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ORIGINAL PAPER

A novel transaminase, (*R*)-amine:pyruvate aminotransferase, from *Arthrobacter* sp. KNK168 (FERM BP-5228): purification, characterization, and gene cloning

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Abstract A novel (*R*)-amine transaminase, which catalyzed (*R*)-enantioselective transamination of chiral amine, was purified to homogeneity from *Arthrobacter* sp. KNK168 (FERM BP-5228). The molecular mass of the enzyme was estimated to be 148 kDa by gel filtration and 37 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis, suggesting a homotetrameric structure. The enzyme catalyzed transamination between amines and pyruvate stereo-specifically. The reaction on 1-methylbenzylamine was (*R*)-enantioselective. Pyruvate was the best amino acceptor, but the enzyme showed broad amino acceptor specificity for various ketone and aldehyde compounds. The apparent K_ms for (*R*)-1-methylbenzylamine and pyruvate were 2.62 and 2.29 mM, respectively. The cloned gene of the enzyme consists of an open reading frame (ORF) of 993 bp encoding a protein of 330 amino acids, with a calculated molecular weight of 36,288. The deduced amino acid sequence was found to be homologous to those of the aminotransferases belonging to fold class IV of pyridoxal-5′-phosphate-dependent enzymes, such as branched-chain amino acid aminotransferases.

Keywords Chiral amine · Biocatalysis · Transaminase · Aminotransferase

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Introduction

Chiral amines are important chemicals as they are the starting materials for the synthesis of optically active pharmaceuticals and agrochemicals. Therefore, the development of economical and efficient production procedures for chiral amines is necessary. Aminotransferases or transaminase has been studied extensively with the intent of applying them either to the kinetic resolution of the racemic amines or the asymmetric amination of the ketone compounds.

ω-Amino acid aminotransferases from *Bacillus megaterium*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* could catalyze the (*S*)-enantioselective deamination of 1-phenyl-3-aminobutane (Stirling 1992). It was reported that these ω-amino acid aminotransferases could be used in the kinetic resolution of racemic amines by enantioselective deamination and in asymmetric synthesis by enantioselective amination (Stirling 1990). Many ω-transaminases, which can be used for the preparation of chiral amines, have been reported (Hanson et al. 2008; Kaulmann et al. 2007; Shin and Kim 1997; Shin and Kim 1999; Shin and Kim 2001; Yun et al. 2004a,b). However, most of the reported enzymes showed (*S*)-enantioselectity. Thus, an (*R*)-enantioselective enzyme has been highly desired. More recently, a trial for searching of (*R*)-amine transaminase activity was reported using in silico screening based on molecular modeling and sequence-based prediction (Höhne et al. 2010).

In previous reports, we isolated a microorganism, *Arthrobacter* sp. KNK168 (FERM-BP-5228), that exhibited (*R*)-enantioselective transaminase activity on secondary amines (Iwasaki et al. 2003) and successfully applied it in the asymmetric synthesis of several kinds of chiral amines, such as (*R*)-dimethoxyamphetamine (DMA), (*R*)-4-methox-

yamphetamine, (*R*)-1-(3-hydroxyphenyl)ethylamine and (*R*)-1-(3-methoxyphenyl)ethylamine (Iwasaki et al. 2006).

In this paper, we describe the purification, characterization, and gene cloning of the (*R*)-transaminase [(*R*)-TA] from *Arthrobacter* sp. KNK168 (FERM BP-5228].

Materials and methods

Chemicals

3,4-Dimethoxyphenylacetone and (*R*)-methylbenzylamine were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). PRO-EX(A12P), acid hydrolysate of corn gluten, was purchased from Bansyu Choumiryou Co., Ltd. (Japan). All other chemicals used in this study were of analytical grade and are commercially available.

Microorganisms and culture conditions

Arthrobacter sp. KNK168 was isolated from soil sample (Iwasaki et al. 2006) and deposited as a collection number of FERM BP-5228 in NPMD (Nite Patent Microorganisms Depositary; http://www.nbrc.nite.go.jp/npmd/e/).

Arthrobacter sp. KNK168 was cultivated with J medium [40 g glycerol, 3 g yeast extract, 20 g PRO-EX(A12P), 1 g NaCl, 5 g KH_2PO_4, 5 g K_2HPO_4, 1 g $MgSO_4{\cdot}7H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.001 g $ZnSO_4{\cdot}7H_2O$, 0.001 g $MnCl_2{\cdot}4H_2O$ in 1 l of the medium, pH 7.2] in a 5-l fermentor (B.E. Marubishi, Japan) as described previously (Iwasaki et al. 2006).

Escherichia coli HB101 carrying pAT28(pNTAT) was cultured in 10 ml of 2Y°°°T medium (15 g Bacto tryptone, 10 g Bacto yeast extract, 5 g NaCl in 1 l of medium, pH 7.2) containing 100 mg/l of ampicillin in a test tube at 37°C for 24 h with shaking. The cells were harvested by centrifugation, then suspended in 10 ml of 100 mM potassium phosphate buffer (pH 7.2), followed by disruption by sonication under cooling. After the cell residue was removed by centrifugation, the resultant supernatant was used as the cell-free extract.

Purification of (*R*)-TA from *Arthrobacter* sp. KNK168

All purification procedures were carried out at 0–5°C.

Step 1: preparation of the cell-free extract

The washed cells obtained from 1.5 l of cultured broth were suspended in 1 l of 20 mM potassium phosphate buffer (pH 6.8) containing 0.01% 2-mercaptoethanol and disrupted with 0.45-mm-diameter glass beads under cooling, and the supernatant was then obtained by centrifugation. Protamine sulfate was added to the supernatant to give a concentration of 50 g/l, and the precipitate was removed by centrifugation.

Step 2: ammonium sulfate precipitation

The cell-free extract was fractionated by ammonium sulfate precipitation. The precipitate obtained at 30–60% saturation was collected by centrifugation and dissolved in 30 ml of 20 mM potassium phosphate buffer (pH 6.8) containing 0.01% (*w*/*v*) 2-mercaptoethanol, and then the solution was dialyzed against the same buffer.

Step 3: DEAE-Sepharose chromatography

The dialyzate was applied to a diethylaminoethyl (DEAE)-Sepharose column (4.4×20 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 20% (*v*/*v*) glycerol and 0.3 M NaCl. The enzyme was eluted with a linear NaCl gradient (0.3–0.5 M).

Step 4: Phenyl-Sepharose chromatography

To the active fractions obtained by DEAE-Sepharose chromatography, ammonium sulfate was added to give a concentration of 0.2 M. The enzyme solution was charged on a Phenyl-Sepharose column (2.2×17 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 20% (*v*/*v*) glycerol and 0.2 M ammonium sulfate and eluted with a linear ammonium sulfate gradient (0.2–0 M).

Step 5: Butyl-Sepharose chromatography

To the active fractions obtained by Phenyl-Sepharose chromatography, ammonium sulfate was added to give a concentration of 0.6 M. The enzyme solution was applied to a Butyl-Sepharose column (2.2×17 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 20% (*v*/*v*) glycerol and 0.6 M ammonium sulfate and eluted with a linear ammonium sulfate gradient (0.6–0.2 M). The active fractions were collected and used as the purified enzyme for characterization.

Molecular mass measurement

The molecular mass of the native enzyme was estimated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Sweden) equilibrated with 50 mM potassium phosphate buffer containing 150 mM NaCl. The molecular mass of the subunit was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel as described by Laemmli (1970).

Enzyme assay

The (*R*)-DMA synthesis activity was assayed as follows. The reaction mixture (200 μl) comprising 20 mM 3,4-dimethoxyphenylacetone, 20 mM (*R*)-1-methylbenzylamine, 20 mM pyridoxal-5′-phosphate (PLP), and the enzyme in 50 mM Tris–HCl buffer (pH 8.5) was incubated at 30°C for 60 min, and then 800 μl of 3.1% (*w*/*v*) trichloroacetic acid was added to the reaction mixture to terminate the reaction. The amount of DMA formed was determined by high-performance liquid chromatography (HPLC) as described previously (Iwasaki et al. 2006). One unit of the enzyme was defined as the amount catalyzing the formation of 1 μmol of DMA per minute.

The (*R*)-1-methylbenzylamine deaminating activity was assayed as follows. The reaction mixture (250 μl) comprising 25 mM (*R*)-1-methylbenzylamine, 25 mM pyruvate, 0.1 mM PLP, and the enzyme in 100 mM Tris–HCl buffer (pH 8.5), was incubated at 30°C for 60 min, and the reaction was stopped by dipping the reaction tube into boiling water. The amount of acetophenone formed was determined by HPLC.

Substrate specificity

Amino donor specificity was assayed as follows. The reaction mixtures (300 μl) comprising 25 mM pyruvate, 25 mM of one of the amino compounds listed in Table 2, 0.1 mM PLP, and the enzyme in 100 mM Tris–HCl buffer (pH 8.5) in a MICRO TUBE (Iwaki Glass, Japan) were incubated at 30°C for 60 min. The reaction was stopped by heating at 100°C for 10 min, and then the amount of alanine formed was determined by HPLC and thin-layer chromatography (TLC).

Amino acceptor specificity was assayed as follows. The reaction mixture (250 μl) comprising 25 mM (*R*)-1-methylbenzylamine, 25 mM of one of the carbonyl compounds listed in Table 3, 0.1 mM PLP, and the enzyme in 100 mM Tris–HCl buffer (pH 8.5) was incubated at 30°C for 60 min. Afterward, the reaction was stopped by heating at 100°C. The amount of acetophenone formed was assayed by HPLC.

Measurement of K_m

The effects of the concentration of 1-methylbenzylamine and pyruvate on the transaminase activity were examined. The enzyme was incubated with one substrate at various concentrations and the other substrate at a fixed concentration of 20 mM. Based on Lineweaver–Burk plotting, the apparent K_ms for 1-methylbenzylamine and pyruvate were calculated, respectively.

N-terminal and partial amino acid sequences

The purified enzyme was digested with TPCK trypsin in 50 mM Tris–HCl buffer (pH 8.0) containing 10 g/l of glycerol, 0.5 g/l of 2-mercaptoethanol, and 10 μM PLP for 24 h at 37°C. The digested fragments of the enzyme were separated by HPLC on a YMC-Pack PROTEIN-RP column (4.6×170 mm; YMC, Japan) equilibrated with 0.1% trifluoroacetic acid. The elution was carried out with a linear acetonitrile gradient (8–80%) at a flow rate of 1.0 ml/min. The amino acid sequences of these peptide fragments and the N-terminal region of the purified enzyme were analyzed on a model 470A gas-phase protein sequencer (Applied Biosystems, USA).

Cloning and sequencing of the (*R*)-TA gene

Chromosomal DNA of *Arthrobacter* sp. KNK168 was partially digested with *Sau*3AI. The partial *Sau*3AI digest was fractionated by agarose gel electrorophoresis, and the fractions containing fragments 6–12 kb in length were recovered from the gel. The DNA fragments were inserted into *Bam*HI-digested pUC19 with a DNA Ligation Kit ver. 2 (Takara-Bio, Japan). *E. coli* JM109 was transformed with the ligated plasmid and plated onto a Luria–Bertani agar plate (10 g Bacto tryptone, 5 g Bacto 1 yeast extract, 10 g NaCl, 15 g Agar in 1 l of the medium) containing 100 mg/l ampicillin, 40 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL), and 100 μM isopropyl-β-D-thiogalactopyranoside.

Degenerate PCR primers were designed on the basis of the N-terminal and internal amino acid sequences. The primers used were forward (5′ cgcggatccgarathgtntayacncayga 3′) and reverse (5′ acyttccattgrtangg 3′) where "n" indicates a, c, t, or g, "r" indicates a or g, "y" indicates c or t. PCR was carried out with these primers using chromosomal DNA of *Arthrobacter* sp. KNK168 as a template. EX Taq DNA polymerase (Takara-Bio, Japan) was used as a DNA polymerase, and the reaction composition was in accordance with the manufacturer's instructions. The amplification conditions were 30 cycles of 1 min at 94°C, 1 min at 44°C, 2 min at 68°C after the initial denaturation for 2 min at 94°C, followed by a final incubation for 5 min at 68°C. The amplified 450-bp DNA fragment was purified by agarose gel electrophoresis and cloned into pT7Blue Vector with a Regular pT7Blue T-vector Kit (Novagen, USA) to give pAT1. Colony hybridization was done in accordance with the standard methods (Sambrook et al. 1989). The probe used was a segment of the (*R*)-TA gene obtained by digestion of pAT1 with *Bam*HI and was labeled with [α-^{32}P]dCTP using the Random Primer DNA Labeling Kit ver. 2 (Takara-Bio, Japan). A positive colony of the recombinant *E. coli* JM109 was obtained from a population

of about 2,000 transformants. The plasmid, pAT11, isolated from the clone cells, had an approximately 8-kb chromosomal DNA fragment. The pAT11 was digested with various restriction enzymes, and the resulting fragments were analyzed to create a restriction enzyme cleavage map. These results suggested that an entire length of the (*R*)-TA gene is contained in the DNA fragment of 1.6 kb resulting from the digestion of pAT11 with *Pst*I. A 1.6-kb DNA fragment was inserted into the *Pst*I site of plasmid pUC19 to give the recombinant plasmid pAT22. A variety of restriction enzymes were reacted with pAT22, and the resultant DNA fragments were inserted into the multi-cloning site of pUC19 to obtain recombinant plasmids. Using these recombinant plasmids, the nucleotide sequence of the 1.6-kb DNA was analyzed.

Construction of an expression vector

To construct an expression plasmid, a 1.0-kb gene fragment comprised of the gene encoding (*R*)-TA and *Nde*I and *Bam*HI linkers was amplified by PCR using pAT11 as a template with the primers 5′-acacatatggcattcagcgccga tacctcc-3′, which was designed to contain the N-terminal region of (*R*)-transaminase gene and an NdeI digestion sequence, and 5′-agaggatcctcagtactgcacaggcgtaag-3′,which was designed to contain the C-terminal region and BamHI digestion sequence. The respective restriction sequences are underlined. The amplified 1.0-kb fragment was digested with *Nde*I and *Bam*HI and then inserted into the *Nde*I–*Bam*HI site of pUCNT (Nanba et al. 1994). The resultant plasmid, pAT28, was introduced into *E. coli* HB101.

Analysis

The protein concentration was measured by the methods of Bradford (1976). The UV–visible absorption spectrum of purified enzyme was measured with a Hitachi U-200 spectrophotometer (Hitachi, Japan) at room temperature. The amount of DMA was determined by HPLC as described previously (Iwasaki et al. 2006). The amount of acetophenone was determined by HPLC with a Finepack C_{18-5} (4.6×250 mm; Nippon Bunko, Japan). The HPLC conditions were methanol/water [20:80 (*v*/*v*)] as a mobile phase, a flow rate of 1 ml/min, and detection at 280 nm. Acetophenone was eluted at 16 min. To measure the amount of alanine, alanine was fluorescent-labeled with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (Dojindo Laboratories, Japan) according to the method of Imai and Watanabe (1981), and the fluorescent-labeled alanine (NBD-Ala) was detected by HPLC with a Finepack C_{18-5} column (4.6×250 mm; Nippon Bunko, Japan). The HPLC conditions were 100 mM potassium phosphate buffer (pH 6.5)/acetonitrile [20/80 (*v*/*v*)] as a mobile phase, a flow rate of 1 ml/min, excitation at 470 nm, and emission at 530 nm. NBD-Ala was eluted at 22 min. The amount of alanine was also measured by TLC. The plate was developed using n-butanol/acetic acid/water [4:1:1 (v/v)]. Alanine was detected using a ninhydrin solution. The optical purity of DMA was determined by HPLC as described in a previous paper (Iwasaki et al. 2003). The nucleotide sequences were analyzed with an ABI373A DNA Sequencer (Perkin-Elmer K.K., USA) using a Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer K.K., Japan).

Results

Purification and some properties of (*R*)-TA from *Arthrobacter* sp. KNK168

Purification of (*R*)-TA from *Arthrobacter* sp. KNK168 is summarized in Table 1. The enzyme was purified 94.3-fold in a 20.8% yield from the cell free extract. The purified enzyme showed a single band with a molecular mass of 37 kDa on SDS-PAGE (Fig. 1). The molecular mass of the native enzyme was about 148 kDa by gel filtration. These results suggested that the enzyme is composed of four identical subunits. The N-terminal amino acid residues of the purified enzyme were sequenced to be XXSADTSEI-VYTHDTGLDYITYSDY (X: not identified), and these were used for the cloning of the gene.

Table 1 Summary of (*R*)-TA purification from *Arthrobacter* sp. KNK168

Step	Total activity[a] (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	90.7	6,480	0.0140	100	1
Ammonium sulfate	59.1	2,240	0.0264	65.2	1.89
DEAE-Sepharose F.F.	38.1	73.8	0.513	42.0	36.6
Phenyl-Sepharose	20.6	17.0	1.21	22.7	86.4
Butyl-Sepharose	18.9	14.3	1.32	20.8	94.3

[a] Activity refers to the (*R*)-DMA synthesis activity

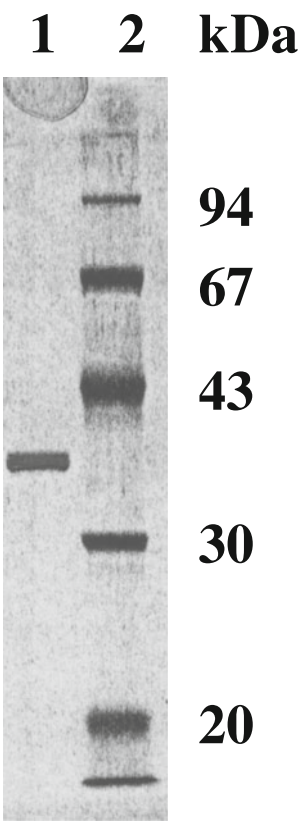


Fig. 1 SDS-PAGE of the purified enzyme. *Lane 1* Purified enzyme; *lane 2* molecular mass standard: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsine inhibitor (20 kDa)

The UV–visible absorption spectrum of (*R*)-TA is shown in Fig. 2. The absorption maxima of the enzyme were observed at 280, 340, and 410 nm. The addition of (*R*)-1-methylbenzylamine to the enzyme solution caused a decrease in absorbance at 410 nm and an increase at 340 nm, suggesting that the enzyme had pyridoxal phosphate (PLP) as the prosthetic group.

The effects of temperature and pH on the enzyme activity are shown in Fig. 3. The optimum temperature was around 30°C, and the activity decreased drastically at temperatures higher than 30°C. The activity was high under weak alkaline conditions, and the optimum pH was in the range between 8.0 and 9.0.

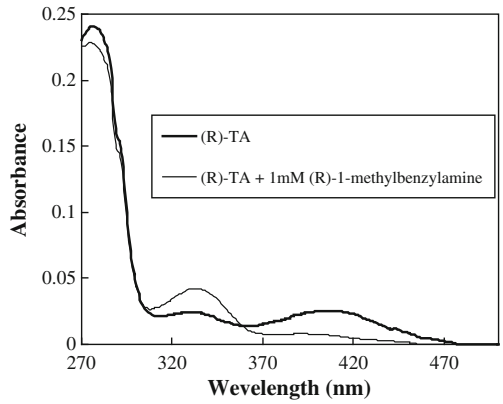


Fig. 2 UV–visible spectrum of the purified enzyme from *Arthrobacter* sp. KNK168

The effects of temperature and pH on the enzyme stability are shown in Fig. 4. The enzyme was stable at 30°C. However, the activity decreased with increasing temperature and was completely lost as a result of incubation at 60°C for 20 min. The enzyme was incubated in the pH range of 4.0–10.0 at 5°C for 3 days, and the residual activity was measured. The enzyme maintained an activity level of more than 90% of the initial level in the pH range of 6.0–8.0.

Substrate specificity

The amino donor specificity of the enzyme was examined with pyruvic acid as an amino acceptor (Table 2). The enzyme showed activity toward aliphatic or arylic chiral amines such as sec-butylamine, 2-aminoheptane, 2-aminooctane, 1-methylbenzylamine, 3,4-dimethoxyamphetamine, and 2-amino-4-phenylbutane. Among these, (*R*)-1-methylbenzylamine was the best amino donor. No detectable activity on (*S*)-1-methylbenzylamine and (*S*)-2-aminoheptane was observed, showing strict (*R*)-enantioselectivity toward the amino donor. Most simple amines, whose nitrogen atoms attached to the terminal carbon atoms, showed little reactivity. Typical substrates of ω-amino acid transaminase, such as β-alanine, 4-aminobutyric acid, 6-aminocaproic acid, putrescine, and taurine, were also inactive.

The amino acceptor specificity of the enzyme was examined with (*R*)-1-methylbenzylamine as an amino donor (Table 3). Pyruvic acid was the best amino acceptor among the carbonyl compounds tested. It is to be noted that several ketone and aldehyde compounds could also serve as amino acceptors.

We carried out further examinations of the substrate specificities (Table 4). No activities were detected on branched-chain amino acids or D-amino acids.

The effects of the substrate concentration of (*R*)-1-methylbenzylamine and pyruvic acid on the enzyme activity were examined. Based on the plotting of Lineweaver–Burk plots, the apparent K_ms for (*R*)-1-methylbenzylamine and pyruvic acid were calculated to be 2.62 and 2.29 mM, respectively .

Effects of chemicals

The effects of various compounds and metals on the enzyme activity were studied (Table 5). As expected, the enzyme was inhibited by carbonyl reagents, such as phenylhydrazine and hydroxylamine, which are known as the inhibitors of PLP-dependent enzymes. The enzyme was not sensitive to sulfhydryl reagents, such as idoacetic acid and *N*-ethylmaleimide.

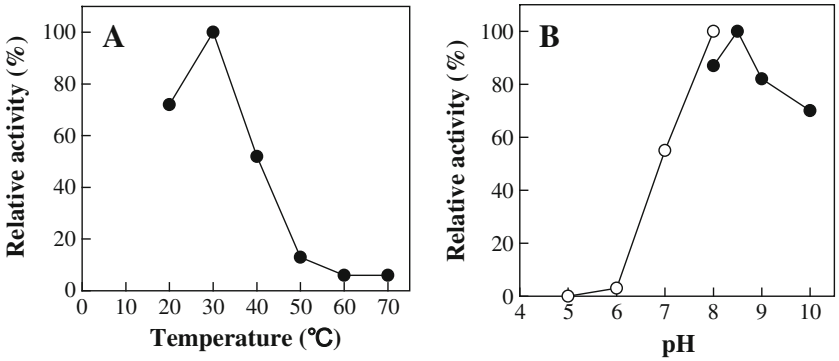


Fig. 3 Effects of temperature and pH on the enzyme activity. **a** The effects of temperature on the (*R*)-1-methylbenzylamine deaminating activity were examined at various temperatures. **b** The effects of pH on the (*R*)-1-methylbenzylamine deaminating activity were examined at various pHs. The buffers used were 100 mM potassium phosphate buffer (*closed circle*) and 100 mM Tris–HCl buffer (*closed circle*)

Synthesis of (*R*)-DMA with purified (*R*)-TA

We previously demonstrated the asymmetric synthesis of chiral amines from prochiral ketones with the cells or cell-free extract of *Arthrobacter* sp. KNK168 (Iwasaki et al. 2006). Here, the synthesis of (*R*)-DMA through asymmetric amination with the purified enzyme was examined. Fifty micromolars of 3,4-dimethoxyphenylacetone and 70 mM (*R*)-1-methylbenzylamine was incubated with the purified enzyme in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.02 mM pyridoxal phosphate at 30°C for 6 h. As a result, 35 mM (*R*)-DMA was formed. The enantiomer excess of (*R*)-DMA was more than 99%.

Cloning and sequence of the (*R*)-TA gene

The nucleotide sequence of 1.6-kb DNA fragment resulting from digestion of pAT11 with *Pst*I was analyzed. The nucleotide sequence containing of (*R*)-TA gene was submitted to DDBJ database (accession no. AB638718). The complete ORF of (*R*)-TA contained a DNA sequence of 993 bp, encoding a polypeptide of 330 amino acids. The calculated molecular weight of this protein was 36,288 Da, which showed a good agreement with molecular weight of 37,000 by SDS-PAGE. The ORF is initiated and terminated with a GTG and a TGA codon, respectively. The putative Shine–Dalgarno sequence was located nine bases upstream from the initiation codon.

The amino acid sequence of (*R*)-TA was compared with other protein sequences by a BLAST search in the databases of NCBI. The enzyme showed homology to aminotransferases belonging to PLP-dependent enzyme of fold class IV (Jansonius 1998), such as branched-chain amino acid aminotransferases (BCATs), throughout the large part of the sequence, and showed the following percentage identities to aminotransferase of fold class IV from the following microorganisms: *Mycobacterium vanbaalenii* PYR-1 (GenBank accession no. ABM15291; 52%), *Hyphomonas neptunium* ATCC15444 (GenBank accession no. ABI75539; 44%), and *Aspergillus fumigatus* Af293 (GenBank accession no. EAL86783; 42%). The linear alignment of (*R*)-TA and the three proteins mentioned above

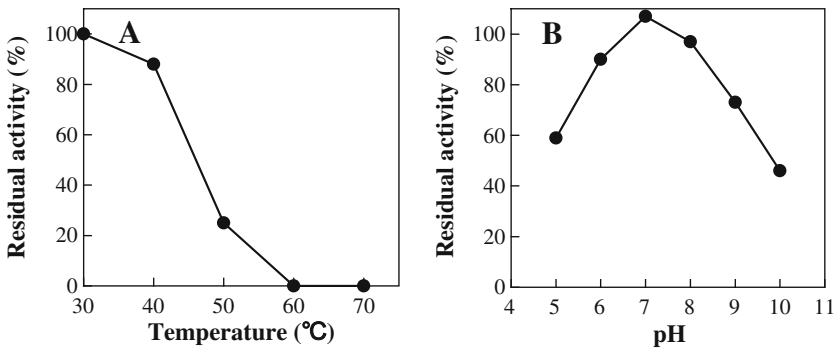


Fig. 4 Effects of temperature and pH on the enzyme stability. **a** The enzyme was incubated in 100 mM potassium phosphate buffer (pH 7.5) at various temperatures for 20 min, and the remaining (*R*)-1-methylbenzylamine deaminating activity was then measured as described in "Materials and methods." **b** The enzyme was incubated at various pH levels for 3 days and the remaining (*R*)-1-methylbenzylamine deaminating activity was then measured as described in "Materials and methods"

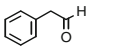
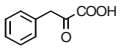
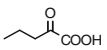
Table 2 Amino donor specificity of (*R*)-TA

Amino donor	Relative activity (%)[a]
Propylamine	0
Butylamine	0
Pentylamine	0
Benzylamine	1
β -Phenethylamine	0
Putressine	0
β -Alanine	0
4-aminobutyric acid	0
6-aminocaproic acid	0
Taurine	0
Isopropylamine	0
rac -sec-Butylamine	1
(*R*)-2-Aminoheptane	40
(*S*)-2-Aminoheptane	0
rac -2-Aminooctane	47
(*R*)-1-Methylbenzylamine	100
(*S*)-1-Methylbenzylamine	0
(*R*)-DMA	39
rac -2-Amino-4-phenylbutane	84
DL-Phenylglycine	0
DL-Phenylalanine	0

[a] Relative activity was calculated based on that of (*R*)-methylbenzylamine, which was taken as 100%

showed that these are homologous to each other (Fig. 5). On the other hand, apparent sequence homologies with (*S*)-selective ω-transaminases from *Alcaligenes denitrificans* Y2k-2 (Yun et al. 2004a,b), *B. megaterium* SC6394 (Hanson

Table 3 Amino acceptor specificity of (*R*)-TA

Amino acceptor	Relative activity (%)[a]	Amino acceptor	Relative activity (%)[a]
	100		13
	4		0
	7		83
	0		16
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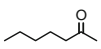
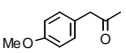
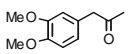
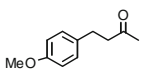
	0		2
	14		1
	7		0
	7		10

[a] Relative activity was calculated based on that of pyruvate, which was taken as 100%

et al. 2008), *Caulobacter crescentus* (Hwang et al. 2008), *Chromobacterium violaceum* DSM 30191 (Kaulmann et al. 2007), and *Vibrio fluvialis* JS17 (Shin et al. 2003) were not observed (data not shown).

Table 4 Activities on α-amino acids

Amino donor	Relative activity	
	Pyruvate	2-Ketoglutarate
(*R*)-Methylbenzylamine	100	0
D-Alanine	–	0
L-Alanine	–	0
DL-Isoleucine	0	0
L-Isoleucine	0	0
D-Leucine	0	0
L-Leucine	0	0
D-Valine	0	0
L-Valine	0	0
D-Phenylalanine	0	0
L-Phenylalanine	0	0
D-Glutamic acid	0	–
D-Aspartic acid	0	0
D-Asparagine	0	0

Table 5 Effects of chemical reagents on the transaminase activity

Compound	Relative activity (%)
None	100
Phenylhydrazine	31
Hydroxylamine	21
Idoacetic acid	109
N-Ethylmaleimide	100
DL-Penicillamine	107
EDTA	86
$CaCl_2$	120
$CuSO_4$	83
$MgSO_4$	119
$HgCl_2$	68
$ZnSO_4$	83
$MnCl_2$	87
$CoCl_2$	73
$BaCl_2$	108
$NiCl_2$	48
$AgNO_3$	66

The (*R*)-1-methylbenzylamine deaminating activity was measured in the presence of 1 mM of one of the compounds listed in the table

[a] Relative activities were calculated based on that without the compound listed in the table, which was taken as 100%

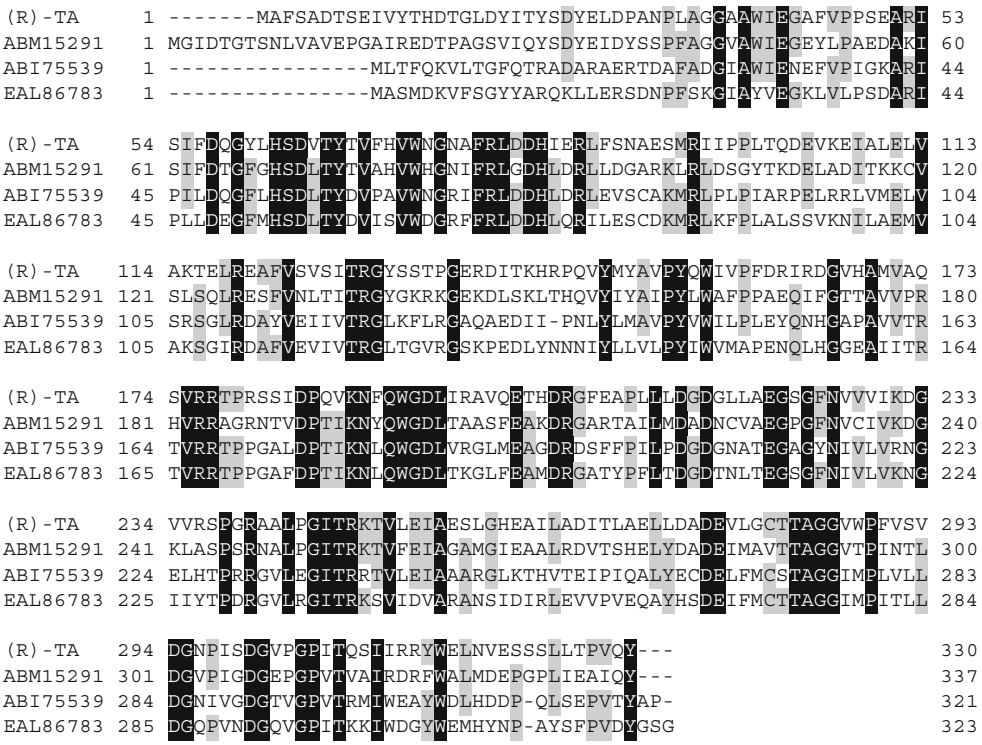


Fig. 5 Sequence alignment of (*R*)-TA and other proteins. Highly conserved residues are in *black* and less strongly conserved residues are in *gray* boxes. Proteins: (*R*)-TA, (*R*)-transaminase in this study; ABM15291, branched-chain amino acid: 2-keto-4-methylthiobutyrate aminotransferase from *Mycobacterium vanbaalenii* PYR-1; ABI75539, aminotransferase, class IV from *Hyphomonas neptunium* ATCC 15444; EAL86783, branched-chain amino acid aminotransferase, putative from *Aspergillus fumigatus* Af293

Expression of (*R*)-TA in *E. coli*

Using the expression vector plasmid pAT28, the (*R*)-TA gene was efficiently expressed in *E. coli*. The specific activity for (*R*)-1-methylbenzylamine deamination of cell-free extract of *E. coli* HB101 carrying pAT28 was 2.5 U/mg protein, which is 13-fold higher than that of cell-free extract of *Arthrobacter* sp. KNK168.

Discussion

In this study, we purified and characterized the (*R*)-amine transaminase [(*R*)-TA], which catalyzes (*R*)-enantioselective transamination, from *Arthrobacter* sp. KNK168. This enzyme was a tetramer composed of four identical subunits, each composed of 330 amino acids with a molecular mass of 36,288 Da. Based on the spectroscopic properties and inhibitory study, the enzyme is supposed to be a PLP enzyme.

The enzyme showed amino donor specificity toward aliphatic and arylic chiral amines. Among the amines tested, (*R*)-1-methylbenzylamine was the best amino donor. However, the enzyme did not act on the (*S*)-enantiomer of 1-methylbenzylamine and 2-aminoheptane, which indicates that the activity of this enzyme is highly (*R*)-enantioselectve. Pyruvate was the best amino acceptor among the carbonyl compounds tested.

The Michaelis–Menten constants for (*R*)-1-methylbenzylamine and pyruvate were 2.62 and 2.29 mM, respectively, indicating that those compounds are good substrates as an amino donor and amino acceptor for (*R*)-TA. However, the enzyme had broad specificity for amino acceptors and utilized a variety of carbonyl compounds as amino acceptors. This feature permits the enzyme to be used for the synthesis of various chiral amines from prochiral ketones. Enantioselective amination of 3,4-dimethoxyphenylacetone into (*R*)-DMA by the purified enzyme suggests that the ketones, used as amino acceptors, will be aminated enantioselectively into the (*R*)-form of the corresponding amines. We previously reported that *Arthrobacter* sp. KNK168 could catalyze the transamination of several kinds of prochiral ketones so as to convert them into the (*R*) form of the corresponding chiral amines (Iwasaki et al. 2006).

Some ω-amino acid transaminases or ω-transaminases were reported to act on chiral amines and to be used for the preparation of chiral amines. In general, most of those enzymes, such as from *A. denitrificans* Y2k-2 (Yun et al. 2004a,b), *Bacillus thuringiensis* JS64 (Shin and Kim 2001), *B. megaterium* SC6394 (Hanson et al. 2008), *C. crescentus* (Koszelewski et al. 2008), *C. violaceum* DSM30191 (Kaulmann et al. 2007), *Klebsiella pneumoniae* LS2F (Shin and Kim 1997), *Pseudomonas putida* ATCC 39213 (Stirling 1992), *Pseudomonas aeruginosa* ATCC 15692 (Stirling 1992), and *V. fluvialis* JS17 (Shin and Kim 2001) possess (*S*)-enantiopreference.

The aminotransferases are classified into four subgroups on the basis of their mutual structural relation. The (*R*)-TA from *Arthrobacter* sp. KNK168 showed homology in its

amino acid sequence to the aminotransferases belonging to PLP-dependent enzyme of fold class IV, such as BCATs. However, this enzyme did not show the activities on branched-chain amino acids and D-amino acids.

Based on the enzymatic properties and the amino acid sequence, it is concluded that (*R*)-TA is a novel enzyme. We termed (*R*)-TA "(*R*)-amine:pyruvate aminotransferase" on the basis of its substrate specificity. To the best of our knowledge, (*R*)-TA is the only naturally occurring (*R*)-enantioselective transaminase whose amino acid and nucleotide sequences were disclosed and enzymatic properties were well elucidated.

Enzyme catalysis is expected to be an alternative to traditional chemical catalysis due to its excellent chemo-, regio-, and enantio-selectivity. Bioprocesses using enzyme catalysis are usually performed under mild conditions and are environmentally friendly as compared with chemical processes. As in the preceding example, alcohol dehydrogenases have been applied in the industrial production of chiral alcohols (Kataoka et al. 2003; Hasegawa et al. 2010). A transaminase is also expected to be the enzyme used for the manufacture of chiral amine compounds. As mentioned above, most transaminases were (*S*)-enantioselective. Therefore, there has been a need for the discovery of a practical transaminase possessing (*R*)-enantioselectivity. (*R*)-TA from *Arthrobacter* sp. KNK168 possesses sufficient reactivity and enantioselectivity for asymmetric synthesis of our target amines such as (*R*)-DMA. We also established a practical production process for them using this enzyme. Thus, (*R*)-TA from *Arthrobacter* sp. KNK168 is a very attractive platform enzyme for the manufacture of various chiral amines. We have disclosed the amino acid and nucleotide sequences of this enzyme in a patent in advance of this report (Yamada et al. 2000). Once the platform enzyme is obtained, it can be evolved to meet the target compounds using protein engineering technologies if necessary. Recently, Christopher made an improvement to (*R*)-TA from *Arthrobacter* sp. KNK168 by mutation and developed it into (*R*)-enantioselective transaminase, which was optimized for the production of sitagliptin by protein engineering methods (Christopher et al. 2010). Protein engineering technologies will play an important role in the development of the bioprocess. However, the discovery of the appropriate platform enzyme is the most important factor in this development. Conventional screening methods, which are based on biodiversity, will serve as a significant role in the development of the bioprocess in the future.

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