* DH5α competent cells were used as a general cloning host for plasmid preparation. *E. coli* Rosetta (DE3) competent cells were used for the heterologous expression of recombinant protein. Q5 High-fidelity DNA polymerase, T_4 DNA ligase and restriction enzymes were purchased from New England Biolab (Beverly, MA, USA). DNA sequencing demonstrated whether the recombinant plasmid was successfully constructed. The plasmid Miniprep kit and genomic DNA extraction kit were purchased from Tiangen (China). The gel extraction kit was from Qiagen (Germany). Racemic lactam was purchased from Sigma-Aldrich (Munich, Germany). Vector pET22b(+) (Invitrogen) was used for the production of recombinant protein. In order to select *E. coli* carrying recombinant plasmids, ampicillin was added to the medium at a final concentration of 50 μg mL^{-1}.

2.2. Construction of the recombinant plasmid

The genomic DNA of *B. thuringiensis* was extracted using a genomic DNA extraction kit. The gene (GeneBank No. YP_035808.1) was amplified from the genomic DNA by standard PCR reaction with the designed forward primer SYJ322B5-F-*Nde*I (5′-CGCCATATGATGAAACA AGCACTATTAATC-3′) and reverse primer SYJ322B5-R-*Xho*I (5′−CCG CTCGAGATCACGATATTGGTCATG-3′). Both *Nde*I and *Xho*I restriction sites were underlined. The amplified gene fragment was purified using a gel extraction kit and digested by *Nde*I and *Xho*I restriction enzymes, and subsequently ligated to the double-enzyme cleaved vector pET22b (+). The ligation product was transformed into the chemical competent cells of *E. coli* DH5α. Positive recombinant plasmids were confirmed by sequencing and purified using a plasmid extraction kit.

2.3. Overexpression and purification of SYJ322B5

The recombinant plasmid harboring the target gene was transformed into *E. coli* Rosetta (DE3) competent cells for overexpression. First, the cells were inoculated into a 50 mL flask with fresh LB liquid medium (containing 50 μg mL^{-1} ampicillin). After incubation at 37 °C overnight under continuous shaking at 220 rpm, cells were transferred into a 200 mL flask with fresh LB medium in the presence of antibiotic ampicillin. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium at a final concentration of 1 mM when its OD600 reached 1.0–1.2. The cells were harvested by centrifugation (6000 rpm for 10 min) after expression at 37 °C for 5 h. The cell pellet was resuspended in lysis buffer with a proper amount of powder lysozyme (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0). Sonication was used to lyse the cells and release the proteins. Insoluble cell debris was removed by centrifugation at 10,000 rpm for 60 min at 4 °C. The supernatant was applied to a nickel NTA column (The target protein was expressed with a 6 His tag at C-terminus). The column was then washed with washing buffer (50 mM Tris, 300 mM NaCl, 30 mM imidazole, pH 8.0) to remove unbound impurities. Then the protein was eluted with 10 mL of elution buffer (50 mM Tris, 300 mM NaCl, 300 mM imidazole, pH 8.0). After elution, the target protein was collected and concentrated by ultrafiltration using Millipore protein concentrator with YM10 membranes (Amicon). For