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Discovery a novel organic solvent tolerant esterase from *Salinispora arenicola* CNP193 through genome mining

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ABSTRACT

An esterase gene, encoding a 325-amino-acid protein (SAestA), was mined form obligate marine actinomycete strain *Salinispora arenicola* CNP193 genome sequence. Phylogenetic analysis of the deduced amino acid sequence showed that the enzyme belonged to the family IV of lipolytic enzymes. The gene was cloned, expressed in *Escherichia coli* as a His-tagged protein, purified and characterized. The molecular weight of His-tagged SAestA is ~38 kDa. SAestA-His_6 was active in a temperature (5–40 °C) and pH range (7.0–11.0), and maximal activity was determined at pH 9.0 and 30 °C. The activity was severely inhibited by Hg^{2+}, Cu^{2+}, and Zn^{2+}. In particular, this enzyme showed remarkable stability in presence of organic solvents (25%, v/v) with log $P > 2.0$ even after incubation for 7 days. All these characteristics suggested that SAestA may be a potential candidate for application in industrial processes in aqueous/organic media.

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1. Introduction

Lipolytic enzymes, including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), catalyze the cleavage and formation of ester bonds [1]. They catalyze the hydrolysis of triacylglycerols in aqueous solutions, the ester synthesis and transesterification under non-aqueous conditions. Lipolytic enzymes are widely employed in food, detergent, dairy, pharmaceutical, textile, biodiesel, and cosmetic industries, and in synthesis of fine chemicals, new polymeric materials, and environment industry [2–5]. Each application requires unique properties with respect to substrate specificity, stability, temperature, and pH dependence or ability to catalyze synthetic ester reactions in organic solvents. As a consequence, the esterases with the above-mentioned functionality gain more attention because of the enzymes could be potentially applied in the restricted reaction conditions [6,7].

Marine covers approximately 70% surface of the earth and has different extreme environments such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the sea water [8]. Marine microorganisms exhibit diverse physiological adaptations for marine environment, which made them capable of producing unique enzymes with extraordinary properties such as high efficiency, alkaline, acidic, alt-tolerant, and cold active. These distinctive characteristics have attracted many researchers to screen enzymes for industrial applications from samples collected from marine environment. Further, more than 99% of marine microorganisms cannot be cultured and only 0.001–0.1% seawater microbe are currently cultivated, which would be beneficial to improve the odds to find novel enzymes [9].

Salinispora is the first obligate marine genus within the order Actinomycetales reported in 2005 [10]. *Salinispora* genus, including three species: *S. arenicola*, *S. tropica*, and *S. pacifica*, is widely distributed in marine environment. *Salinispora* strains were found to be the prolific resources of novel secondary metabolites and enzymes. No less than 10 kinds of novel secondary metabolites with unique structure and bioactivities were currently found from them [11,12]. However, owing to *Salinispora* strains are slow growing, the enzymes from them have not been well studied.

With the ongoing development of genome sequencing technology, the increasing availability of DNA sequence data made biologists can deduce all the proteins that are likely produced in a given bacteria, and brought enzymes research into the genomic era [13,14]. Microbial genome mining has been a rapidly developing approach to discover new and novel enzymes including pectinase [15], nitrile hydratase [16], α-amylase [17], glucoamylase [17], vitamin D3-specific hydroxylase [13], fluorinases [18], as well as a mandelonitrile hydrolase [19] were found. However,

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there are still no any study reports about the enzymes discovery from obligate marine actinomycete. Therefore, genome mining approach was used to mine esterase gene from obligate marine actinomycete strain *S. arenicola* CNP193 genome sequence. As a result, a novel esterase gene was found. The cloning, expression, purification, and biochemical characterization of the esterase is also described herein.

2. Materials and methods

2.1. Bacterial strains and chemicals

The *S. arenicola* CNP193 was kindly provided from Dr. Paul R Jensen at Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, USA. The genome of *S. arenicola* strain CNP193 was deposited in the Joint Genome Institute's Integrated Microbial Genomes (IMG) database, http://img.jgi.doe.gov/cgi-bin/w/main.cgi (accession number: 2518285552).

Escherichia coli DH5α and *E. coli* BL21 (DE3) were purchased from Takara (Takara Mirus Bio Inc. Dalian, China) and used as cloning and expression host respectively. The pGEM®-T Easy vector, pET28a, and Ni–NTA (Ni^{2+}-nitrilotriacetate) His Tag Kit were purchased from Promega (Peking, China). *E. coli* strains were grown in Luria–Bertani (LB) broth with or without 1.8% agar at 37 °C. Sodium ampicillin (100 μg/ml) was added as necessary.

2.2. Genome mining and bioinformatics analysis for esterase-encoding sequences

Candidate esterase genes were screened on gene function (annotation-derived). Then, all the sequences were confirmed again by protein BLAST in GenBank. Multiple sequence alignments were performed with the ClustalW program [20] and phylogenetic analysis was done with MEGA5 [21]. Signal peptide was analyzed using program SignalP (http://www.cbs.dtu.dk/services/SignalP/).

2.3. Recombinant DNA methods

Chromosomal DNA of *S. arenicola* CNP193 was prepared as described by Maldonado et al. [22]. Standard methods were used for the isolation of plasmid DNA, restriction digestion, ligation, cloning, and transformation [15].

2.4. Protein expression and detection

Single cells of *E. coli* BL21 harboring the cloned esterase gene was grown at 30 °C on a rotary shaker of 250 rpm in 5 ml of Luria–Bertani (LB) medium containing 100 μg/ml ampicillin. The starting pH of the broth was adjusted to 7.0. The overnight cultures were diluted 100-fold in fresh LB medium, grown to OD600 ≅ 0.5 under the same culture conditions and then induced with 0.5 mM IPTG for 6 h. The bacterial cells were harvested and disrupted by sonication. The crude extract was centrifuged at 12,000 × *g* for 20 min at 4 °C to separate the cell debris. The clear supernatant of cell lysate was used for protein characterization. Protein concentration was determined by Bradford assay [23].

2.5. Protein purification

Ni–NTA His Tag Kit was used to purify the recombinant enzyme. The prepared sample was loaded to the NTA resin column (18 × 100 mm). The column was then eluted with a linear imidazole gradient (100 ml of 0–300 mM in buffer A of the Kit). The molecular mass and homogeneity staining of the purified recombinant esterase were estimated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and the protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250.

2.6. Enzyme assay

Esterase activity was determined using a modification of a previous method [1]. *p*NPB (*p*-nitrophenyl butyrate) used as the substrate was dissolved in acetonitrile at a concentration of 10 mM. Subsequently, ethanol and 50 mM Tris–HCl buffer (pH 9.0) were added to a final composition of 1:4:95 (V/V/V) of acetonitrile/ethanol/buffer, respectively. Sample (0.3 ml) and 0.9 ml substrate solution were mixed well. After 15 min of incubation at 30 °C, esterase activity was measured by monitoring the change in absorbance at 405 nm that represents the amount of released *p*NP (*p*-nitrophenol). All measurements were carried out under first-order reaction conditions. Control reactions containing no enzyme were utilized to account for any spontaneous hydrolysis of the substrates tested. All the assays were performed in triplicate. One unit of esterase activity was defined as the amounts of enzyme releasing 1 μmol *p*NP per min under the assay conditions.

2.7. Characterization of the recombinant enzyme

Biochemical characterizations were determined with purified SAestA-His_6 (1 mg/ml). All experiments were performed in triplicate and the average values were calculated. All the activity assays, unless stated otherwise, were performed at standard condition (30 °C, pH 9.0 in 50 mM Tris–HCl buffer).

Substrate specificity of the enzyme was determined with the method mentioned above using *p*-nitrophenyl fatty acid esters, of varying chain length (C2, C4, C8, C12, C16 and C18). The final concentration of substrates, dissolved in isopropanol, was all 10 μmol/ml.

Optimum pH of esterase was determined by measuring the hydrolytic activity in 50 mmol/l various buffer systems (pH 4.0–11.0) at 30 °C for 30 min. Buffer systems were used as follows: sodium acetate (pH 4.0–6.0), potassium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.0), and glycine–NaOH (pH 9.0–11.0). The stability of recombinant esterase to pH was investigated by detecting the residual activity after incubating the esterase in the buffers with different pH values at 30 °C for 1 h.

The effect of temperature on esterase activity was determined by assaying the enzyme activity at various temperatures ranging from 10 to 50 °C. The thermostability of esterase was investigated by detecting the residual activity after incubating the purified recombinant esterase at a temperature range of 10–50 °C for up to 3 h. The activity is represented as a percentage of the maximum activity.

The effect of metal ions and detergents on esterase activity was studied by preincubating the esterase with 1 mM of the ions, or 1% (either w/v or v/v) detergents, for 1 h at 30 °C. The residual esterase activity was measured by the *p*NPB method. The control contained no addition of the test compounds.

The effects of organic solvents with different log *P* values at 25% (v/v) concentration on the activity and stability of the purified esterase were investigated by previously method with slight modifications [24]. Log *P* was used as the quantitative measure of the solvent polarity. It is the logarithm of the partition coefficient of the solvent in a defined 1-octanol–water mixture [25].

One milliliter of organic solvent was added to 3.0 ml of esterase with 0.1 mg/ml protein concentration in screw capped tubes and incubated at room temperature (25 °C), 200 rpm for 1 day or 7 days. Residual activity was expressed as a percentage of the initial activity without incubation.

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