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Characterization of a (*R*)-selective amine transaminase from *Fusarium oxysporum*

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ABSTRACT

Amine transaminases are prominent biocatalysts in the production of chiral amines which are indispensable building blocks in asymmetric synthesis. In this study, a new (*R*)-enantioselective amine transaminase from *Fusarium oxysporum* (ATFo) was identified by genome mining. ATFo possibly evolved from a branched chain amino acid aminotransferase (BCAT) with key amino acids being changed, which belong to one of the three groups of pyridoxal 5′-phosphate dependent enzymes class IV (PLPDE_IV). The gene of the amine transaminase was functionally expressed and the protein was then purified with a molecular mass of approximately 36 kDa. The purified ATFo demonstrated high stereoselectivity towards (*R*)-enantiomer of α-phenethylamine and other analogues, which clearly indicated its (*R*)-selectivity. The optimal temperature and pH for the activities of ATFo were 25 °C and 7.0, respectively. Addition of Mn^{2+} and Zn^{2+} could greatly enhance the enzyme activity. In addition, the specific activities and stereoselectivities of these ATFo toward various amino donors and amino acceptors were determined. Compared to (*S*)-selective amine transaminase, the (*R*)-selective counterpart has been less studied. Given their pivotal role in asymmetric biocatalysis, it is of great importance to find more (*R*)-selective amine transaminases with ability or potential for synthesis of the target compounds. Thus, the discovery of the (*R*)-selective amine transaminase ATFo is a valuable contribution to the currently small toolbox of these enzymes.

1. Introduction

Amine transaminases are biocatalysts of great significance for the production of chiral amines which represent a series of compounds used in biochemistry [1–4]. The enzyme catalyzes the transfer of an amino group from an amino donor onto a carbonyl moiety, utilizing pyridoxal-5′-phosphate (PLP) as cofactor [5,6] (Fig. 1). Amine transaminases have been used as an attractive approach to synthesize optically pure amines due to excellent optical purity, high yield, broad substrate specificity, and environmental friendliness. Given the fact that they play a pivotal role in asymmetric biocatalysis, the discovery and identification of appropriate new amine transaminases with potential applications is of vital importance.

Classification of transaminases has recently been reviewed [7]. Compared to (*S*)-selective amine transaminases [8–10], only a few (*R*)-selective counterparts were studied [11–16]. Among these (*R*)-selective amine transaminases ((*R*)-ATs), the three dimensional structures of the ones from *Aspergillus terreus* and *Aspergillus fumigatus* were solved to have an insight into the enantioselectivity and explore the catalytic mechanism of (*R*)-selective amine transaminase [17,18]. Besides, researchers made great efforts to engineer the enzymes by means of molecular modeling and structure-activity relationship [19–24]. Issues such as unfavorable equilibrium constant and inability to accept larger molecules could be addressed. For instance, the remarkable work was done by engineering an (*R*)-selective amine-transaminase from *Arthrobacter* sp. The results showed that the mutant enzyme was able to accept the bulky substrate [3]. Although, the rational search for more applicable (*R*)-ATs is still ongoing for the reason that it will build the basis for further and extensive applications of these enzymes.

At the beginning, BLASTP (Basic Local Alignment Search Tool Protein sequences) search using the amino acid sequence of the (*R*)-selective amine transaminases from *Arthrobacter* sp. KNK 168 as template was performed. Analysis of the results indicated that the sequences standing out with different degree of similarities could be divided into three categories. These were annotated as pyridoxal 5′-phosphate dependent enzymes class IV (PLPDE_IV) (aminotransferase class IV), putative branched chain amino acid aminotransferase (BCAT), and D-alanine aminotransferase (D-AAT_like), respectively [25–30]. In our study, one hypothetical protein FOXB_13959 from fungal *Fusarium oxysporum* annotated as PLPDE_IV (designated as ATFo) was chosen for

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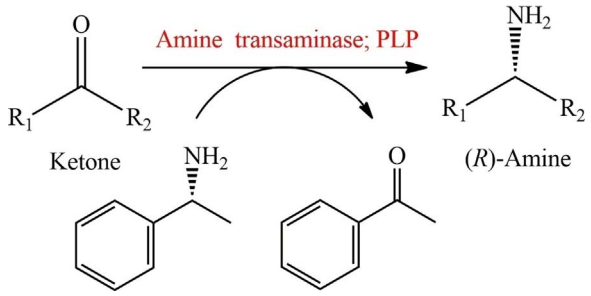

Fig. 1. Asymmetric synthesis by amine transaminase.

further experimental identification and confirmation. ATFo was cloned, overexpressed, and purified to homogeneity. The amine transaminase activity and enantioselectivity were experimentally confirmed. In addition, the substrate specificity of the enzyme was investigated by determining its reactivity toward a variety of amines as donors and ketones as accepters.

2. Materials and methods

2.1. Chemicals and microorganisms, and molecular biology tools

Chemical reagents, unless stated otherwise, were purchased as analytical grade from Beijing Beihua Fine Chemicals Co., Ltd. (Beijing, China). *F. oxysporum* (CGMCC No. 3.6864) was supplied by CGMCC (China General Microbiological Culture Collection Center, Beijing, China). *Fusarium oxysporum* was incubated at 25 °C for 24 h with shaking at 200 rpm in PDA medium containing 200 g/L potato,20 g/L glucose, 15 g/L agar powder. The genomic DNA of *F. oxysporum* was prepared using fungal genomic DNA extraction kit according to manufacturer's handbook. Restriction enzymes, T4 DNA ligase and Q5 High-Fidelity DNA polymerase, were purchased from New England Biolabs (Beverly, MA, USA). The gel extraction kit was from Qiagen (Germany). The plasmid extraction kit and fungal genomic DNA purification kit were from Tiangen (China). His-tagged protein purification columns Ni-NTA Superflow Cartridges were purchased from QIAGEN Gmbh (Hilden, Germany).

2.2. Cloning and expression of ATFo

The ATFo gene (GeneBank No. EWZ43325.1) was amplified from genomic DNA of *F. oxysporum* by standard PCR with designed forward primer FW-ATFo (5′-GGAATTCCATATGGCGACCATGCAGGAAATC TTC-3′) and reverse Primer RW-ATFo (5′-CCCAAGCTTTGGCTCG TAACTAATCTTGGTTG-3′) .The primers were designed to incorporate *Nde*I and *Hin*dIII restriction sites (underlined). Then the amplified gene fragments were gel-purified and inserted into the expression vector pET-22b (+). *Escherichia coli* DH5α cells were transformed with the recombinant plasmid. The positive plasmid pET22-ATFo was confirmed by sequencing and purified using a plasmid extraction kit (Qiagen). Subsequently, *E. coli* Rosetta (DE3) cells were transformed with the recombinant plasmid pET22-ATFo. To induce the expression, 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the medium when its optical density at 600 nm reached 1.0–1.2. The medium used for bacteria cultivation and protein expression was LB medium.

The result cells were harvested after incubation at 16 °C for 20 h by centrifugation (3500 × *g* for 20 min).

2.3. Purification of the recombinant ATFo

The collected cells were suspended in Tris-HCl buffer I (50 mM Tris-HCl, 300 mM NaCl, pH 7.8) and then disrupted on ice using a sonicator. Cell debris were removed by centrifugation at 12,000 × *g* for 60 min at 4 °C. The supernatant was applied to a Ni-affinity column that had been equilibrated with 20-ml Tris-HCl buffer I. After binding for 15 min, the unbound enzymes were washed with Tris-HCl buffer II (50 mM Tris-HCl, 300 Mm NaCl, 40 mM imidazole, pH 7.8) to remove impurities. At last, the bound enzyme was eluted with Tris-HCl buffer III (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.8), and the pure ATFo proteins were collected. To remove the imidazole present in the elution buffer, the fractions were concentrated and dialyzed against phosphate buffer (pH 7.6) using a Superdex 200 colum (GE Healthcare). The whole purification was carried out at 4 °C. The purity of ATFo was investigated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using the BCA Protein Assay Kit (Pierce) with bovine serum albumin (BSA) as a standard. SDS-PAGE was carried out with a 6% polyacrylamide stacking gel and a 15% polyacrylamide separating gel [31]. A protein marker (14–100 kDa) was used for determination of relative molecular weight.

2.4. Enzyme assay

Enzyme activity of ATFo was determined as described below. The reaction was carried out at 25 °C in 1 mL potassium phosphate buffer (50 mM, pH 7.0) containing 20 mM (*R*)-α-phenethylamine (or (*S*)-α-phenethylamine), 20 mM sodium pyruvate, 0.1 mM pyridoxal phosphate (PLP), and an appropriate amount of purified enzyme. These parameters were modified in order to investigate the behavior of the enzyme in different reaction conditions if necessary. All the experiments were conducted with three replicates. The activity was estimated according to the production of acetophenone during the first 30 min. The reaction was terminated by adding equal volume of ethyl acetate. After extraction, the organic phase was analyzed by High Performance Liquid Chromatography (HPLC) using an Agilent C18 column (250*4.6 mm, Agilent, USA) with a mobile phase of acetonitrile/ultrapure water (50/50, v/v) at a flow rate of 0.6 mL/min. The UV absorbance of produced acetophenone performed at 254 nm.

2.5. Effect of pH and temperature on enzyme activity

In order to investigate the effect of temperature on pure enzyme activity, the reaction was carried out at different temperatures ranging from 15 °C to 50 °C at pH 7.0 using (*R*)-α-phenethylamine as amino donor and sodium pyruvate as amino acceptor. The optimal pH of purified enzyme was determined at the pH varied from 6.0 to 12.0 (Potassium phosphate buffer was used for pH range from 6.0 to 8.0, Tris buffer was used for pH range from 9.0 to 12.0) in prepared buffer at optimal temperature. The thermostability of ATFo was assessed by measuring the residual activity after incubating the enzyme at varying temperatures from 25 °C to 65 °C for 30 min under the standard reaction condition using the method described above. Relative activities (%) were calculated using the maximal activity as a control (100%).

2.6. Effect of metal ions on enzyme activity

The effect of metal ions on enzyme activity was monitored by adding various metal ions to reaction mixture. The reaction was performed containing 1 mM metal ions (K^+, Ca^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) under the standard reaction condition. Relative activities (%) were calculated using the activity determined without adding any metal ion as a control (100%).

2.7. Kinetic parameter determination of ATFo

Kinetic constant measurements for both (*R*)-α-phenethylamine and sodium pyruvate were performed at 25 °C. The reactions for the kinetic constant measurement of (*R*)-α-phenethylamine were carried out in 1 mL potassium phosphate buffer (50 mM, pH 7.0) containing 20 mM sodium pyruvate, 0.1 mM PLP, varied concentration of (*R*)-α-