J. Microbiol. Biotechnol. (2004), **14**(1), 153–157

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enhanced Stability of Tyrosine Phenol-Lyase from *Symbiobacterium toebii* by DNA Shuffling

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Received: August 4, 2003
Accepted: September 16, 2003

Abstract Tyrosine phenol-lyase (TPL) is a useful enzyme for the synthesis of pharmaceutical aromatic amino acids. In the current study, sequential DNA shuffling and screening were used to enhance the stability of TPL. Twenty-thousand mutants were screened, and several improved variants were isolated. One variant named A13V, in which the 13th amino acid alanine was substituted by valine, exhibited a higher temperature and denaturant stability than the wild-type TPL. The purified mutant TPL, A13V, retained about 60% of its activity at 76°C, whereas the activity of the wild-type TPL decreased to less than 20% at the same temperature. Plus, A13V exhibited about 50% activity with 3 M urea, while the wild-type TPL lost almost all its catalytic activity, indicating an increased denaturant tolerance in the mutant A13V. It is speculated that the substitution of Val for the Ala in the β-strand of the N-terminal arm was responsible for the heightened stabilization, and that the current results will contribute to further research on the structural stability of TPL.

Key words: Tyrosine phenol-lyase, thermostability, denaturant tolerance, DNA shuffling

Pyridoxal phosphate-dependent tyrosine phenol-lyase (TPL, E.C.4.1.99.2) catalyzes the α,β-elimination, β-replacement, and racemization of L-tyrosine, and the synthesis of L-tyrosine from pyruvate, ammonia, and phenol by the reverse reaction of α,β-elimination [3, 6, 7]. Due to its broad substrate specificity, TPL has attracted much attention as a biocatalyst for the enzymatic synthesis of useful pharmaceutical aromatic amino acids, such as L-tyrosine and L-DOPA, which is a therapeutic agent for the treatment of Parkinsons disease [2, 4, 9, 16]. However, the stability of the TPL is also a critical factor in the use of TPL as a biocatalyst, as most substrates, including phenol and pyrocatechol, are strong protein denaturants [11, 12, 15]. Therefore, microorganisms that are more stable against aromatic substrates need to be isolated. It is also well known that thermostable enzymes exhibit an enhanced tolerance against aromatic detergents.

A previous study by the current authors isolated an obligatory symbiotic thermophile, *Symbiobacterium toebii*, that could grow at 60°C and produce thermostable TPL, and the TPL of *Symbiobacterium toebii* was cloned and overexpressed [5, 10, 13]. It was also found that the thermostable TPL from *Symbiobacterium toebii* was much more stable against pyrocatechol than the TPL from *Citrobacter freundii* [10]. Accordingly, based on the need to enhance the thermostability and denaturant tolerance of TPL during the enzymatic synthesis of L-tyrosine from phenol, pyruvate, and ammonia, the current study attempted to increase the stability of TPL by DNA shuffling and describes the properties of the mutant TPL.

Materials and Methods

Materials

All the chemicals used in the current study were commercial products. L-Tyrosine was purchased from Junsei Chemicals (Japan), the sodium bicarbonate, 4-aminoantipyrine, potassium ferricyanide, and other chemicals from Sigma-Aldrich (U.S.A.), the restriction enzymes from New England Biolabs (U.K.), and the DNase I from Sigma (U.S.A.).

Construction of Mutant Library

The TPL from *Symbiobacterium toebii* was generated from the pHLT1-TTPL constructed previously by the authors of this work. The TPL gene was amplified using a PCR with two primers, the TPn of an N-term primer (5'-

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ATGAATTCACCCTGGGCGGAACC-3') and the TPc of a C-term primer (5'-CAAAACAGCCAAGCTTATTAGCT-3'), respectively. The forward primer contained an *Eco*RI recognition site, while the reverse primer contained an *Hin*dIII recognition site for convenient subcloning of the TPL gene into pHCE19T(II). The amplified gene was purified from 1% agarose gel using a QIAquick gel extraction kit (QIAGEN, U.S.A.), then digested with DNase I. About 50 bp-long fragments were purified from 2% agarose gel and reassembled using a PCR (94°C, 30 sec; 53°C, 30 sec; 72°C, 30 sec; 30-60 cycles) without primers. After diluting the reassembled DNA fragments up to 40-fold, they were amplified using a PCR with two primers, TPn and TPc. All the PCR procedures were performed using *Taq* polymerase (*Ex. Taq* Version Premix, TaKaRa, Japan) under the conditions recommended by the supplier. The amplified DNA fragments (1.4 kb) were purified from the agarose gel and then digested with *Eco*RI and *Hin*dIII. Next, the shuffled genes were cloned into the constitutive expression vector pHCE19T(II) and transformed into *E. coli* DH5α by electroporation (GenePulser II Electroporation System, Bio-Rad, U.S.A.). The transformants were plated on Luria-Bertani (LB) plates containing ampicillin (100 μg/ml) and grown overnight at 37°C.

Screening of Mutant Library

The TPL variants were primarily screened using the colorimetric method for phenol detection in a 96-well plate. The variants were grown in an LB-medium in a 96-well plate overnight at 37°C, then harvested by centrifugation at 2,500 rpm for 10 min. Thereafter, the pellets were suspended in a 50 mM potassium phosphate buffer (pH 7.2) and heat-treated at 75°C for 30 min to remove any heat-labile proteins. To detect the enzyme activity of the TPL, saturated L-tyrosine was added to the heat-treated samples and reacted at 75°C. After 20 min of reaction, 1.5% $NaHCO_3$, 0.85% 4-aminoantipyrine, and 5.4% $K_3Fe(CN)_6$ solution were added in that order. The positive variants in the mutant library were identified, based on an increased red intensity.

The plasmids harboring the mutant TPL genes were isolated from the transformants exhibiting a strong red color due to the action of the mutant TPL, and the nucleotide sequences of the selected genes were determined using Sanger's method (ABI PRISM 3100 Automatic DNA Sequencer, Applied Biosystems, U.S.A.).

Production and Purification of Mutant TPL

The recombinant *E. coli* DH5α harboring either plasmid pHCE19T(II)-TTPL or pHCE19T(II)-A13V was cultivated in an LB-amp medium overnight, and the cells were harvested by centrifugation at 5,000 rpm for 10 min and washed with 100 mM potassium phosphate buffer (pH 8.0). After centrifuging and suspending the cells in the same buffer containing 2 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF), they were disrupted by sonication with a SONICATOR W-380 (Heat Systems-Ultrasonics, U.S.A.). The cell lysate was obtained by centrifugation at 13,000 rpm for 20 min, then the supernatant was heated at 60°C for 30 min to remove any heat-labile *E. coli* proteins and cooled on ice. After removing the protein aggregates by centrifugation at 13,000 rpm for 20 min, the soluble proteins were precipitated by the addition of ammonium sulfate up to 30-35% saturation. Next, the precipitates were collected by centrifugation at 13,000 rpm for 20 min, then the pellets were dissolved with 50 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a Resource Q column (6 ml; Pharmacia, Sweden) preequilibrated with 50 mM Tris-HCl (pH 8.0), then the enzyme was eluted with a linear gradient of potassium chloride formed from 0 to 1 M at room temperature (flow rate: 1 ml/min). The active fractions were collected and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). The protein concentration was measured by Bradford's dye binding method using a protein assay kit (Bio-Rad Lab., Richmond, U.S.A.) with bovine serum albumin as the standard [14].

Enzyme and Protein Assay

The TPL activity was assayed by measuring the amount of phenol formed from L-tyrosine. The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.2), 0.2 mM L-tyrosine, 0.1 mM PLP, and an appropriate amount (5-10 μg) of the enzyme in a total volume of 1 ml. The reaction was carried out at 60°C for 10 min and stopped by the addition of 5 M potassium hydroxide, then 1.5% $NaHCO_3$, 0.85% 4-aminoantipyrine, and a 5.4% $K_3Fe(CN)_6$ solution were added in that order. The amount of phenol formed was determined by measuring absorbance at 505 nm.

Thermostability and Denaturant-Tolerance of Enzyme

To determine the thermostability of the enzyme, the purified enzyme solution was heated in 50 mM potassium phosphate buffer (pH 7.2) for 30 min at various temperatures, and the remaining activity was measured. To determine the denaturant-tolerance of the enzyme, the purified enzyme was incubated with 0-7 M urea at 25°C. After 18 h of incubation, appropriate samples were taken and the residual activity was measured. In each experiment, the same amount of purified enzyme was used for the comparison between the wild-type and mutant TPL.

RESULTS AND DISCUSSION

Shuffling and Screening of Mutant TPL from *S. toebii*

DNA shuffling was carried out to improve the stability of TPL, as described in Materials and Methods. To assess the

mutagenesis rate of the DNA shuffling protocol and the fidelity of every shuffled mode, unscreened clones were randomly selected for sequencing (data not shown). The overall mutation rate of the current library was within 0.05-0.5%, making it useful for screening.

After amplifying the shuffled genes using a PCR, 1.4 kb of amplified DNA fragments digested with *Eco*RI and *Hin*dIII were ligated with a high-level constitutive expression vector, pHCE19T(II), and the resulting plasmids were transformed into *E. coli* DH5α to construct a mutant library. Among the variants, about 20,000 clones were selected and their TPL activities were measured using the colorimetric method for phenol detection after heat-treatment at 75°C for 30 min in a 96-well plate. Among the 20,000 clones, about 0.2% of the clones revealing a higher activity than the wild-type were selected from the first screening. In order to precisely determine the stability increase of the mutant clones, a second-round screening was performed using the cell lysate and the purified enzyme. Thereafter, one variant exhibiting the highest activity was finally selected from the first screened pool and its DNA was sequenced (Fig. 1). The DNA sequence analysis revealed that the 38th nucleotide was changed from cytosine (C) to thymine (T), resulting in the substitution of valine (V) for alanine (A) in the 13th amino acid, and the resultant mutant TPL was named A13V.

Expression and Purification of Mutant TPL, A13V, in Recombinant *E. coli*

To express the mutant TPL, *E. coli* DH5α containing pHCE(II)A13V harboring the mutant TPL gene was cultured in 1 l of an LB medium containing 100 μg/ml of ampicillin at 37°C without any inducer. After cultivating overnight, the mutant TPL was expressed in more than 50% of the total protein, whereupon the transformant exhibited TPL activity, and the purified mutant TPL revealed a molecular weight of about 50 kDa on 12% SDS-PAGE (Fig. 1).

To purify the mutant TPL, the A13V cultured recombinant *E. coli* was sonicated and the soluble fraction was collected. TPL activity was retained in the soluble fraction and exhibited more than 90% of the total activity of the crude extract after incubation at 60°C for 30 min, and after up to 80% of the heat-liable proteins was removed (Fig. 1). After heat treatment of the crude extract, the TPL was purified to a greater than 99% purity by ammonium sulfate

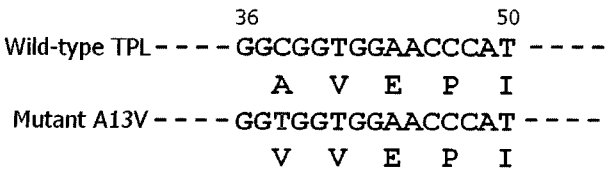

Fig. 1. Alignment of mutant A13V with wild-type TPL.

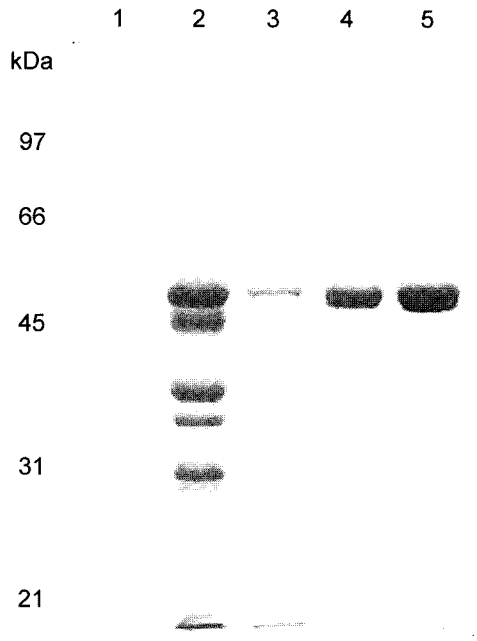

Fig. 2. Purification of A13V from *E. coli*.
Lane 1, M.W. marker; lane 2, cell-extract of *E. coli* DH5α harboring pHCE19T(II)-A13V; lane 3, heat-treated supernatant; lane 4, pellets precipitated with 30% $(NH_4)_2SO_4$; lane 5, purified enzyme after Resource Q column chromatography.

precipitation and ion-exchange column (Resource Q) chromatography. It was also found that A13V sustained activity longer than the wild-type TPL when stored at 4°C (data not shown).

The current authors previously reported on the kinetics of the wide-type TPL from *S. toebii* for which the K_m, V_{max}, and k_{cat}/K_m of L-tyrosine were 0.19 mM, 2.1 mmol/min/mg, and 9.5 $s^{-1}mM^{-1}$, respectively [10]. The mutant TPL, A13V, also revealed similar kinetic parameters, supporting the theory that the substitution of valine for alanine affected the enzyme's stability rather than its activity. Thus, it is expected that the current study will contribute to further research on the structural stability of TPL.

Physical Characteristics of Mutant TPL, A13V

The purified enzyme was incubated at various temperatures for 30 min in a 50 mM potassium phosphate buffer, and the remaining TPL activity was measured to evaluate the thermostability (Fig. 3). In previous research by the current authors, the wild-type TPL from *Symbiobacterium toebii* was found to be stable up to 70°C [10]. Similarly, in the current study, when the enzymes were incubated at various temperatures for 30 min, the wild-type TPL from *Symbiobacterium toebii* remained stable up to 70°C, then rapidly lost its activity above 70°C. However, the mutant TPL, A13V, still retained about 80% of its activity at 74°C, then gradually lost its activity above this temperature. While the wild-type TPL exhibited half of its activity at

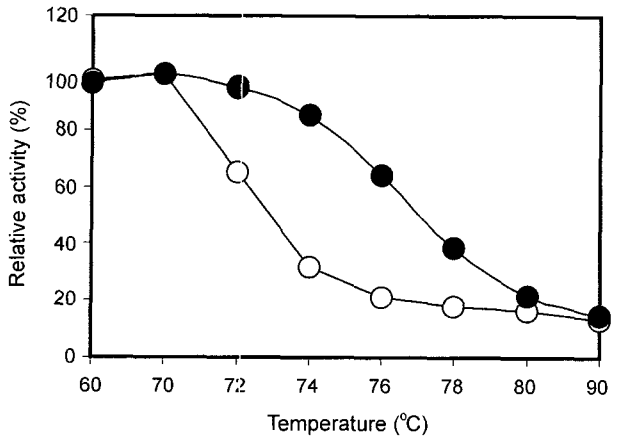

Fig. 3. Thermostability of wild-type TPL (open circle) and mutant A13V (filled circle).
See Materials and Methods for details.

73°C, the mutant TPL, A13V, exhibited half of its activity at about 77.4°C, indicating that the thermostability of A13V was about 4.3°C higher. This result supports the theory that the substitution of the 13th amino acid in TPL with valine instead of alanine enhanced the thermostability of the enzyme. Antson *et al.* [1] reported that the TPL of *C. freundii* was a tetramer and the binding site of the coenzyme, pyridoxal-5-phosphate (PLP), was lysine at position 257 (K257). They also reported that a single subunit consisted of four compartments, an N-terminal arm, small and large domains, and a link region of two domains. On the basis of these results, the substitution of the 13th amino acid in TPL with valine instead of alanine (A13V) was the apparent site in the N-terminal arm that formed a contact region between the subunits by generating an antiparallel β-strain structure. As such, the A13V substitution in the contact region of the N-terminal arm possibly increased the binding force between the subunits, thereby enhancing the thermostability of the TPL.

This substitution also influenced the denaturant tolerance of the TPL. As shown in Fig. 4, A13V exhibited half of its activity on about 3 M of urea, whereas the wild-type TPL almost lost its activity on the same concentration of urea, representing a 50% increase in denaturant tolerance from 2 M (wild-type TPL) to 3 M (mutant A13V).

Furthermore, it is speculated that the substitution of Val for Ala located in the β-strand of the N-terminal arm was also responsible for the heightened stabilization.

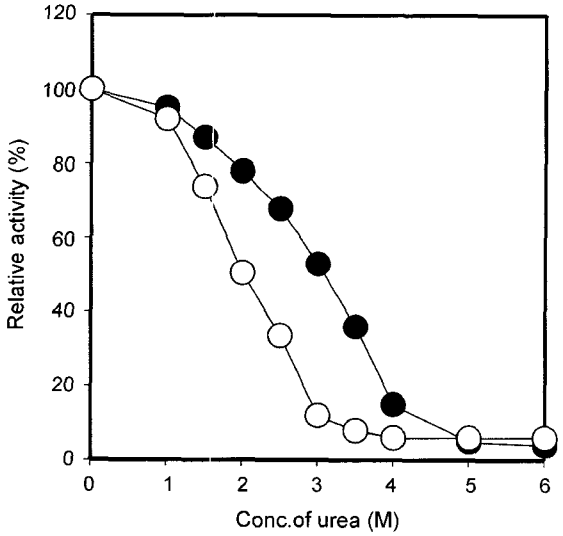

Fig. 4. Denaturant tolerance of wild-type TPL (open circle) and mutant A13V (filled circle).
See Materials and Methods for details.

Acknowledgments

This work was supported by grant CCW0100311 from the Korean Ministry of Commerce, Industry, and Energy and also partially supported by National Research Laboratory (NRL) program grant NLM0020123 from the Korean Ministry of Science and Technology.

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