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Efficient synthesis of the intermediate of abacavir and carbovir using a novel (+)-γ-lactamase as a catalyst

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ABSTRACT

The enantiomers of 2-azabicyclo[2.2.1]hept-5-en-3-one (γ-lactam) are key chiral synthons in the synthesis of antiviral drugs such as carbovir and abacavir. (+)-γ-Lactamase can be used as a catalyst in the enzymatic preparation of optically pure (−)-γ-lactam. Here, a (+)-γ-lactamase discovered from *Bradyrhizobium japonicum* USDA 6 by sequence-structure guided genome mining was cloned, purified and characterized. The enzyme possesses a significant catalytic activity towards γ-lactam. The active site of the (+)-γ-lactamase was studied by homologous modeling and molecular docking, and the accuracy of the prediction was confirmed by site-specific mutagenesis. The (+)-γ-lactamase reveals the great practical potential as an enzymatic method for the efficient production of carbocyclic nucleosides of pharmaceutical interest.

The enantiomers of 2-azabicyclo[2.2.1]hept-5-en-3-one (γ-lactam) are extremely versatile synthons in the preparation of carbocyclic nucleoside analogues which possess therapeutic properties such as antibiotic activity and antiviral activities.[1–3] The optically pure enantiomer (−)-γ-lactam has attracted much more attentions during last two decades due to its great potential as a precursor in the synthesis of carbovir and abacavir which are active against HIV.[4,5] The hydrolyzed (+)-γ-lactam amino acid product is also used in the synthesis of pharmaceutical targets such as melogliptin[6], MK-0812[7] and piperidinium compound[8] (Scheme 1). Many methods have been exploited for the preparation of (−)-γ-lactam. The enzymatic synthesis method proved to be an efficient alternative to classical chemical method because of its high stereoselectivity and low cost.

(+)-γ-Lactamase was defined on the basis of its enantioselectivity on (*rac*)-γ-lactam. It enantioselectively catalyzed the hydrolysis of (+)-γ-lactam, and then the optical (−)-γ-lactam was obtained. The kinetic resolution of γ-lactam by (+)-γ-lactamases has been a popular method for the preparation of the optically pure (−)-γ-lactam since the first wild type strain containing (+)-γ-lactamase was screened.[9]

Up to now, only five (+)-γ-lactamases have been purified and characterized in *Escherichia coli* expression system.[10–14] In our previous report, we discovered a (+)-γ-lactamase gene from *Bradyrhizobium japonicum* USDA 6 by sequence-structure guided genome mining.[15] (+)-γ-lactamase activity was detected by high-performance liquid chromatography (HPLC). In this letter, the overexpression, purification and characterization of the (+)-γ-lactamase was reported. The active site of the (+)-γ-lactamase was studied by homologous modeling and molecular docking method. The preparation of pure (−)-γ-lactam with high yield (>49.9%) and ee value (>99.8%) was achieved.

According to the sequence (GenBank accession no. BJ6T_02120), primer P1 and P2 (Table 1) were designed for the amplification process of the (+)-γ-lactamase gene. The gene was cloned into the expression vector pET-28a with a 6×His tag at the N terminus. The recombinant protein was overexpressed in *E. coli* Rosetta under the induction of 0.08 M isopropyl-β-D-thiogalactopyranoside (IPTG). The harvested cells were sonicated and followed by Ni-affinity column chromatography and gel filtration by using a standard purification procedure. The gene encoded 504 amino acid residues with a theoretical molecular weight of 49 kDa. The relative molecular mass was about 50 kDa on native SDS–PAGE after gel filtration (Fig. 1, lane 3), which meant that the native protein appeared as a monomer.

The physical properties of the enzyme were investigated. The standard assay mixture (0.5 mL) contained 50 mM Tris–HCl buffer (pH 7.5), 4.6 mM γ-lactam and 4.8 μM purified enzyme. The activity was determined by a Daicel Chiralpak AD-H column which was eluted with a mobile phase consisting of 93% *n*-hexane and 7% isopropanol (volume ratio) at a flow rate of 0.75 mL/min. The UV absorbance of the eluted γ-lactam was measured at 230 nm. One unit of (+)-γ-lactamase was defined as the amount of enzyme

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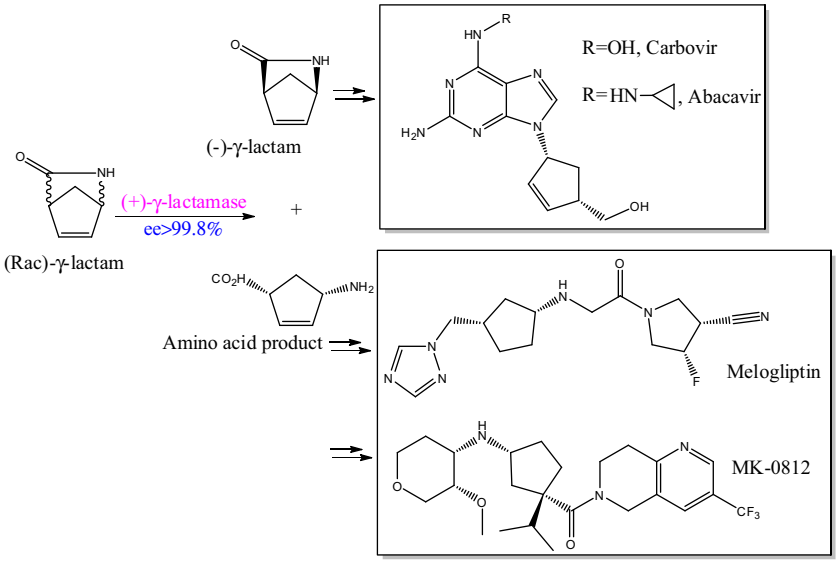

Scheme 1. Kinetic resolution of (*rac*)-γ-lactam catalyzed by (+)-γ-lactamase and corresponding hydrolyzed product used for the generation of drugs and clinical candidates.

Table 1
Primers used in this study

Name	DNA sequence (5′–3′)	Description
P1	GGAATTCCATATGACAGTTGTCCTTCCCA	Amplification upstream
P2	AAGGAAAAAAGCGGCCGCTCAATCTTCTTCCAGTC	Amplification downstream
P3	CGTCGCGCTGGCGGACAACATCATG	Cys145Ala mutation upstream
P4	CATGATGTTGTCCGCCAGCGCGACG	Cys145Ala mutation downstream
P5	ATTCGGCCGGCGGCGCGTCCTCCGGCT	Ser171Ala mutation upstream
P6	AGCCGGAGGACGCGCCGCCGGCCGAAT	Ser171Ala mutation downstream
P7	CGACCAGGGCGGCGCGATCCGCATGCCGTCTT	Ser195Ala mutation upstream
P8	AAGACGGCATGCGGATCGCGCCGCCCTGGTCG	Ser195Ala mutation downstream

required to hydrolyze 1 μmol substrate in 1 h. The optimum temperature of (+)-γ-lactamase was determined by incubating the assay mixtures at temperatures ranging from 20 to 60 °C. The enzyme exhibited highest activity at 45 °C (Fig. 2A). Thermostability studies were performed by measuring the residual activity after 1 h of pre-incubation in standard assay mixture at various temperatures (20–60 °C). After being incubated at 50 °C for 1 h, enzyme activity was quickly lost (Fig. 2B). This demonstrated that the enzyme was unstable at high temperature.

The optimum pH for (+)-γ-lactamase was measured at 45 °C. The reaction was carried out at buffer Tris–HCl pH 4.0–10.0. The purified enzyme exhibited an optimum activity at pH 8.0. Activity was almost lost at pH 4.0 (Fig. 2C). To determine the pH stability of the (+)-γ-lactamase, the enzyme was incubated in different buffers mentioned above at 30 °C for 1 h and then the residual activity was assayed under the standard conditions. The results show that the enzyme is quite stable when the pH values range from 5.0 to 10.0 (Fig. 2D).

We also tested the effects of different ions on the enzyme. From the results we found that no metal ions tested in the experiments enhanced enzymatic activity. In fact, Cu^{2+}, Zn^{2+}, and Mg^{2+} almost completely inhibited the activity (Fig. 3).

The kinetic parameters were determined by Lineweaver–Burk plot. The kinetic parameters were studied at the concentrations of the substrate ranging from 3 to 11 mM. The reaction was stopped at 10, 20, 30, 40, and 50 min at corresponding concentrations. The K_m of the enzyme was 7.08×10^{-4} M, and the V_{max} was 3.73×10^{-9} M/s.

To date, the catalytic mechanism of (−)-γ-lactamase from *Aureobacterium* sp. was elucidated by protein structure resolution.[16] It involved in the turn-over of the (−)-γ-lactam by the (−)-γ-lactamase and the formation of a stable tetrahedral intermediate at the active site which agreed with the mechanism proposed

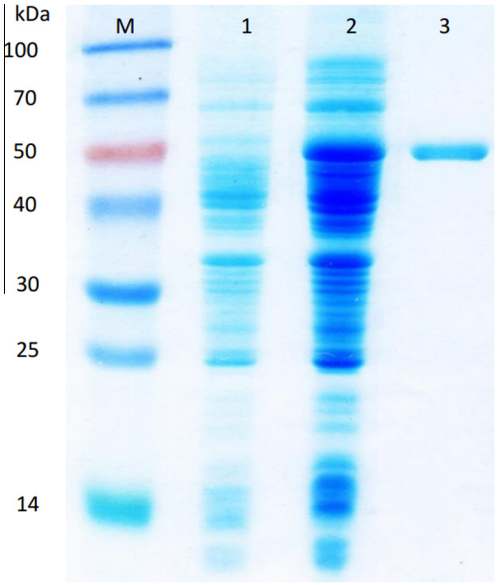

Figure 1. SDS–PAGE analysis of (+)-γ-lactamase from *B. japonicum* USDA 6. M, protein marker; lane 1, *E. coli* Rosetta (DE3) cells transformed with empty pET-28a vector; lane 2, *E. coli* Rosetta (DE3) cells transformed with pET-28a-(+)-γ-lactamase vector; lane 3, purified (+)-γ-lactamase.