Enzyme and Microbial Technology 56 (2014) 1–7

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

A new role for penicillin acylases: Degradation of acyl homoserine lactone quorum sensing signals by *Kluyvera citrophila* penicillin G acylase

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ARTICLE INFO

Article history:
Received 14 June 2013
Received in revised form 5 December 2013
Accepted 11 December 2013
Available online 19 December 2013

Keywords:
*Kc*PGA
Quorum quenching
Acyl homoserine lactones
CV026

ABSTRACT

Use of penicillin acylases for the production of semi-synthetic penicillins is well-known. *Escherichia coli* penicillin G acylase (*Ec*PGA) has been extensively used for this purpose; however, *Kluyvera citrophila* penicillin G acylase (*Kc*PGA) is assumed to be a better substitute, owing to its increased resilience to extreme pH conditions and ease of immobilization. In the present article we report a new dimension for the amidase activity of *Kc*PGA by demonstrating its ability to cleave bacterial quorum sensing signal molecules, acyl homoserine lactones (AHL) with acyl chain length of 6–8 with or without oxo-substitution at third carbon position. Initial evidence of AHL degrading capability of *Kc*PGA was obtained using CV026 based bioassay method. Kinetic studies performed at pH 8.0 and 50 °C revealed 3-oxo-C6 HSL to be the best substrate for the enzyme with V_{max} and K_m values of 21.37 + 0.85 mM/h/mg of protein and 0.1 + 0.01 mM, respectively. C6 HSL was found to be the second best substrate with V_{max} and K_m value of 10.06 + 0.27 mM/h/mg of protein and 0.28 + 0.02 mM, respectively. Molecular modeling and docking studies performed on the active site of the enzyme support these findings by showing the fitting of AHLs perfectly within the hydrophobic pocket of the enzyme active site.

1. Introduction

Penicillin acylases (PAs) have been recognized as enzymes of tremendous industrial importance for more than 50 years now. PAs provide us with a greener route to obtain the essential beta-lactam (6-APA) nucleus using which various semi-synthetic penicillins are prepared (Scheme 1(i)). Penicillin acylases belong to the structural super family of N-terminal nucleophilic hydrolases (Ntn-hydrolases). They are classified into different groups based on the substrate specificity shown by the enzyme. Many organisms have been implicated in the production of both penicillin G and penicillin V acylase, including bacteria, fungi and actinomycetes. In the recent past penicillin G acylase produced by *Kluyvera citrophila* (*Kc*PGA) has received more attention compared to *Escherichia coli* PGA (*Ec*PGA) due to its numerous industrial process-friendly properties, namely increased resilience to harsh conditions and ease of immobilization [1].Like other PGAs, *Kc*PGA is also produced as a pro-peptide which auto-proteolytically gets cleaved into an α and β chains of 209 and 555 amino acid residues, respectively, which then eventually forms a heterodimer [2]. Although the mechanism of action of PGA for commercial use is widely understood, much uncertainty surrounds its in vivo role in the organism. It is proposed that PGA may function during the free-living mode of the organism to degrade aromatic molecules like phenyl acetylated compounds and generate carbon source.

In the present study a novel and very fascinating property of *Kc*PGA has been highlighted which may alter our present perception of the ultimate application of this enzyme and its tangible role in nature. The present report talks about the ability of this enzyme to cleave bacterial communication signal molecules named as acyl homoserine lactones (AHLs) with great efficiency (Scheme 1(ii)). Single cell bacteria have lately been shown to communicate with their own kind using small diffusible signal molecules in a density dependent manner and this phenomenon has been recognized as quorum sensing (QS) [3].Quorum sensing based transcription regulation has been shown to regulate a variety of important cellular functions like mating, virulence against the host, antibiotic production, competence development and range of other phenomenon. AHLs, the largest and the most well characterized class of signal molecules, mediate communication amongst Gram negative

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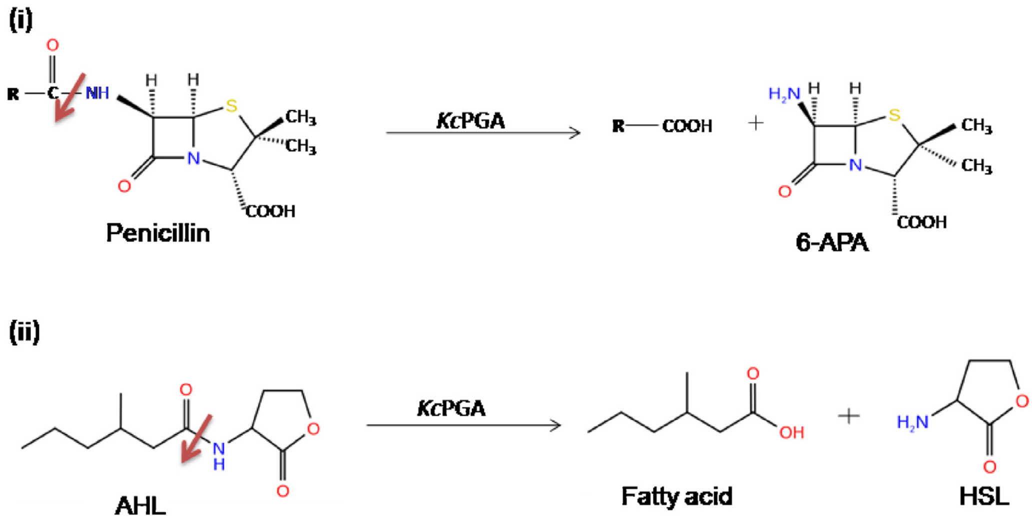
http://dx.doi.org/10.1016/j.enzmictec.2013.12.010



Scheme 1. Reaction catalyzed by *Kc*PGA: the red arrow shows the site of action of enzyme. (i) A schematic representation of penicillin deacylation by *Kc*PGA (ii) A shematic representation of the digestion of AHLs by *Kc*PGA as reported in this manuscript. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bacteria and a diminished accumulation of these molecules impedes intercellular communication leading to a state of communication blackout. This forms the basis of the phenomenon known as quorum quenching (QQ) [4]. The quorum quenching approach aims at reducing the expression of quorum sensing mediated phenotypes without any other damaging influence on the producer organism. Thus, quorum quenching has been touted to be the future in the development of sustainable antibacterial therapeutics, since there is a decidedly reduced propensity for development of bacterial resistance against this approach considering that it does not impose any 'life or death' selective pressure on the target organism [5].

Of the many probable ways to quench quorum sensing, enzymatic methods have received increasing amounts of attention since the year 2003 [6]. Two types of enzymes that have been shown to degrade acyl homoserine lactones are AHL lactonases and AHL acylases. Several AHL lactonases have been discovered and studied in detail, but in comparison AHL acylases have been a less studied cluster. AHL acylases show great degree of similarity with Ntn-hydrolase family of enzymes. PvdQ is one of the best characterized AHL acylase obtained from *Pseudomonas aeruginosa* and active only against long chain AHLs, shows 24% similarity with *Ec*PGA [7]. Analysis of crystal structure of PvdQ AHL acylase shows the presence of αββα core structure which is a characteristic of all members of Ntn-hydrolase superfamily, including *Kc*PGA. AHL acylases reported to date differ greatly in their substrate specificities acting exclusively against one type or group of similar types of molecules [8]. In the present report AHL degrading capability of a well-recognized penicillin G acylase, *Kc*PGA has been shown for the first time. *Kc*PGA specifically degrades medium chain length AHLs (C6–C8) efficiently, which has been validated by using chemical, biological, and in-silico approaches.

2. Materials and methods

2.1. 2.1Culture conditions used for the microorganisms

Chromobacterium violaceum tn5 mutant CV026, obtained as kind gift from Dr. Paul Williams, University of Nottingham, was grown at 30 °C in Luria-Bertani broth supplemented with 100 μg/ml ampicillin and 30 μg/ml kanamycin.

2.2. Cloning, over expression and purification of enzyme KcPGA

A 2562 bp gene fragment covering the region from 12 nucleotides upstream from the start codon and 12 nucleotide downstream of pac gene was amplified from chromosomal DNA of *K. citrophila* DMSZ 2660 (ATCC 21285) by gene (Accession No-M15418) specific primers using components from KOD polymerase kit and generalized PCR conditions. The amplified PCR product with desired restriction site near the ends was ligated to expression vector pET26b (+) and protein was expressed in BL21 DE3 pLysS. Purification of expressed recombinant C-terminal histidine tagged *Kc*PGA, was performed by affinity chromatography using Ni+- Sepharose beads followed by gel filtration on Sephacryl S-200. The fractions containing *Kc*PGA protein were checked for the presence of PGA activity using the modified method of Shewale et al. [9] which is based on colorimetric assay proposed by Bombstein and Evans [10]. Purity of the positive fractions was checked on SDS PAGE. Details of cloning, overexpression, crystallization, and preliminary X-ray analysis of *Kc*PGA is reported [11].

2.3. Deacylation of N-acyl homoserine lactone by KcPGA (HSL-OPA assay)

This assay was used to determine the amidohydrolase activity of *Kc*PGA against AHLs. Free amino acid in the form of the HSL moiety released during the deacylation reaction was estimated using *o*-phthaldialdehyde (OPA, Sigma–Aldrich, India) in 0.1 M Na-borate. OPA stock was prepared by adding 4 mg of OPA in 0.1 ml of ethanol with 5 mg of dithiothreitol (DTT) dissolved in 4.9 ml of 0.1 M Na-borate buffer pH 9.0. OPA (*o*-phthaldialdehyde) is a primary amine-reactive fluorescent detection reagent. Deacylation of AHL compounds with *Kc*PGA results in the formation of HSL, which can be detected following the reaction of its primary amine group with OPA. AHL-degrading product was immediately mixed with 100 μl of *o*-phthaldialdehyde solution and then the mixture was incubated for 2 min at 25 °C to prepare the fluorescent derivative of the released HSL. The quantitative estimate of HSL released as a result of AHL degradation reaction is estimated by measuring the OD of fluorescence at 340 nm, which is proportional to HSL released. A standard plot of pure HSL (Sigma) in the concentration range of 0.1–1 mM was plotted for calculating the amount of HSL released after enzymatic degradation.