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The cloning and characterization of one novel metagenome-derived thermostable esterase acting on *N*-acylhomoserine lactones

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ABSTRACT

A novel gene (designated as *est816*) encoding an esterase was isolated from a Turban Basin metagenomic library with a functional screening method. Sequence analysis revealed that *est816* encoded a protein of 271 amino acids with a predicted molecular mass (Mr) of 29.9 kDa and was expressed in *Escherichia coli* BL21 (DE3) in soluble form. The optimum pH and temperature of the recombinant Est816 were 7.5 and 60 °C, respectively. The enzyme was stable in the pH range of 5.0–9.0 and at temperatures below 50 °C. The residual activity of Est816 was 47.7% when stored at 25 °C for 5 months. The enzyme could hydrolyze a wide range of ρ-nitrophenyl esters, but its best substrate is ρ-nitrophenyl acetate with the highest activity (364 U/mg). It could also degrade medium to long-chain AHLs at the concentration of 1 mM in half an hour with more than 90% degradation efficiency. This is the first report to construct one metagenomic library from Turban Basin to obtain one esterase, which belongs to family V esterases/lipases and has AHL-lactonase activity. The recombinant enzyme displayed broad substrate spectrum, high activity and thermostability. These excellent properties make it an attractive enzyme for quorum quenching.

1. Introduction

Quorum sensing is a mechanism of bacterial gene regulation based on cell-density involving intercellular communication via diffusible chemical signal molecules [1–3]. In diverse gram-negative bacteria, the best described signal molecules are the *N*-acylhomoserine lactones (AHLs) that consist of a homoserine lactone ring linked via a saturated or unsaturated acyl chain (generally between 4 and 18 carbons) and without or with a keto or hydroxy substituent at the C3-position [1,3–6]. As the microbial density increases, the concentration of AHLs in the environment also increases to surpass a certain threshold which triggers target cells to coordinate gene expression and regulate the production of virulence factors [1,7]. Genes encoding these virulence factors controlled by quorum sensing regulation include swarming motility [8], antibiotic biosynthesis [9,10], exotoxin and extracellular degradative enzymes production [11,12], plasmid transfer [13], and biofilm development [14,15]. Traditionally, bacterial infections are treated by applying antibiotic agents which has resulted in the development of antibiotic resistance [16], and made antibiotic agents ineffective in controlling diseases [17]. In contrast to traditional treatment, the disruption of the AHL-mediated quorum sensing mechanisms, known as quorum quenching, aims to suppress the expression of virulence rather than to disinfect the organisms. Therefore, quorum sensing is a potential therapeutic target on bacterial virulence, and quorum quenching is supposed to be a promising strategy to attenuate virulence and thus control bacterial infections [18].

Besides the use of small molecule inhibitors of quorum sensing [19,20], enzymatic degradation of the quorum sensing signal molecules is a good method of quorum quenching and has been reviewed recently [21,22]. So far, only two groups of enzymes that are specific for AHL degradation are studied. Both of the degradation products above are no longer active, thus disrupting the AHL-mediated quorum sensing [23]. Members of the AHL-acylases inactivate signals by cleaving the acyl chain hydrolyze the amide linkage of AHLs, but display high substrate specificities based on the lengths of the AHL acyl chains [24,25]. While the AHL-lactonases family inactivates AHLs by hydrolyzing the lactone bond, it is noticed that they have broader AHL substrate spectrum [26,27]. Transgenic plants expressing AHL lactonase exhibited significantly enhanced resistance to the infection of *P. carotovorum* subsp. carotovorum [28]. Bacteria harboring the qsdA gene (encoding for AHL-lactonase) from *Rhodococcus* can interfere very efficiently with quorum-sensing-regulated functions [29]. The oral administration of an AHL lactonase $AiiA_{AI96}$ is efficient for the control of *Aeromonas hydrophila* [30]. Thus, it can be seen that AHL-lactonases are valuable for developing quorum-quenching procedures.

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Thermostability and storage stability of enzyme are vitally important properties, which endow enzymes considerable potential applications in many practical applications [31,32]. However, the report on detailed property of thermostable AHL-lactonases is meager. Soil microorganisms were the major source for enzymes of industrial importance [33]. Metagenomics, one culture-independent technique, has been complemented or replaced by culture-dependent methods, which theoretically provides access to the collective nucleic acids of all native microorganisms present in the studied environment [34]. Metagenomics was used to broaden the sources to grub novel thermostable AHL-lactonases. The Turban Basin is the hottest area in China. And the land surface temperature is more than 82 °C for a certain period in summer. Therefore, most of the native microorganisms possess thermotolerant characteristics, and enzymes released by these microorganisms are likely to be thermostable. In this study, we constructed a metagenomic library from Turban Basin soil, and cloned an esterase with a functional method. The property characterization of this enzyme was detailed. This esterase with thermostability could degrade medium to long-chain AHLs at the concentration of 1 mM in half an hour with more than 90% degradation efficiency. These features demonstrate that this enzyme is potential to be applied to disrupt quorum sensing.

2. Materials and methods

2.1. Chemicals and materials

The AHLs used in this study, *N*-butyryl-DL-homoserine lactone (C_4-HSL), *N*-hexanoyl-DL-homoserine lactone (C_6-HSL), *N*-octanoyl-DL-homoserine lactone (C_8-HSL), *N*-decanoyl-DL-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-DL-homoserine lactone (C_{12}-HSL), *N*-tetradecanoyl-DL-homoserine lactone (C_{14}-HSL), *N*-(3-oxooctanoyl)-DL-homoserine lactone ($3OC_8$-HSL) and all ρ-nitrophenyl esters were purchased from Sigma. Bromcresol purple was purchased from Aladdin. All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated. *Bam*HI, *Eco*RI, T4 DNA ligase and PrimeSTAR® HS DNA Polymerase were purchased from TaKaRa (Dalian, China) and used according to the recommendations of the manufacturer. E.Z.N.A. Plasmid Mini Kit and E.Z.N.A. Gel Extraction Kit were purchased from OMEGA (Norcross, USA).

2.2. Bacterial strains and plasmids

Escherichia coli DH5α and *E. coli* BL21 (DE3) (Novagen, Madison, USA) were used as the host for gene cloning and protein expression, respectively. The pUC118 (TaKaRa, Dalian, China) and pET-28a (+) (Novagen, Madison, WI, USA) were used to construct metagenomic libraries and express the target protein, respectively. *E. coli* transformants were grown in Luria-Bertani (LB) broth with appropriate antibiotics at 37 °C.

2.3. Metagenomic library construction and AHL-lactonase screening

A metagenomic library was constructed using the topsoil samples (3–10 cm) from Turban Basin. The total DNA was extracted based on a method described previously [35]. Routine DNA manipulations were carried out according to standard techniques. The purified DNA was partially digested with *Bam*HI. DNA fragments within 3.0–10 kb were ligated into *Bam*HI-digested pUC118, and the ligated products were transformed into *E. coli* DH5α. To screen positive clones rapidly and precisely, the new culture medium was designed. The transformed cells were plated onto LB agar plates containing 50 μg/mL ampicillin, 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and 100 μM 5-bromo-4-chloro-3-indolyl caprylate (X-caprylate). After incubation at 37 °C for 24 h, clones with blue color were selected. Then clones with blue color were further tested for the ability to hydrolyze C_8-HSL confirmed by high performance liquid chromatography analysis. Only one transformant (clone-pUC118A) with AHL-lactonase activity was obtained.

2.4. Sequencing and analysis of AHL-lactonase gene

The recombinant plasmid (pUC118A) was sequenced on ABI 377 DNA sequencer. The deduced amino acid sequence analysis and open reading frame search were performed with BLAST program provided by NCBI. And multiple alignments among similar enzymes were conducted by Clustal W software.

2.5. Cloning, expression and purification of AHL-lactonase

The putative AHL-lactonase gene was amplified from the pUC118A plasmid by using the primers and to introduce *Bam*HI and *Eco*RI restriction sites for cloning in the pET-28a (+). The following primers were used: fw (5′-CGCGGATCCATGCCGCATGTAGAGAACGA-3′; the *Bam*HI cutting site is underlined) and rv (5′-CCGGAATTCTCAGGACACCAATGAAGCTTCTCGA-3′; the *Eco*RI cutting site is underlined). The PCR product was digested with *Bam*HI/*Eco*RI, and then ligated into *Bam*HI/*Eco*RI digested expression vector pET-28a (+), and transformed into *E. coli* BL21 (DE3) cells (Stratagene). The transformants were plated onto LB agar containing 50 μg/mL kanamycin. Transformed cells were grown in a 250 mL flask containing 50 mL of LB (50 μg/mL kanamycin) at 37 °C until the cell concentration reached OD_{600} of 1.1, then induced with 0.5 mM IPTG. After incubation at 30 °C for 8 h with shaking at 220 rpm, cells were harvested by centrifugation (6000 × *g*, 10 min) at 4 °C and suspended in binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris–HCl, pH 7.9). The cells were disrupted by sonication, and the supernatant was collected by centrifugation (13,000 × *g*, 10 min) at 4 °C. The sample was loaded onto a Ni-NTA His·Bind column pre-equilibrated with binding buffer. Then the column was washed with binding buffer and washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris–HCl, pH 7.9). Finally, the bound protein was eluted with eluting buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The fractions containing the recombinant protein Est816 were collected and stored at −20 °C.

2.6. Determination of molecular mass

The molecular mass of the denatured protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 12% SDS-PAGE was prepared by the method of Laemmli [36]. Proteins were stained with Coomassie brilliant blue G-250. The molecular mass of the enzyme subunit was estimated using protein marker as standards, Rabbit muscle phosphorylase B (97,200 Da), bovine serum albumin (66,409 Da), ovalbumin (44,287 Da), carbonic anhydrase (29,000 Da), Soybean Trypsin Inhibitor (20,100 Da), Hen egg white Lysozyme (14,300 Da).

2.7. Determination of substrate specificity

As our research, Est816 belonged to esterase family. Above all, AHLs were very expensive. And therefore, general substrates of esterases were chosen to determine substrate specificity of this enzyme. Substrate specificity against different ρ-nitrophenyl esters was determined using ρ-nitrophenyl acetate (C2), ρ-nitrophenyl butyrate (C4), ρ-nitrophenyl caprylate (C6), ρ-nitrophenyl caproate