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Single-step purification and immobilization of γ-lactamase and on-column transformation of 2-azabicyclo [2.2.1] hept-5-en-3-one

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ABSTRACT

We prepared two constructs of the γ-lactamase gene from *Sulfolobus solfataricus* P2 and heterogenously expressed these as two separate proteins. The first construct had His tags fused at both the N and C terminals of the γ-lactamase gene (double fusion enzyme). The second had a single His tag fused at the N terminal of the gene (single fusion enzyme). Enterokinase treatment of the two fusion enzymes produced two additional proteins. The kinetic parameters, absorption capacity on nickel-chelating agarose, and immobilization stability of the four proteins were compared. The double fusion enzyme was chosen as the on-column transformation catalyst. It was purified to electrophoretic homogeneity on nickel-chelating agarose and immobilized on the same matrix in the meantime. The optimal temperature of the immobilized enzyme was 10 °C higher than that of the free enzyme. The optimal pH was also 2.0 units higher. The immobilized enzyme maintained approximately 80% of its maximum activity at pH 5–11. The activity of the free enzyme decreased rapidly at pH values below 6.0 or above 8.0. The immobilized enzyme was used for on-column transformation reactions for 5 h each day. It retained approximately 75% of its original activity after repeated transformation cycles over a period of 30 days.

1. Introduction

γ-Lactamase (EC 3.5.2.-) is a type of amidase. Amidases (EC 3.5.1.4) cleave carbon–nitrogen bonds by transferring an acyl group to water to form free acids and ammonia. Amidase substrates include short-chain amides [1], unsaturated amides [2], cyclic amides [3], fatty acid amides [4], and amino acids [5]. Amidases are divided into two different groups based on the presence of a signature sequence. Amidases lacking the signature sequence have been isolated from *Pseudomonas aeruginosa* [6], *Brevibacterium* sp. [7], *Methylophilus methylotrophus* [8], and *Helicobacter pylori* [9]. Amidases having the signature sequence, which is characterized by a GGSS(S/G)GS sequence in a conserved stretch of about 130 amino acids at the center of the protein [10], have mainly been isolated from archaea such as *Methanococcus jannaschi* [11], *Archaeoglobus fulgidus* [12], and *Pyrococcus horikoshi* [13].

γ-Lactamase from *Sulfolobus solfataricus* MT4 has been thoroughly characterized because it is a wide spectrum signature amidase [14]. In addition to the γ-lactamase activity, the enzyme can also transform nitriles into the corresponding organic acid. The dual activity is believed to be due to the presence of a Cys-cisSer-Lys catalytic site in addition to the classical catalytic residues Ser-cisSer-Lys [15]. An interesting property of the 110-kDa dimeric enzyme is that it reversibly associates into an octamer in a pH- and temperature-dependent reaction [16]. Although γ-lactamase has been obtained in the form of single crystals by the hanging-drop method and has been studied at a low temperature using synchrotron radiation [17], the 3D structure of the protein has not yet been elucidated.

γ-Lactamase from *S. solfataricus* MT4 is a good candidate enzyme for the resolution of racemic compounds because it is easy to purify and has high thermostability and enantioselectivity [18]. Previous studies have shown that this enzyme could be used for the resolution of (*rac*)-2-azabicyclo [2.2.1] hept-5-en-3-one (γ-lactam). γ-Lactam is an important synthon in the synthesis of carbocyclic nucleosides such as the anti-HIV agent (−) carbovir [19].

In our research works, we are always trying to find some enzymatic catalysts that suit for the industrial application of the resolution of γ-lactam [20,21]. These enzymes should show high enantio-selectivity and stability. Therefore, we cloned and expressed γ-lactamase from the *S. solfataricus* P2 strain, which is closely related to the *S. solfataricus* MT4 strain, because the archaeal enzymes often show excellent thermo-stability. In the mean time, we also attempt to develop a simple, efficient and

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feasible transformation method for large-scale production of the substrate.

In this paper, we developed a method by which the double fusion enzyme was immobilized on the Ni-NTA agarose matrix during purification steps. By using this method the time for the enzyme immobilization was shorten, which probably means cost reduction in industrial application. We found that the double fusion enzyme which had His tags fused at both the N and C terminals was immobilized well on the Ni-NTA agarose matrix, and the immobilized enzyme was more stable than the free fusion enzyme in terms of both temperature and pH stability. The immobilized enzyme retained most of its activity and had a relatively constant efficiency when used for on-column transformations. In addition, γ-lactamase showed absolute enantioselectivity to (+)-γ-lactam. In conclusion, we believe that on-column transformation with the double fusion enzyme, reported in this paper, is a promising method for the industrial preparation of (−)-γ-lactam.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture media

Escherichia coli DH5α cells were used for the cloning studies. *E. coli* rosetta (DE3) cells were used for protein expression. pET30a (+) (Invitrogen, California, USA) was used for standard cloning and expression in *E. coli*. The *E. coli* strains were routinely grown in LB medium or on LB agar plates at 37 °C. To select bacterial strains carrying the appropriate recombinant plasmids, 35 μg chloramphenicol ml^{-1} or 50 μg kanamycin ml^{-1} was added to the medium.

2.2. Extraction and purification of DNA

Plasmids were isolated using the TIANprep Mini Kit (Tiangen, Beijing, PR China) according to the protocol provided by the manufacturer. DNA was purified using the TIANgel Midi Kit (Tiangen, Beijing, PR China) according to the manufacturer's instructions.

2.3. Cloning of γ-lactamase

The primers were designed based on the genomic DNA sequence annotation of *S. solfataricus* P2 deposited in GenBank (AE006849).

The sense primer (Ssg fwd) was 5′-CATGCCATGGGAATTAAGTTACCCAC-3′; the *Nco*I site is underlined.

The antisense primer (Ssg rwd) for the single fusion enzyme (Fig. 1, protein 3) was 5′-CCTCGAG***TTA***TTTTTTGATTCTCTCAAATAC-3′; the termination codon is shown in bold italics, and the *Xho*I site is underlined.

The antisense primer (Ssgnt rwd) for the double fusion enzyme (Fig. 1, protein 1) was 5′-CCTCGAGTTTTTTGATTCTCTCAAATAC-3′; the *Xho*I site is underlined.

PCR was performed with the Red-*Pfu* DNA Polymerase (Biocolors, Beijing, China). The thermal cycling program was as follows: an initial cycle of predenaturation at 94 °C for 5 min (1st step), followed by 30 cycles of denaturation at 94 °C for 1 min (2nd step), annealing at 53 °C for 1 min (3rd step), and extension at 72 °C for 3 min. This was followed by a final extension at 72 °C for 10 min. The PCR-amplified γ-lactamase gene was inserted into the *Nco*I and the *Xho*I restriction sites of the pET30a vector. Primers Ssg fwd and Ssg rwd were used to construct the single fusion enzyme gene into pET30a vector (protein 3 gene, Fig. 1), while primers Ssg fwd and Ssgnt rwd were used to construct the double fusion enzyme into pET30a vector (protein 1 gene, Fig. 1).

2.4. Expression of γ-lactamase in E. coli rosetta (DE3) cells

The constructs were transformed into *E. coli* rosetta (DE3) cells and grown overnight at 37 °C. Stock culture (5 ml) was transferred into fresh 500-ml culture medium and grown until the optical density at 580 nm (OD 580) was 0.8. Isopropylthiogalactoside (IPTG) was then added to a final concentration of 1.0 mM, and after incubation for another 2 h, the cells were harvested by centrifugation (4000 × *g* for 10 min).

2.5. Single-step purification and immobilization of the γ-lactamase fusion proteins on a nickel (Ni)-chelating column

Purification of the γ-lactamase fusion protein on the Ni-chelating column was carried out using the protocol and buffer supplied by Novagen (New Jersey, USA). Cells from the expression culture (500 ml) were suspended in 40 ml binding buffer (50 mmol l^{-1} NaH_2PO_4, 300 mmol l^{-1} NaCl, and 10 mmol l^{-1} imidazole, (pH 8.0)) and sonicated. The supernatant was applied to a 1-ml Novagen His

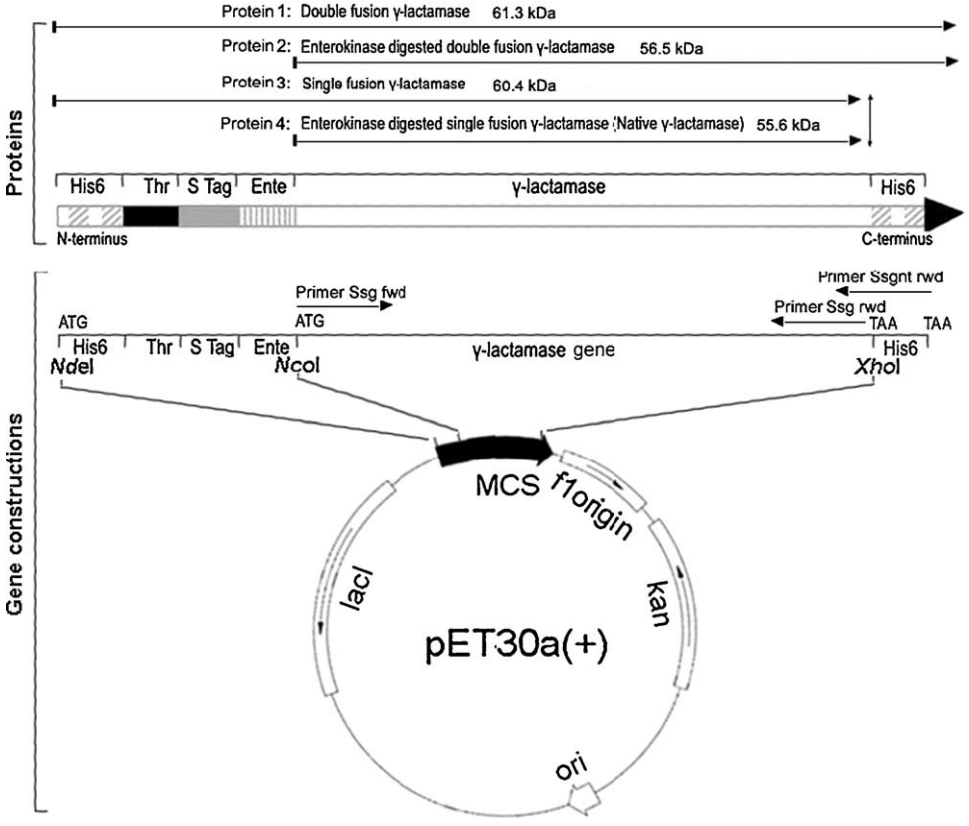

Fig. 1. Construction of different γ-lactamase genes in the pET30a (+) plasmid and the proteins expressed by these constructs. Protein 1: Double fusion enzyme, γ-lactamase with His tags fused to both the N and C terminals. Protein 2: Enterokinase-digested double fusion enzyme. Protein 3: Single fusion enzyme, γ-lactamase with a His tag fused at the N terminal. Protein 4: Enterokinase-digested single fusion enzyme (native γ-lactamase).