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Characterization of an arylsulfatase from a mutant library of *Pseudoalteromonas carrageenovora* arylsulfatase

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ABSTRACT

A library of *Pseudoalteromonas carrageenovora* arylsulfatase mutants was constructed by introducing random mutagenesis using error-prone PCR. After screening, one mutant strain was obtained whose arylsulfatase had improved thermal stability. Protein sequence analysis revealed one amino acid substitution of H260L. The mutant arylsulfatase (named H260L) retained higher residual activity than wild-type enzyme (named WT) after incubation at 45, 50, 55 and 60 °C for 60 min. Thermal inactivation analysis showed that the half-life ($t_{1/2}$) value at 55 °C for H260L was 40.6 min, while that of WT was 9.1 min. When *p*-nitrophenyl sulfate was used as a substrate, the optimal reaction temperature and pH for the mutant enzyme were 55 °C and pH 8.0, respectively. H260L was stable over the pH range of 6.0–9.0. Inhibition assay with EDTA indicated that metal ions play an important role during the catalytic process of the mutant enzyme. The desulfation ratio against agar of *Gracilaria lemaneiformis* was 82%.

1. Introduction

Sulfatase acts on organophosphates to free inorganic acid. According to the mechanisms, sulfatase can be divided into α-ketoglutarate-dependent dioxygenase superfamily, metallo-β-lactamase-ralated sulfatase, and arylsulfatase [1]. Arylsulfatase catalyzes the hydrolysis of the arylsulfate esters to aryl compounds and inorganic sulfate [2]. It widely spreads in many organisms including animals, plants, and microorganisms. A number of arylsulfatases of microbial origin have been characterized. These include arylsulfatases from *Aspergillus oryzae* [3], *Klebsiella aerogenes* [4], *Klebsiella pneumoniae* [5], *Marinomonas* sp. [6], *Pseudoalteromonas carrageenovora* [7], *Pseudomonas aeruginosa* [8,9], *Salmonella typhimurium* [10], *Serratia marcescens* [11], *Sphingomonas* sp. [12], *Streptomyces* sp. [13], and *Thermotoga maritima* [14]. Arylsulfatase plays an important role in cancer detection [15], increasing the inorganic sulfate of soil [16], and improving the quality of agar [6].

Agar, the main cell wall structure polysaccharide of red algae, is composed of agarose and agaropectin [17]. Agarose is the gelling component and comprises a linear chain of alternately linked 3,6-anhydro-α-L-galactose and D-galactose by α-1,3 and β-1,4 glycosidic bonds [17]. Agaropectin has the same backbone units with some hydroxyl groups of 3,6-anhydro-α-L-galactose residues replaced by sulfoxy, methoxy or pyruvate residues [17]. The sulfate groups in C6 position of galactosyl residues form L-galactose-6-sulfate [18]. The sulfate groups in agar usually weaken the gel strength due to the avoidance of the cross-linked structure during gelation [19]. The chemical and enzymatic methods can be applied to remove the primary sulfate groups of the galactopyranosyl unit in agar. The alkali treatment is a tranditional way which has been wildly used in the agar industry [20]. However this method has some drawbacks, such as the reduction of agar yield, the formation of brown color in product, having difficulty to control the agar quality, and the environmental pollution. In contrast, arylsulfatase has the potential application in agar desulfation and improvement of the agar quality [6,12,14,21]. The enzymatic method has many

Abbreviations: H260L, mutant arylsulfatase H260L; WT, wild-type arylsulfatase; pNPS, p-nitrophenyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; β-ME, β-mercaptoethanol; DTT, dithiothreitol; SD, standard deviation.

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advantages, such as high specificity, mild reaction conditions, and low environmental pollution.

The recombinant arylsulfatase from *Pseudoalteromonas carrageenovora* has activity towards *p*-nitrophenyl sulfate (*p*NPS) and activity in desulfation of agar [7,21]. The thermostability of this enzyme is not reported [7]. The arylsulfatase gene (984 bp) from *Pseudoalteromonas carrageenovora* was also cloned and expressed in *E. coli* in our laboratory. We found that the recombinant enzyme was not stable above 45 °C. In order to use the arylsulfatase from *Pseudoalteromonas carrageenovora* as a biocatalyst in agar desulfation, it is desirable to improve its thermostability. In this study, we used error-prone PCR based directed evolution to improve the thermostability of *Pseudoalteromonas carrageenovora* arylsulfatase and then the biochemical properties of the mutant enzyme were characterized.

2. Materials and methods

2.1. Construction of E. coli containing the wide-type Pseudoalteromonas carrageenovora arylsulfatase gene

Pseudoalteromonas carrageenovora arylsulfatase contains a signal peptide with 18 amino acids length. We cloned the 930 bp of arylsulfatase gene except the signal peptide gene part aiming to increase the solubility of the recombinant arylsulfatase. The gene was amplified from the bacterial genomic DNA using the primers ars-F (5-CGCGGATCCTTTACGTTTAACGGCAGC-3′) and ars-R (5′-CCCAAGCTTGCGTTTTAGTTCGTAAC-3′) (*Bam* HI and *Hin* dIII sites are underlined). After digestion with Bam HI and Hin dIII, the amplicon was inserted into the pET-28a(+) vector (Novagen) and the resulting construct was named pET-28a-ars. Once the sequence of the gene was confirmed by sequencing, the recombinant plasmid was introduced into *E. coli* BL21 (DE3).

2.2. Construction of random mutant library of Pseudoalteromonas carrageenovora arylsulfatase

The random mutant library was generated by the error-prone PCR method using the above two primers ars-F and ars-R. Error-prone PCR was done in 50 μl volume containing 5 μl 10 × buffer, 0.2 μM primers, 0.5 mM dTTP, 0.5 mM dGTP, 0.1 mM dATP, 0.1 mM dCTP, 1 U r*Taq* polymerase (TaKaRa), 7 mM $MgCl_2$, and 2 ng template DNA of recombinant plasmid pET-28a-ars. The thermal cycling parameters were: 95 °C for 5 min; 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min (35 cycles); 72 °C for 10 min. After digestion with *Bam* HI and *Hin* dIII, the PCR products was inserted into the pET-28a(+) vector to generate a recombinant plasmid library. The plasmids were transformed into freshly prepared *E. coli* BL21 (DE3) competent cells to generate the mutant library.

2.3. Screening the library for mutant with improved thermostability

A two-step screening protocol was used for the selection of arylsulfatase mutant with improved thermostability. First, the transformants in the library were replicated on Luria-Bertani (LB) agar plates containing 50 μg/ml kanamycin, and incubated at 37 °C for 16 h. Then these transformants were transferred individually using toothticks to two sets of LB agar plates and incubated at 37 °C for 10 h. One set of plates for screening were supplemented with kanamycin (50 μg/ml) and 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and another set of plates for storage were supplemented with kanamycin (50 μg/ml). After the screening plates were treated at 50 °C for 90 min, 0.4% (w/v) agar with 5 mM *p*-nitrophenyl sulfate (*p*NPS) was covered on the plates. Transformants showing arylsulfatase activity (resulting in yellow colonies) were selected.

In the second round screening, the above selected individual variant was selected from the above storage agar plates and grown in 5 ml LB medium with 50 μg/ml kanamycin at 37 °C. After the OD_{600} reached 0.6, enzyme expression was induced by adding 0.05 mM IPTG. After incubation at 16 °C for 16 h, the cells in 5 ml culture were harvested by centrifugation (6000 × *g*, 5 min) at 4 °C and washed with 50 mM Tris-HCl (pH 7.5) to eliminate the pH shift caused by the remaining medium. The cell pellet was resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5), and then the cells were lysed by intermittent sonication on ice. After centrifugation at 10,000*g* for 20 min at 4 °C, the supernatant was collected as the sample. After the sample was treated at 55 °C for 30 min, the residual activity of arylsulfatase was determined by the method described below. The variant showing higher enzymatic thermostability than the parent was selected, and the enzyme gene sequence of the variant was sequenced by Invitrogen Trading (Shanghai) Company.

2.4. Sequence and structure analysis of the mutant derivative

The gene and protein sequence was analyzed by using DNAMAN 5.1 software (Lynnon BioSoft, Canada). The three-dimensional structural model of arylsulfatase was generated using the Modeller 9.16 software [22]. The model quality was analyzed using the PROCHECK program [23]. CDOCKER of arylsulfatase and *p*NPS was performed using Discovery Studio 2016 (BIOVIA, USA). The PyMol Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA, USA) was used for the visualization and analysis of the structure.

2.5. Expression and purification of recombinant enzymes

200 ml of the mutant or wild-type transformant was grown in LB medium containing 50 μg/ml kanamycin at 37 °C until the OD_{600} reached 0.5, then IPTG was added to a final concentration of 0.05 mM. After incubation at 16 °C for 16 h, the induced cells were harvested by centrifugation at 6000 × *g* for 5 min. Purification of the His-tagged protein was conducted using Ni sepharose 6 Fast Flow (GE Healthcare Life Sciences) affinity chromatography under the native conditions according to the method described before [24]. The eluate was dialyzed twice against 50 mM Tris-HCl buffer (pH 7.5). The protein concentration was determined by Bradford method [25] with bovine serum albumin as the standard. The homogeneity of the purified enzyme and its molecular mass were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed according to the standard procedures [26].

2.6. Arylsulfatase activity assay

Arylsulfatase activity was assayed according to the method of Kim et al. [12] with slight modifications. Purified enzymes were used unless otherwise mentioned. Briefly, the reaction mixture contained 20 μl of enzyme solution (0.4 μg) and 80 μl of substrate solution which was 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM *p*-nitrophenyl sulfate (*p*NPS, Sigma). After incubation at 55 °C for 10 min, the reaction was terminated by adding 25 μl of 5 M NaOH. The amount of *p*-nitrophenyl released was determined by measuring the absorbance at 410 nm with a spectrophotometer (Cary 50, USA). One unit of arylsulfatase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenyl per minute under the assay conditions.