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Screening and Purification of a Novel Transaminase Catalyzing the Transamination of Aryl β-Amino Acid from *Mesorhizobium* sp. LUK

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Abstract *Mesorhizobium* sp. LUK, which utilizes 3-amino-3-phenylpropionic acid as the sole source of nitrogen with high enantioselectivity (E(*S*)>100), was isolated using enrichment culture. The enzyme involved in the utilization of (*S*)-3-amino-3-phenylpropionic acid was confirmed to be a transaminase and was purified by 235-folds with a specific activity of 0.72 U/mg. The molecular weight of the purified protein was ca. 47 kDa and the active enzyme was determined as a dimer on gel filtration chromatography. The N-terminal sequence was obtained from the purified protein. Spontaneous decarboxylation of produced β-keto acids was observed during the chiral resolution of 3-amino-3-phenylpropionic acid.

Key words: Transaminase, β-amino acid, 3-amino-3-phenylpropionic acid, *Mesorhizobium* sp. LUK

β-Amino acids exist in nature as metabolites in mammalian and in lower organisms and also as a component of peptidic or nonpeptidic molecules [4, 9]. The pharmacological activities and the relative stability of β-amino acids in peptidic molecules have been attracting increasing attention for the synthesis of β-amino acids [1, 11, 12, 14]. Achiral and enantioselective syntheses of β-amino acids have been extensively studied chemically [7, 9, 13] and biocatalytically [10]. Among the biocatalytic methods, we have focused on the study of transaminases, as the transaminase reaction showed rapid reaction rates without the requirement for external cofactor regeneration, and it allows asymmetric synthesis from prochiral ketone compounds [17, 18]. As the aryl β-amino acids are the important chemical intermediates for pharmaceuticals, the transamination of 3-amino-3-phenylpropionic acid has been introduced by yet uncharacterized cell extracts from *Alcaligenes eutrophus* and *Variovorax paradoxus*, respectively [2]. Although we have reported an ω-transaminase of *Acaligenes denitrificans* that can catalyze the transamination of β-amino acids [22], its substrate specificity is strictly limited to small aliphatic substrates.

We performed an enrichment culture to isolate new bacteria that can catalyze the transamination of aryl β-amino acid. Enrichment cultures were prepared with domestic soil samples using minimal medium containing 10 mM racemic 3-amino-3-phenylpropionic acid as the sole nitrogen source [22]. After several rounds of enrichment culture, we found a strain showing the ability to use β-amino acid as a nitrogen source. This strain was designated as strain LUK and deposited to the Korea Culture Center of Microorganism (KCCM-10752P). Strain LUK was identified as *Mesorhizobium* sp. by sequencing 16S rRNA. The top three sequences using BLAST showed to have 99% 16S rRNA sequence identity with an uncultured bacterium gene for the 16S rRNA of RB379 (identity=1,348/1,357, gi: 81230222), *Mesorhizobium* sp. 'Smarlab BioMol-2302657' 16S rRNA gene (identity=1,318/1,321, gi: 29568986), and *Mesorhizobium* sp. casi-1 16S rRNA gene (identity=1,342/1,357, gi: 45686251).

Cell extract from strain LUK (0.005 U/ml, final concentration) was used for the resolution of 1 ml of 10 mM racemic 3-amino-3-phenylpropionic acid, using 10 mM pyruvate as the amine acceptor. One unit (U) of enzyme activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol of L-alanine from 10 mM racemic 3-amino-3-phenylpropionic acid and 10 mM pyruvate for 1 min in 100 mM phosphate buffer (pH 7.0) at 37°C. Separation of alanine and each enantiomer of racemic 3-amino-3-phenylpropionic acid was usually achieved on a C_{18} Symmetry column (Waters, MA, U.S.A.) with a Waters HPLC system after the derivatization of sample with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [6]. Samples were eluted with 20 mM of

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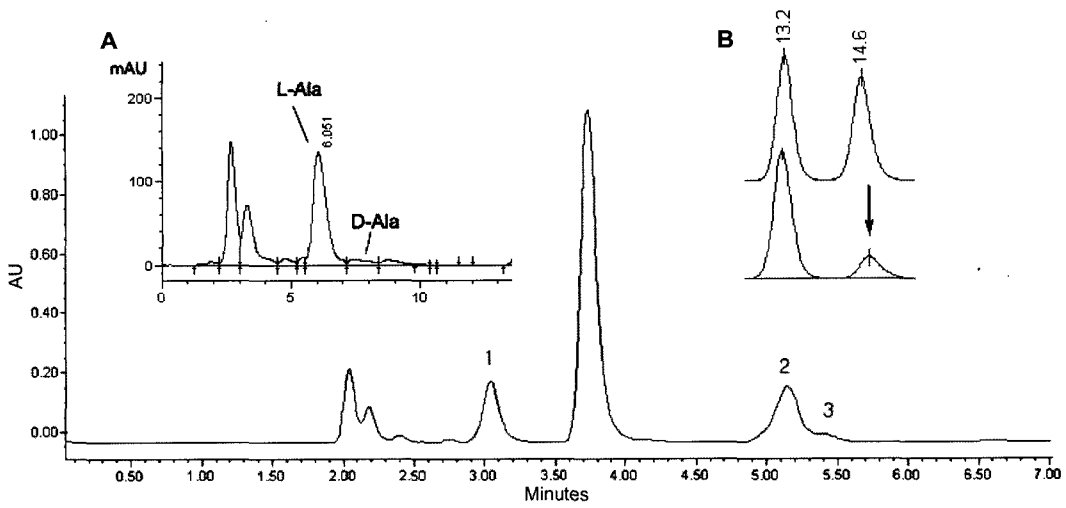

Fig. 1. Chirality analysis after the derivatization of reaction mixture with cell extract.
(1) Alanine; (2) (*R*)-3-amino-3-phenylpropionic acid; and (3) (*S*)-3-amino-3-phenylpropionic acid. In small window **A**, formation of L-alanine was analyzed, separately. In small window **B**, selective decrease of (*S*)-3-amino-3-phenylpropionic acid (14.6 min) was analyzed from the reaction mixture with purified protein. In this window, samples were analyzed on Supelco Discovery HSF5 (4.6×150 mm, 5 μm). Columns were eluted with a mixture of potassium phosphate buffer (pH 6.4, 20 mM) and acetonitrile (2:1, v/v) at 1 ml/min. However, alanine was not observable in this condition, because its peak was overlapped with impurity from GITC.

phosphate buffer (pH 6.3) and acetonitrile [60:40 (v/v)] with flow rate of 1 ml/min, observing at 250 nm. Analyzing the reaction mixture, we found that the reaction showed enantioselectivity toward (*S*)-3-amino-3-phenylpropionic acid (peak 3, Fig. 1) without a significant decrease in (*R*)-3-amino-3-phenylpropionic acid (peak 2, Fig. 1). High enantioselectivity (E>100) was observed, which is beneficial for efficient use as a biocatalyst (Fig. 2).

These results suggested that the strain LUK has β-amino acid transaminase activity using aromatic β-amino acid as

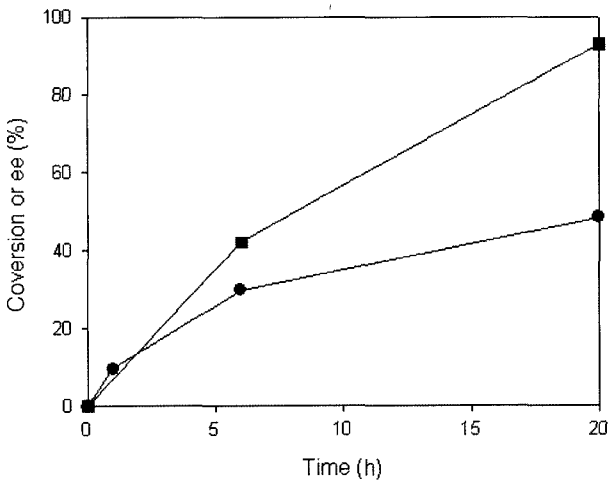

Fig. 2. Chiral resolution of racemic 3-amino-3-phenylpropionic acid using the cell extract of strain LUK.
(●) Conversion and (■) ee (%) from the reaction mixture with 5 mM of racemic 3-amino-3-phenylpropionic acid using 0.05 U of cell extract.

the the amino donor with (*S*)-enantiospecificity. To investigate whether or not the enzyme activity for the kinetic resolution of the racemic 3-amino-3-phenylpropionic acid is really transamination, the enzyme activity was examined using the crude extract of strain LUK in the presence of typical inhibitors of PLP-dependent enzyme [20]. The enzyme activity was inhibited by gabaculine, hydroxylamine, and aminooxyacetic acid. In addition, equivalent amounts of L-alanine (peak 1 and the small window of Fig. 1) were produced according to the consumed pyruvate and in stoichiometric amount of the reacted 3-amino-3-phenylpropionic acid. To analyze each enantiomer of alanine, a 66:34 (v/v) mixture of 20 mM of phosphate buffer (pH 6.3) and acetonitrile was used as eluent (Fig. 1A), since both enantiomers were not separable in the previous condition. Therefore, the enzyme involved in the kinetic resolution was confirmed to be a kind of transaminase with enantioselectivity to (*S*)-3-amino-3-phenylpropionic acid, producing L-alanine from pyruvate.

The transaminase showing activity to aryl β-amino acids has not been reported as being purified, thus we tried to purify the protein from the strain LUK. The strain LUK was generally grown at 30°C in LB medium with 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ (LBMC), to which 5 mM of racemic 3-amino-3-phenylpropionic acid and 1 g/l of yeast extract were added (supplemented LBMC) for the production of aromatic β-transaminase. Brief optimization of the culture media showed that the total activity of cell extract was ca. 10-folds higher in this supplemented LBMC than that grown in the same volume of plain LBMC. For further purification of the protein, strain LUK was precultured at

30°C in 50 ml of LBMC for 48 h in a 200-ml conical flask. Four liters of supplemented LBMC medium was inoculated with the preculture and the cultures were grown in a BioflolI (New Brunswick Scientific Inc., New Brunswick, NJ, U.S.A.). The air flow rate was 1.5 vvm and the agitation rate was 350 rpm while maintaining the temperature at 30°C. About 30 g of wet cell pellets were obtained by harvesting late-logarithimic phase cells at 8,000 ×*g* for 20 min.

For the purification of the transaminase, 15 g of wet cell pellets were resuspended in 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM of dithiothreitol (DTT), to give a final protein concentration of 15–25 mg/ml. To make cell extracts, the cells were broken by two passages through a French pressure cell at 18,000 pounds/square inch and the debris was removed by centrifugation at 17,000 ×*g* for 20 min. All purification steps were carried out at 4°C and were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Column chromatography was performed with the AKTA system (GE Healthcare Europe GmbH, Germany). The supernatant was fractionated by ammonium sulfate precipitation between 28–42% saturation. The fraction was loaded in a Q-Sepharose FF column (1.6 mm×16 cm, GE Healthcare) pre-equilibrated with 20 mM Tris-HCl (pH 7.4), and eluted with 20 mM Tris-HCl (pH 7.4) by using a 0–0.2 M linear gradient of KCl. Active fractions were pooled and concentrated with an Amicon PM-10 ultrafiltration unit (MWCO, 10 kDa). The concentrated active fraction was applied to a HiTrap Phenyl HP (1 ml, GE Healthcare) pre-equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 0.5 M ammonium sulfate. The proteins were eluted using a 0–100% linear gradient with 50 mM potassium phosphate buffer (pH 8.0) at 1.0 ml/min flow rate. The active fractions were pooled and concentrated with an Amicon PM-10 ultrafiltration unit. The transaminase was purified by these above procedures in 235-folds with 1.1% recovery yield (Table 1). The purified enzyme showed the same enantioselectivity with cell extract, as shown in Fig. 1B. The protein gave a near homogeneous single protein band on SDS-PAGE with an estimated molecular mass of 47 kDa (Fig. 3). The molecular mass of the native protein was estimated to be ca. 90 kDa by gel filtration chromatography, which suggested a dimeric structure of native protein. The isoelectric point of the native protein was determined to be pI 5.1.

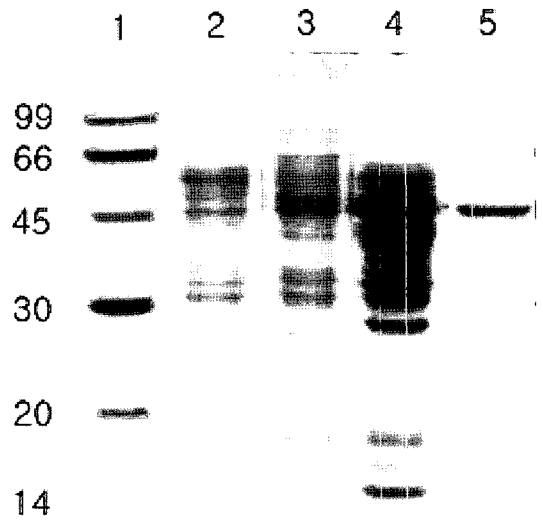

Fig. 3. SDS-PAGE of protein samples from the different stages of purification of transaminase from *M.* sp. LUK.
Proteins were separated on a 10% polyacrylamide gel in the presence of 1% SDS. Lanes; (1) Molecular weight standard proteins (from the top, 97.4, 66, 45, 31, 21.5, 14.4 kDa), (2) cell extract, (3) ammonium sulfate fraction (28–42%), (4) after Q Sepharose, and (5) after HiTrap Phenyl HP.

To determine the N-terminal amino acid sequence, the protein band of the enzyme purified on a 10% SDS-PAGE gel was transferred to PVDF membrane using a Trans-blot Cell system (Bio-Rad). The band corresponding to the enzyme was excised and used for automated Edman degradation with a Procise 492 clc protein sequencer (Applied Biosystems, CA, U.S.A.) at the Institute of Korea Basic Science Institute (Seoul Center, Korea). The sequence of 23 amino acid residues (MNEPIGEPXRSPAXDTAEK-AQXI) was obtained from N-terminal sequencing of the purified transaminase. However, it was unable to classify this protein according to the obtained sequence [3, 8, 15] because of the relatively diverse N-terminal sequences of the transaminase family. It is interesting that the new transaminase was found in *Mesorhiozobium* sp., because this strain belongs to collective rhizosphere bacteria that perform nitrogen-fixing symbioses with leguminous plants [19, 23]. A brief search of reference sequences on NCBI showed that one of the rhizobia, *Mesorhizobium loti* MAFF303099, has a relatively large number of transaminases (83 reference sequences, summation of the number of protein functionally annotated transaminases and putative/

Table 1. Purification of aromatic β-transaminase from *M.* sp. LUK.

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification fold
Cell extract	1,620	0.00303	4.91	100	-
Ammonium sulfate (28–42%)	261	0.0114	2.96	60	3.7
Q Sepharose FF	13.4	0.165	2.21	45	54
HiTrap Phenyl HP	0.076	0.72	0.054	1.1	235

probable transaminases) comparing to *Pseudomonas putida* KT2440 (58 reference sequences), *Salmonella enterica* subsp. *enterica* serovar Typhi Ty2 (33 reference sequences) and *Bacillus subtilis* subsp. *subtilis* str. 168 (45 reference sequences). This suggested that the relatively large pool of functionally diverse transaminases could be available from rhizosphere bacteria.

In this article, we report the screening of a new transaminase that can catalyze the chiral resolution of aryl β-amino acids. We also successfully purified the protein to near homogeneity and determined its N-terminal sequence, but the sequence data was still not enough to characterize the protein. However, this is the first report of a purified protein showing transaminase activity to aryl β-amino acids. The chiral resolution of β-amino acids is quite interesting, as the chiral resolution of β-amino carboxylic acid produced β-keto carboxylic acid as a reaction product. This β-keto carboxylic acid can readily lose the carboxyl group due to spontaneous decarboxylation [5]. Separation of racemic 3-amino-3-phenylpropionic acid, 3-keto-3-phenylpropionic acid, and acetophenone was achieved with the underivatized aliquot of sample using a C_{18} Symmetry column with an isocratic elution with water and acetonitrile [90:10 (v/v), 0.15% (w/v) trifluoroacetic acid, 1 ml/min], observing at 250 nm. Our analysis of the reaction mixture with racemic 3-amino-3-phenylpropionic acid in the middle of the reaction showed the generation of 3-keto-3-phenylpropionic acid along with acetophenone, a decarboxylated product from the corresponding β-keto acid (Fig. 4). This can be advantageous for the resolution reaction, by shifting the reaction equilibrium to the forward reaction. As transaminases usually have substrate and product inhibition by various ketone compounds [16, 21], it would be interesting to follow the reaction profile with a different temperature that can change the degree of decarboxylation from the product β-ketocarboxylic acid.

Partial 16S rRNA Sequence

The partial 16S rRNA sequence of *Mesorhizobium* sp. LUK was determined at the Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea) and was deposited in the GenBank nucleotide sequence database under accession No. DQ839129.

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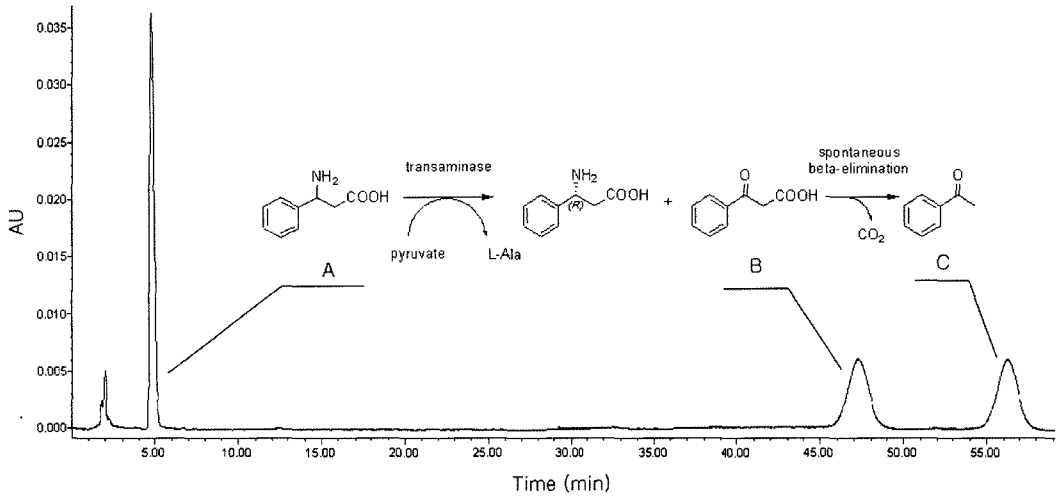

Fig. 4. Reaction scheme and the analysis of reaction products.
Racemic 3-amino-3-phenylpropionic acid (**A**), 3-keto-3-phenylpropionic acid (**B**), and acetophenone (**C**).

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