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Effects of catalytic site mutations on active expression of phage fused penicillin acylase

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ABSTRACT

Penicillin G acylase (EC 3.5.1.11) is 86-kDa large heterodimeric protein comprising two peptide A 23-kDa and peptide B 62-kDa, produced by intein-mediated auto-splicing of a 92-kDa precursor. Since penicillin G acylase was potentially employed in the preparation of a wide range of semi-synthetic β-lactam antibiotics from acyl side-chain precursors and β-lactam nucleus, directed evolution of penicillin acylase using phage display technology for extending its novel specificity is an interesting topic both of industry and academic. We fused the penicillin acylase to fd phage coat protein III and used pIII secretion signal sequence instead of penicillin acylase, which coupled gene and enzyme on phage particle and will be useful for directed evolution of penicillin acylase. Western blotting and enzyme activity assay were performed to demonstrate penicillin acylase has been functionally displayed on phage surface. Owing to the intimate association of enzyme activity and precursor processing in penicillin acylase, alterations of protein residues to make a phage library should be careful not to lead to processing defects. By site-directed mutagenesis, we have then identified effect of Ser B1 and Asn B241 variants on post-translational maturation of phage fused penicillin acylase.
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1. Introduction

Penicillin G acylase (EC 3.5.1.11) was originally used to catalyze the hydrolysis of natural penicillins to yield 6-amino-penicillanic acid (6-APA) as the common nucleus of a range of semi-synthetic penicillins (Shwale et al., 1990). It was further used in kinetically controlled synthesis of a few of β-lactam antibiotics from acyl side-chain precursor (an ester or amide) and β-lactam nucleus (Kasche, 1986). However, it was a major challenge for the industrial implementation of the enzymatic synthesis of more beta-lactam antibiotics to extend penicillin G acylases to novel specificity (Roa et al., 1994; Jager et al., 2008).

Directed evolution of enzymes has proved to be highly efficient way to optimize an enzyme to perform desired catalytic function. This process at its fundamental level involves three major steps. Protein expression is the first step which involves expressing high levels of stable and functional enzymes using recombinant gene technology. Making a library is the second step which involves generating one or more genetic changes in a population of gene sequence. Screening proteins with evolved function is the third step which involves determining which mutated enzyme performs better catalytic functions than before genetic changes were made. The process of directed evolution is typically iterative, where improved genes with their encoded enzymes are further evolved to perform the desired function at an even higher level (Brakmann, 2001).

Phage display provides a protein expression method for the directed evolution of enzyme. Initially this technique has been extensively used for the isolation of binding proteins and their encoding genes from large libraries. By selecting for improved binding to transition-state analogs, modest improvements have been obtained for catalytic antibodies displayed on phage. Specialized selections using reactive substrates, inhibitors, active site ligands, and reactive products have also been used in attempts to select for improved catalysts (Hoess, 2001).

Recently, penicillin acylase has become the focus of interest from the view point of fundamental enzymology since it was recognized as a new protein structure superfamily called Ntn-hydrolyase (N-terminal nucleophile) (Brannigan et al., 1995; Oinone and Rouvine, 2000; Virden, 1990). Proteasomes, glutamine 5-phosphoribosyl-pyrophosphate amindotransferase, glutarylamidase (cephalosporin acylase), aspartylglucosaminidase, etc. have been categorized into this family with penicillin acylase. Although the amino acid sequence homology is almost completely absent, the common fold of this superfamily shares a four-layered αββα-core structure. All of proteins in this family go through intein-mediated auto-splicing from inactive precursor and gen-

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Table 1
List of oligonucleotide primers.

YE 1	5′-AGC TTA CCT G GTGCAC AG GAG CAG TCG TCA AGT GAG-3′
YE 2	5′-GGT CGG TAC C GCGGCCGC ACG CCC CTC AAT CCC TCT-3′
BN241X1	5′-GTC GGG ATA TAT TGC TAA CTG G NNK AA TTC TCC-3′
BN241X2	5′-GGA GAA TT MNN C CAG TTA GCG ATA TAT CCC GAC-3′
BW240C1	5′-CGG GAT ATA TTG CTA AC TGT A ACA ATT CTC CCC-3′
BW240C2	5′-GGG GAG AAT TGT T ACA GT TAG CAA TAT ATC CCG-3′

erate an active N-terminal nucleophile at the junction of two anti-parallel β-sheets as single catalytic residue. Structural comparison of Ntn-hydrolases revealed similar catalytic machinery, however, the substrate binding and oxyanion hole varied partially.

According to the determination of crystal structure and catalytic mechanism of penicillin G acylase, it is 86-kDa large heterodimeric protein comprising two peptide A 23-kDa and peptide B 62-kDa, produced by intein-mediated auto-splicing of a 92-kDa precursor. Mechanism of penicillin G acylase-catalyzed reaction suggested that the catalytic residue is the N-terminal serine of B subunit which attacks the carbonyl of the peptide bond. The oxyanion that is formed in the transition state is stabilized by BN241 and BA69. After collapse of the transition state, the acyl-enzyme is formed, which can be cleaved by water or a different nucleophile yielding the acid or the condensation product (Duggleby et al., 1995; McVey et al., 2001). However, there is a different mechanism for post-translational processing, in which BS1 is mutated to BC1, the precursor is processed but there is no enzymatic activity (Choi et al., 1992). The challenge for phage display of penicillin acylase is whether this large protein goes through post-translational processing and becomes enzymatic activity after making mutant libraries. Since phage display is powerful protein expression tool for directed evolution of penicillin acylase in vitro (Verhaert et al., 1999), we reported penicillin G acylase from *Escherichia coli* was functionally displayed on phage surface. To explore the limits of post-translational processing to phage display of penicillin acylase mutations, we made mutants of the active site BN241 and investigated its effect on intein-mediated post-translational processing.

2. Materials and methods

2.1. Cloning and expression of penicillin acylase on phage

Penicillin acylase gene in chromosomal DNA extracted from *E. coli* Migula 1895 (encoding penicillin acylase gene as same as ATCC 11105) was amplified by Expand High Fidelity PCR system (Roche) using the two primers YE1 and YE2 (Table 1) with Alw441 and Not1 cloning sites respectively (restriction site are underlined). The generated fragment containing penicillin acylase gene was then cloned into the phage display vector fd-Dog carrying tetracycline resistance by fusion to the coat protein pIII. *E. coli* TG1 (Stratagene) was used as host cell for recombinant plasmid and gene expression. The penicillin acylase phages were produced in *E. coli* TG1 in LB medium containing Tetracycline (7 μg/ml final concentration) at 23 °C 2 days under stirring. After centrifugation for 10 min at 5000 rpm (3800 × *g*), the cell pellet was discarded and phages from supernatant were precipitated by addition of 1/5 volume of PEG/NaCl (20% (v/v) PEG 6000, 2.5 M NaCl) and incubation at 4 °C for 1 h. The precipitated phages were centrifuged at 10,000 rpm (15,000 × *g*) for 30 min and the phage pellet was resuspended in PBS buffer. The solution was then filtered through a 0.44 μm filter (Millipore) to remove the remaining cell debris. The filtrated solution was precipitated with PEG/NaCl and the resulting phages were resuspended in PBS buffer. The concentration of phage was measured by monitoring the absorbance at 265 nm with a Beckman spectrometer (estimated phage concentration (number/ml) = 6.0×10^{12} × absorbance value at 265 nm).

2.2. Site-directed mutagenesis

Mutant penicillin acylase phage encoding mutant gene at B:241 (using Primer BN241X1 and BN241X2 (Table 1)) and B:240 (using Primer BW240C1 and BW240C2) was generated by PCR mediated standard procedure using Stratagene Quick Change XL Site-Directed Mutagenesis Kit. The nucleotide sequence of the cassette was confirmed by double standard DNA sequencing to ensure the mutant genes.

2.3. SDS-PAGE and Western blotting

About 10^{12} phages were denatured by boiling for 5 min in the presence of SDS-page sample buffer. The 25 μl samples were loaded on a SDS-10% (w/v) polyacrylamide gel and separated in BioRad Mini-Protein 3. After electrophoresis, the proteins were transferred onto nitro-cellulose membrane (Amersham Phamacia) using Bio-Rad Trans-Blot semi-Dry. The proteins were detected with a rabbit anti-pIII antibody (Mo Bi Tech) and anti-penicillin acylase antibody followed by anti-mouse and anti-rabbit antibody IgG conjugated with HRP (Amersham Phamacia). The antibodies were diluted by 1:1000 in PBS buffer containing 1% (w/v) dried milk. Then it was dyed by Peroxidase Stain Kit for Immuno-blotting (Nacalai Tesque, Inc.).

2.4. Assay of catalytic activity

For the determination of kinetic parameters of K_m and k_{cat}, the conversion of 2-nitro-5-[(phenyl-acetyl)amino]benzoic acid (NIPAB) (Sigma) were determined by measuring initial velocities at different substrate concentration. The hydrolysis of NIPAB was monitored by measuring the increase in absorbance at 400 nm, in Beckman U640 spectrophotometer. All measurements were performed by purified penicillin acylase phage at 25 °C in 50 mM potassium phosphate buffer, pH 7.0. Rates were calculated by using molar extinction coefficient of 5-amino-2-nitrobenzoic acid which is 9.09 mM^{-1} cm^{-1}.

3. Results

3.1. Functional display of penicillin acylase on phage

Penicillin acylase from *E. coli* ATCC 11105 is 86-kDa large heterodomic protein comprising two peptide A 23.8-kDa and peptide B 62.2-kDa, produced by intein-mediated auto-splicing of a 92-kDa precursor (Hewitt et al., 2000; Kasche et al., 1999; Xu et al., 1999). We fused the penicillin acylase coding sequence (A subunit–intein–B subunit) to the gene of fd phage coat protein III, using pIII secretion signal sequence instead of penicillin acylase. In order to verify active penicillin acylase has been displayed on phage, we tested SDS-PAGE and Western blotting and analyzed the kinetics using purified penicillin acylase phage. When western blotting using monoclonal anti-pIII antibody, besides a major band corresponding to free pIII at 62-kDa (Gray et al., 1981) a higher molecular weight band corresponding to penicillin acylase B subunit fused pIII was observed (Fig. 1). Western blotting detection with multiclonal anti-penicillin acylase antibodies showed 62-kDa band of mature penicillin acylase subunit B (Figure 2).

Measurement of NIPAB hydrolysis is a standard method of penicillin acylase kinetics (Svedas et al., 1997; Alkema et al., 1999). The value for k_{cat} and K_m of NIPAB hydrolysis by penicillin acylase phages were determined using classic initial rates measurements at various NIPAB concentrations (Table 2). Comparing penicillin acylase kinetic parameters found phages ($k_{cat} = 15\ s^{-1}$ and $K_m = 30\ \mu m$) as same order as *E. coli* (Alkema, 1999) ($k_{cat} = 16.2\ s^{-1}$ and $K_m = 11.3\ \mu m$), which indicated at least one penicillin acylase

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