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Biochemical characterization of thermostable ω-transaminase from *Sphaerobacter thermophilus* and its application for producing aromatic β- and γ-amino acids

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ABSTRACT

An (*S*)-ω-transaminase from the thermophilic eubacterium *Sphaerobacter thermophilus* was expressed and functionally characterized. The enzyme showed good stability at high temperature and in the presence of various substrates. Substrate specificity analysis showed that the enzyme had activity towards a broad range of substrates including amines, β- and γ-amino acids. The purified enzyme showed a specific activity of 3.3 U/mg towards *rac*-β-phenylalanine at 37 °C. The applicability of this enzyme as an attractive biocatalyst was demonstrated by synthesizing optically pure β- and γ-amino acids. Among the various beta and gamma amino acids produced *via* asymmetric synthesis, (*S*)-4-amino-4-(4-methoxyphenyl)-butanoic acid showed highest analytical yield (82%) with excellent enantiomeric excess (>99%).

1. Introduction

Production of optically pure β- and γ-amino acids is of high pharmacological significance as they are often used as precursors in bioactive compounds and for generating hybrid peptides [1,2]. For example, aromatic β-amino acids are used in pharmaceutically relevant compounds such as CCR-5 receptor antagonist maraviroc used for treating HIV infection [3]. Similarly, γ-amino acids are key components of natural products such as hapalosin [4], dolastatin [5] and caliculins [6]. Of late, due to environmental concerns, industries are increasingly looking towards biocatalysts for manufacturing compounds in an environmentally sustainable manner. Attractive features such as simplicity in inserting chirality and selectivity with regard to regio- and stereochemistry in molecules has made biocatalyst an appealing alternative to chemical synthesis. Over time, ω-transaminases (ω-TA) has emerged as an important class of enzymes for the production of optically pure amines and unnatural amino acids including β- and γ-amino acids owing to their characteristic features such as broad substrate specificity, high enantioselectivity, high turnover number, and non-requirement for regenerating external cofactors [7–9]. An enzyme can be broadly classified as ω-TA if it transfers an amino group from an amino donor onto the carbonyl moiety of an amino acceptor, in which at least one of the two substrates is not α-amino acid or α-keto acid [10]. Since this enzyme from *Sphaerobacter thermophilus* satisfies the above definition, it can be classified as ω-TA. Until now, only few TAs has shown activity towards both aromatic β- and γ-amino acids [11].

Usually ω-TAs work in mild reaction condition and are susceptible to reaction conditions such as high temperature, exposure to organic solvents and other substrates that are often used in industries. To enhance the utility of ω-TAs for industrial applications, tolerance towards high temperature and organic solvents are highly desirable. Recently, a number of industrial bio-catalytic enzymes were developed by employing protein engineering approaches [12]. An excellent example demonstrating the power of protein engineering is the production of sitagliptin by researchers from Merck and Codexis using a (*R*)-ω-TA mutant [13]. After performing 11 rounds of mutations containing 27 mutations on (*R*)-ω-TA from *Arthrobacter* sp., the enzyme was able to operate at elevated temperature (45 °C) and in the presence of high concentration of DMSO (50%). The best variant was utilized for high

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concentration reaction (200 g/L) to generate sitagliptin with 92% assay yield and *ee* >99.95%. Though protein engineering can be utilized to develop industrial enzymes, adopting this approach can be complex as it may require high throughput screening method to identify improved variants from thousands of candidates. Recently, stability of enzymes particularly ω-TAs were enhanced by incorporating unnatural amino acids [14,15]. However, this method is also complex and expensive to be adopted for industrial scale [16].

Earlier, an *in silico* approach was developed by Hohne et al. in which an algorithm based on rational assignment of motifs were utilized to identify novel (*R*)-ω-TAs from NCBI protein database [17]. Another approach to develop industrial enzymes is by identifying thermostable enzymes which are tolerant enough to withstand industrial process conditions. To the best of our knowledge, most reported ω-TAs to date was not sourced from thermophiles [8–10]. Thermophiles which have an optimal growth temperature beyond 60 °C constitute an abundant repository for identifying relevant thermostable enzymes. Compared to enzymes from mesophiles, thermostable enzymes are excellent tools for biotransformation due to its intrinsic properties such as high operational stability, high tolerance towards organic solvent, prolonged storage and low possibility of microbial contamination [18,19]. These characteristics make thermostable enzymes ideal for industrial applications. Recently, our group utilized a ω-TA from a thermophile- *S. thermophilus* (ω-TAST) as a reporter protein for incorporating unnatural amino acids [15]. In this work, ω-TAST was biochemically characterized and its utility in synthesizing β- and γ- amino acids was demonstrated.

2. Materials and methods

2.1. Chemicals

β-ketoesters, β-amino acids (**9–15**), GITC (2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate), lipase (Catalog No. L1754), PLP (Pyridoxal 5′-phosphate hydrate), dimethyl sulfoxide (DMSO), amino donors and amino acceptors were obtained from Sigma-Aldrich. *rac*-γ-amino acids and γ-keto acids were synthesized as described elsewhere [11]. Isopropyl-β-d-thiogalactopyranoside (IPTG) was obtained from Carbosynth.

2.2. Identification of ω-TA gene

Previously reported ω-TA from *Polaromonas* sp. JS666 (ω-TAPO) which had shown activity towards aromatic β- and γ-amino acids was taken as the query sequence and was searched against protein sequences from different thermophile organisms [11,20]. The selected gene (WP_012871332) was categorized as a class III aminotransferase and it showed 41% identity and 90% query coverage with the query sequence.

2.3. Enzyme expression and purification

ω-TAST gene was codon-optimized (Supplementary information) and synthesized with C-terminal His-tag by Bioneer Corporation, South Korea. ω-TAST gene was then inserted into the IPTG inducible pET24ma expression vector at *Nde*I and *Xho*I restriction sites. The plasmid was then introduced into the *Escherichia coli* (BL21) cell using Hanahan method [21] and the transformants were grown at 37 °C in 1 L LB containing 100 μg/ml of kanamycin. When the OD_{600} reached 0.5–0.6, IPTG was added to a final concentration of 0.5 mM. After 7 h of induction at 37 °C, the over-expressed cells were centrifuged at 4000 rpm for 20 min at 4 °C. Subsequently, the cell pellet were resuspended and washed twice in 10 mL volume of 20 mM Tris–HCl buffer (pH 8.0) containing 20 μM pyridoxal 5-phosphate (PLP), 2 mM EDTA, 1 mM PMSF and 10% glycerol. The suspension was then subjected to ultrasonic disruption with SONICS VCX-750 (2 s pulse, 5 s wait, amplitude 22) for 20 min at 4 °C. The sonicated cell was then centrifuged at 16,000 rpm for 30 min. The C-terminal His6-tagged fusion protein was purified at 4 °C on a Ni-NTA agarose resin obtained from Qiagen (Hilden, Germany). Briefly, the crude extract was passed directly over a column containing 3 mL of Ni-NTA agarose resin. The column was then washed with 50 mL of phosphate buffer (pH 8.0) containing 20 mM imidazole and the C-terminal His6-tagged protein was eluted with phosphate buffer (pH 8.0) containing 200 mM imidazole. The eluted solution containing purified protein was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 20 μM PLP and concentrated using an Amicon PM-10 ultrafiltration unit. Glycerol was added to the purified enzyme solution (25%) and it was stored at −20 °C for further characterization studies and enzyme synthesis. The SDS gel of purified ω-TAST and ω-TAPO are shown in Supplementary information (Fig. S1).

2.4. Site-directed mutagenesis

The R36A mutant of ω-TAST was constructed by using EZchange site-directed mutagenesis kit from Enzynomics®. The oligonucleotides used as primers for the mutant R36A were (forward, 5′-CAGGCGGCAATACAGCGACCACTGTTTATAG-3′ and reverse, 5′-CTATAAACAGTGGTCGCTGTATTGCCGCCTG-3′). Next, the gene was inserted into the expression (pET24ma) and subsequently transformed into DH5α and BL21 cells. The mutant was confirmed after sequencing the gene in Cosmo Genetech, South Korea.

2.5. Enzyme assay

The deamination of ω-TAST on various substrates were determined by performing a 1 mL enzyme reaction containing 100 mM Tris–HCl buffer (pH 8.0), 10 mM amino donor, 10 mM pyruvate, 0.1 mM PLP and ω-TAST (0.01–0.1 mg/ml) at 37 °C for 30 min. The amination of ω-TAST on various substrates were determined by performing a 1 mL enzyme reaction containing 100 mM Tris–HCl buffer (pH 8.0), 10 mM amino acceptor, 10 mM *rac*-**β**-Phe (**9**), 0.1 mM PLP and ω-TAST (0.01–0.1 mg/ml) at 37 °C for 30 min. For amino acceptor specificity analysis, substrate and product inhibition, *rac*-β-Phe was used as the amino donor while (*S*)-α-phenyl-ethylamine (**3**, (*S*)-α-PEA) was used as amino donor for determining thermostability analysis, effect of pH and temperature. Enzyme reactions were stopped after 30 min by adding 10% perchloric acid (50% v/v) before centrifuging (13,000 rpm, 15 min) and analyzing the samples in HPLC. One unit of enzyme activity was defined as the amount of enzyme that depleted 1 μmol pyruvate/*rac*-**β**-Phe in 1 min. In enzymatic reactions where (*S*)-α-PEA was used as amino donor, enzyme activity was measured based on the production of acetophenone. To determine the kinetic parameters of ω-TAST towards 4-oxo-4-phenylbutanoic acid (**G1**), initial rate were measured with different concentration of 4-oxo-4-phenylbutanoic acid (0–25 mM) and 50 mM (*S*)-α-PEA.

2.6. Analytical methods

The conversion analysis of β- and γ-amino acids (Fig. 1) were measured using HPLC with a Crownpak CR (Daicel Co., Japan) column at 210 nm with an elution of pH 1.5 perchloric acid solution (0.6 mL/min). Each enantiomer of β-amino acids was not separated in this analytical condition but the exact conversion could be calculated. Quantitative chiral analysis of β-amino acids was performed using a C18 Symmetry column (Waters, MA) with a Waters HPLC system at 254 nm after the derivatization of sample with GITC. Separation of each enantiomer was achieved through an isocratic elution with a mixture of 45–55% methanol and 45–55% water (0.1%