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Efficient enzymatic synthesis of ampicillin by mutant *Alcaligenes faecalis* penicillin G acylase

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ABSTRACT

Semi-synthetic β-lactam antibiotics (SSBAs) are one of the most important antibiotic families in the world market. Their enzymatic synthesis can be catalyzed by penicillin G acylases (PGAs). In this study, to improve enzymatic synthesis of ampicillin, site-saturating mutagenesis was performed on three conserved amino acid residues: βF24, αR146, and αF147 of thermo-stable penicillin G acylase from *Alcaligenes faecalis* (Af PGA). Four mutants βF24G, βF24A, βF24S, and βF24P were recovered by screening the mutant bank. Kinetic analysis of them showed up to 800-fold increased k_{cat}/K_m value for activated acyl donor D-phenylglycine methyl ester (D-PGME). When βF24G was used for ampicillin synthesis under kinetic control at industrially relevant conditions, 95% of nucleophile 6-aminopenicillanic acid (6-APA) was converted to ampicillin in aqueous medium at room temperature while 12% process time is needed to reach maximum product accumulation at 25% enzyme concentration compared with the wild-type Af PGA. Consequently, process productivity of enzymatic synthesis of ampicillin catalyzed by Af PGA was improved by more than 130 times, which indicated an enzyme viable for efficient SSBAs synthesis.

1. Introduction

β-Lactam antibiotics are wildly used anti-infection drugs in the world. Semi-synthetic β-lactam antibiotics (SSBAs) are the most important family of β-lactam antibiotics (Chandel et al., 2008; Parmar et al., 2000; Peñalva et al., 1998). They were produced by the coupling of the acyl side chain with β-lactam moiety like 6-APA (Bruggink et al., 1998). Chemical syntheses of SSBAs were developed since early 1960s. Nowadays, they have dominated the industrial production of SSBAs for high yield obtained (Wegman et al., 2001). But the factors like harsh reaction conditions, complicated process steps, and large volume of organic solvent needed, make them environmental unsustainable (Bruggink et al., 1998; Sheldon, 2008). Enzymatic synthesis of SSBAs is an environmental friendly alternative and mainly catalyzed by penicillin G acylase (PGA: EC 3.5.1.11) and less-studied α-amino ester hydrolase (AEH: E.C. 3.1.1.43) (Wegman et al., 2001). Enzymatic synthesis of SSBAs can be carried out under thermodynamic control, with a non-activated acyl side chain, or kinetic control (Fig. 1), which utilizes an activated side chain (mainly an ester or amide). In general, kinetically controlled synthesis provides higher yield (40–60%), but still relatively low in comparison with the traditional chemical method (Bruggink et al., 1998; Sheldon, 2008). Although productivity of this process can be improved by adding water-miscible organic solvents, excess acyl donor and high substrate concentration (Aguirre et al., 2006; Deaguero and Bommarius, 2014; Illanes et al., 2007; Kallenberg et al., 2005), and kinetic parameters of enzymes are the most important since low yield can be mainly ascribed to enzyme-catalyzed initial hydrolysis of the activated acyl donor and the secondary hydrolysis of antibiotic product (Marešová et al., 2014). Ideally, PGA used in this process should have high catalytic activity for its unnatural substrate: activated acyl donor, high *S*/*H* ratio (initial speed of antibiotic product synthesis to initial

Abbreviations: SSBA, semi-synthetic β-lactam antibiotic; PGA, penicillin G acylase; Af PGA, *Alcaligenes faecalis* PGA; D-PGME, D-phenylglycine methyl ester; 6-APA, 6-aminopenicillanic acid; AEH, α-amino ester hydrolase; 7-ADCA, 7-aminodesacetoxycephalosporanic acid; *S*/*H*, synthesis to hydrolysis; PAA, phenylacetic acid; Ec PGA, *Escherichia coli* PGA; NIPAB, 2-nitro-5-[(phenylacetyl) amino]-benzoic acid; D-PG, D-phenylglycine; D-PGA, D-phenylglycinamide; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

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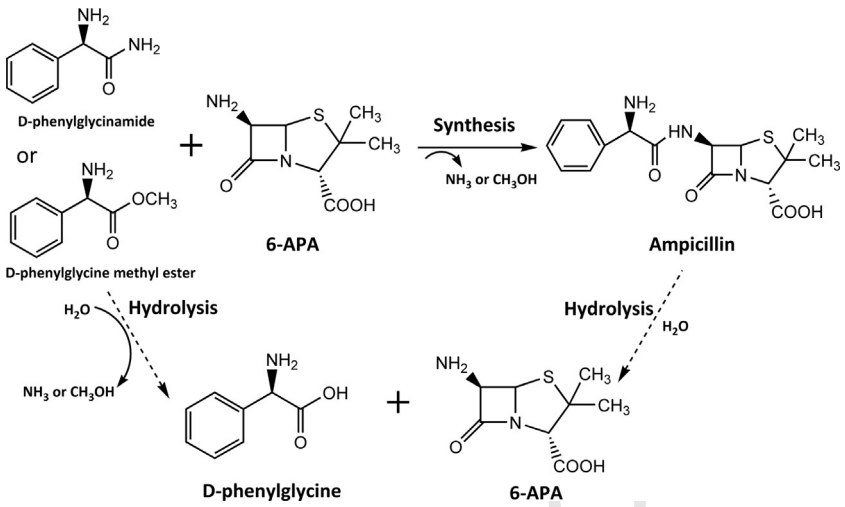

Fig. 1. Scheme for enzymatic synthesis of ampicillin under kinetic control. Undesired side reactions: hydrolysis of D-phenylglycine methyl ester to D-phenylglycine and hydrolysis of ampicillin to 6-APA (6-aminopenicillanic acid) and D-phenylglycine are shown.

speed of byproduct free acid formation), and low activity for antibiotic product. However, for natural PGAs, relatively low *S*/*H* ratio is observed and k_{cat}/K_m values of them for activated acyl donor are usually 10 times lower than those of antibiotic product (Alkema et al., 2002a; Alkema et al., 2002b; Bečka et al., 2014; Gabor et al., 2005). Moreover, they are prone to be inhibited by residual phenylacetic acid (PAA) in substrate 7-aminodesacetoxycephalosporanic acid (7-ADCA) and 6-APA (about 0.3–0.5%, w/w) (Alkema et al., 2002a; Švedas et al., 1997).

Protein engineering is commonly used to tailor wild-type enzymes for industrial purposes (Bornscheuer et al., 2012). But until now, limited progress has been made to improve PGA-catalyzed kinetically controlled synthesis of SSBAs (Deaguero, 2011). To our knowledge, this could be ascribed to several reasons. Firstly, the participation of amino acid residues close to the active site in autoproteolytic activation process of PGAs (Done et al., 1998; Hewitt et al., 2000; McVey et al., 2001). Usually, amino acid residues close to the active site, like βPhe24, αArg145, and αPhe146 of *Escherichia coli* PGA (Ec PGA), were selected because of assumed interaction with substrate and participation in reaction process. But mutations on these sites might lead to inefficient protein expression. Secondly, reliable high-throughput screening methods lack (Jager et al., 2008). It is difficult to screen PGA libraries for mutants with improved SSBAs synthesis since proper sampling time is hard to be determined for the transient maximum product accumulation, different expression and activity level of mutant enzymes. Moreover, higher *S*/*H* ratio of reported mutant enzymes were usually gained at the expense of catalytic activity for acyl donor (Alkema et al., 2002a; Alkema et al., 2002b; Gabor and Janssen, 2004; Jager et al., 2007; Jager et al., 2008). For example, both rational and combinatorial designs were performed on the most-studied Ec PGA, but low k_{cat}/K_m values for D-PGA or D-PGME were usually observed for the selected mutant enzyme (Alkema et al., 2002a; Alkema et al., 2002b; Jager et al., 2008). The similar results have been reported by semi-random mutagenesis on penicillin acylase PAS2 and DNA shuffling of several PGAs (Jager et al., 2007; Gabor and Janssen, 2004). Also, a majority of mutant enzymes gained by site-directed mutations of PGA from *Bacillus megaterium* showed decreased synthetic activity (Wang et al., 2007). Moreover, to synthesize diastereomerically pure ampicillin from racemic PGME, mutagenesis of Ec PGA aimed at high selectivity for D-PGME was also performed (Deaguero et al., 2012).

Af PGA is one of the three frequently used PGAs for industrial production of SSBAs under kinetic control (Marešová et al., 2014). Af PGA was in homology with the commonly used Ec PGA while some unique properties were observed (Braiuca et al., 2003; Švedas et al., 1997; Verhaert et al., 1997). For example, a unique intramolecular disulfide bridge makes Af PGA more thermo-stable (Verhaert et al., 1997). Until now, few protein engineering was performed on Af PGA to improve SSBAs synthesis. And recently, crystallographic structures of Af PGA have been reported (Varshney et al., 2012), but with low resolution (3.5 Å for PDB 3ML0.ENT and 3.3 Å for PDB 3K3 W.ENT). This prevents the intimate study of spatial arrangement and residues interactions around the active site and makes rational design of Af PGA difficult (Varshney et al., 2012; Tishkov et al., 2012). Consequently, in this study, three conserved amino acid residues close to the active site: βF24, αR146, and αF147 of Af PGA were selected for site-saturating mutagenesis. After two rounds of screening, four mutants were selected and purified. Their kinetic properties were investigated. The process of ampicillin synthesis at industrially relevant conditions was also studied later.

Table 1
Primers used for site-saturation mutagenesis and his tag adding.

Primers	Sequence (5′→3′)[a]	Restriction sites
αR146 forward	TTGG GTC GGG TCT ATG GCT AAT NNK TTC TCC GAC ACG AAT CTG GAAG	–
αR146 reverse	CTTC CAG ATT CGT GTC GGA GAA MNN ATT AGC CAT AGA CCC GAC CCAA	–
αF147 forward	GTC GGG TCT ATG GCT AAT CGC NNK TCC GAC ACG AAT CTG GAA GTGA	–
αF147 reverse	TCAC TTC CAG ATT CGT GTC GGA MNN GCG ATT AGC CAT AGA CCC GAC	–
βF24 forward	CTG ATC AAT GGC CCG CAG NNK GGC TGG TAC AAC CCG GCT	–
βF24 reverse	AGC CGG GTT GTA CCA GCC MNN CTG CGG GCC ATT GAT CAG	–
His-tag forward	CATG CC ATG GTG AAA GGG CTG GTT CGT ACAG	NcoI
His-tag reverse	CCCAAGCTTCTAGTGATGGTGATGGTGATG AGGCTGAGGCTGAATCAAAAGC	HindIII

[a] N = A, G, C, T (equivalent amounts); K = G, T (equivalent amounts); and M = A, C (equivalent amounts).

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