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ORIGINAL PAPER

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Purification, characterization, and molecular cloning of a novel amine:pyruvate transaminase from *Vibrio fluvialis* JS17

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Abstract A transaminase from *Vibrio fluvialis* JS17 showing activity toward chiral amines was purified to homogeneity and its enzymatic properties were characterized. The transaminase showed an apparent molecular mass of 100 kDa as determined by gel filtration chromatography and a subunit mass of 50 kDa by MALDI-TOF mass spectrometry, suggesting a dimeric structure. The enzyme had an isoelectric point of 5.4 and its absorption spectrum exhibited maxima at 320 and 405 nm. The optimal pH and temperature for enzyme activity were 9.2 and 37°C, respectively. Pyruvate and pyridoxal 5′-phosphate increased enzyme stability whereas (S)-α-methylbenzylamine reversibly inactivated the enzyme. The transaminase gene was cloned from a *V. fluvialis* JS17 genomic library. The deduced amino acid sequence (453 residues) showed significant homology with ω-amino acid:pyruvate transaminases (ω-APT) from various bacterial strains (80 identical residues with four ω-APTs). However, of 159 conserved residues in the four ω-APTs, 79 were not conserved in the transaminase from *V. fluvialis* JS17. Taken together with the sequence homology results, and the lack of activity toward β-alanine (a typical amino donor for the ω-APT), the results suggest that the transaminase is a novel amine:pyruvate transaminase that has not been reported to date.

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Introduction

Transaminase has been studied extensively due to a high potential for use in the production of amino acids and chiral amines (Stirling 1992; Taylor et al. 1998; Chao et al. 1999). It is present ubiquitously in microorganisms as well as higher organisms, and plays an important role in amino acid metabolism (Christen and Metzler 1985; Stirling 1992). Despite many advantages, such as broad substrate specificity, high turnover rate, and high stability (Taylor et al. 1998), its industrial use has been limited, mainly due to the low equilibrium constant of the transaminase reaction. However, successful transaminase processes for production of unnatural amino acids and D-amino acids have been demonstrated recently (Bartsch et al. 1996; Stinson 1997; Taylor et al. 1998; Chao et al. 1999). In the latter cases, at least one of the products was removed to overcome the equilibrium problem by coupling other enzymes or by using whole cell biotransformation.

Transaminases can be classified into α- and ω-transaminase according to the relative position of the amino group to be transferred with respect to the carboxyl group of the substrate (Yonaha and Yonaha 1980; Yonaha et al. 1983a, 1983b; Christen and Metzler 1985; Yonaha et al. 1987). Unlike α-transaminase, which acts only on the α-amino group of α-amino acids, ω-transaminase transfers amino group on non-α positions, such as the β-amino group of β-alanine. Among the ω-transaminases, only ω-amino acid:pyruvate transaminase (ω-APT) has been reported to show catalytic activity toward aliphatic amines not bearing a carboxyl group (Yonaha and Yonaha 1980; Yonaha et al. 1983a, 1983b; Christen and Metzler 1985; Yonaha et al. 1987). In this case, the equilibrium constant of the reaction is high enough not to limit the reaction (Shin and Kim 1997, 1998). This fact makes the ω-APT an attractive biocatalyst for use in the production of chiral amines. However, there has been no report on biochemical characterization and molecular cloning of a transaminase showing amino donor specificity toward

arylic chiral amines such as α-methylbenzylamine (α-MBA) and 1-aminoindan.

In a previous study, we screened and isolated several microorganisms showing transaminase activity towards arylic chiral amines (Shin and Kim 2001). Among them, *Vibrio fluvialis* JS17 showed the highest specific activity in asymmetric synthesis as well as kinetic resolution in producing chiral amines (Shin and Kim 2001; Shin et al. 2001). In this report, we present the purification, characterization, and gene cloning of the transaminase from *V. fluvialis* JS17. Based on the comparison of amino acid sequence and amino donor specificity, the enzyme proved to be a novel activity that has not been reported previously.

Materials and methods

Media and cultivation conditions

Cells of *V. fluvialis* JS17 from LB agar plates were suspended in 50 ml LB medium and were cultivated for approximately 12 h at 37°C. Medium A (1.8 l) was inoculated with the culture broth and the cells were cultivated for 1 day. The resulting culture broth was transferred to 30 l medium A. The cultivation was performed with a Biostat U (Braun, Melsungen, Germany) for 1 day at 37°C and 500 rpm. Medium A is composed of 10 mM (S)-α-MBA, 100 mM glycerol, 50 mM potassium phosphate (pH 7.0), 1 g/l $MgSO_4 \cdot 7H_2O$, 0.2 mM $CaCl_2$, and trace metal components as described previously (Shin and Kim 1997).

Purification of the transaminase

All purification steps were carried out at 4°C and were monitored by SDS-PAGE. Column chromatography was performed with a Biologic HR system (Bio-Rad, Hercules, Calif.).

The cell pellet harvested from 30 l culture broth was suspended in 550 ml 100 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and 0.01% (v/v) 2-mercaptoethanol, and was then subjected to ultrasonic disruption for 20 min. The supernatant solution obtained after centrifugation (17,000 *g*, 30 min) was employed as cell-free extract. Solid ammonium sulfate was added carefully to the cell-free extract with gentle stirring until 35% saturation was reached. After equilibration for 30 min, the supernatant after centrifugation was taken and ammonium sulfate was added again to 55% saturation. The solution was allowed to equilibrate for 30 min and the resulting precipitate was collected after centrifugation. The precipitate was dissolved in 100 mM potassium phosphate buffer (pH 7.0) and dialyzed overnight against 50 mM bis-Tris buffer (pH 6.0) containing 20 μM pyridoxal 5′-phosphate (PLP). The dialyzed ammonium sulfate fraction was loaded onto a Q-Sepharose FF column (10 mm×30 cm, Pharmacia, Uppsala, Sweden) pre-equilibrated with 50 mM bis-Tris buffer (pH 6.0). After washing the column with the same buffer, elution was carried out with a linear gradient from 0 to 40% NaCl solution (1 M). The active fractions were collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate and 20 μM PLP. The dialysate was applied to a butyl-Sepharose FF column (10 mm×30 cm, Pharmacia) pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. Elution was carried out with a linear gradient from 1 to 0 M ammonium sulfate. The active fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 20 μM PLP.

Gel electrophoresis

SDS-PAGE was carried out with 10% polyacrylamide gel as described elsewhere (Daniel and Edelstein 1996). Isoelectric focusing (IEF) was performed with a 5% disc-type polyacrylamide gel (pH gradient ranging from 5 to 7). Proteins were visualized by Coomassie blue staining.

Enzyme assay

All the enzyme assays were carried out at 37°C and pH 7. One unit of transaminase was defined as the amount that catalyzed the formation of 1 μmol acetophenone in 1 min at 50 mM (S)-α-MBA and 10 mM pyruvate. The typical reaction volume was 200 μl and the reaction was stopped by adding 75 μl 16% (v/v) perchloric acid.

Analytical methods

Acetophenone was analyzed using a μ-Bondapak reverse phase column (Waters, Milford, Mass.) with isocratic elution of acetonitrile/water (50/50, v/v). UV detection was carried out at 205 nm. L-Alanine was analyzed after derivatization with *o*-phthalaldehyde using a Novapak column (Waters) as described elsewhere (Dennis et al. 1979). Pyruvate was analyzed with an Aminex HPX-87H column (Bio-Rad). An aqueous solution of 5 mM H_2SO_4 was used as eluent at a flow rate of 0.6 ml/min.

Molecular mass determination, N-terminal sequencing, and internal peptide sequencing

To determine the apparent molecular mass of the transaminase, the purified enzyme was subjected to gel filtration chromatography using a Bio-Silect SEC 250-5 column (0.78×30, Bio-Rad) at a flow-rate of 0.7 ml/min with elution using 50 mM potassium phosphate (pH 7.0) containing 150 mM NaCl. The subunit molecular mass was determined by MALDI-TOF mass spectrometry with a Biflex IV system (Bruker, Bremen, Germany). The matrix solution contained a mixture of acetonitrile:methanol (7:3) saturated with sinapinic acid. The purified protein solution (0.5 μl) was mixed with 0.5 μl matrix solution and deposited on the target plate. Signals from 100 shots were acquired. For N-terminal amino acid sequencing, 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 transaminase was excised and used for automated Edman degradation with a Procise 491 sequencer (Applied Biosystems, Foster City, Calif.). In the case of internal peptide sequencing, the purified enzyme was digested with sequencing-grade trypsin (Promega, Madison, Wis.) and the resulting peptides were fractionated on an Aquapore reverse-phase column (Applied Biosystems) using a Micro-gradient LC system (Applied Biosystems). The N-terminal amino acid sequences of collected peptide fragments were determined using the same protein sequencer.

Effect of additives on enzyme stability

Enzyme solutions containing predetermined concentrations of additives were incubated at 37°C after being filtered through a syringe filter (0.2 μm cut-off) to maintain sterility. Aliquots of the enzyme solution were periodically added to a reaction solution containing 50 mM (S)-α-MBA and 10 mM pyruvate, and the initial reaction rate was measured by analyzing the acetophenone produced. The half-life of the enzyme was calculated by fitting the initial rate data to the first-order enzyme inactivation equation.

Construction of the genomic library

Chromosomal DNA of *V. fluvialis* JS17 was partially digested with *Sau*3AI to achieve DNA fragments of 30–40 kb. The fragments were ligated into the cosmid vector pOJ446 (Bierman et al. 1992) digested with *Bam*HI and *Hpa*I. The ligation mixture was packaged in vitro using a lambda packaging system (Stratagene, La Jolla, Calif.) and transfected into *Escherichia coli* XL1-Blue MRF. Colonies were selected on LB agar plates containing apramycin (100 μg/ml).

Construction of the probe for colony hybridization

Degenerate PCR primers were designed on the basis of N-terminal- and one of the internal transaminase-peptide sequences. Primers used were forward (5′ AAIAARCCICARISITGGGA 3′) and reverse (5′ CCRAARAAIGCRTGRTAICCIGG 3′) where I, R, and S indicate inosine, A or G, and C or G, respectively. PCR was performed with these primers using genomic DNA of *V. fluvialis* JS17 as a template. The PCR product was ligated into the vector pGEM-T (Promega) and the DNA sequence was analyzed by ABI 3100 (Perkin-Elmer Biosystems, Foster City, Calif.). The PCR product was used as a probe after being labeled with [α^{32}P]dCTP using a random-labeling kit (Promega).

Screening and isolation of the transaminase gene

Positive clones were identified from the cosmid library by colony hybridization with the ^{32}P-labeled oligonucleotide probe using standard methods (Sambrook et al. 1989). One of these clones (pHD1) was further characterized. Southern blot analysis revealed that the plasmid contained a 3.7 kb *Pst*I fragment that hybridized to the oligonucleotide probe. This fragment was ligated with *Pst*I-digested pGEM-3. The ligation mixture was transformed into *E. coli* XL1-Blue MRF and subjected to DNA sequencing using the ABI 3100 DNA sequencer (Perkin-Elmer). The deduced amino acid sequence of the transaminase was analyzed by BLAST searches of the databases at the National Center for Biotechnology Information. Multiple alignments of the amino acid sequences of the homologous proteins were performed with the CLUSTAL multiple sequence alignment program (Higgins and Sharp 1988).

Construction of the expression vector and expression of the transaminase gene in E. coli

The coding region of the transaminase gene was amplified by PCR using forward (5′ GCTGACCATATGAACAAACCGCAAAGC-TGGGA 3′) and reverse (5′ GGGTCGGGATCCGTCAGGT-CAGGCAACCTCGG 3′) primers designed to subclone the entire transaminase gene into *Nde*I/*Bam*HI-cleaved pET24ma expression vector (kindly donated by Hiroshi Sakamoto, Pasteur Institute, Paris). The plasmid was introduced into *E. coli* BL21 and the transformant was grown in LB broth containing 50 μg/ml kanamycin at 37°C. When the OD_{600} reached 0.75, IPTG was added to 1 mM. After 5 h induction, the cells were harvested and disrupted by sonication. After centrifugation, enzyme activity of the cell-free extract was measured to evaluate expression efficiency.

Results

Purification and characterization of the transaminase

The transaminase was purified 49-fold with 42% recovery yield (Table 1) after ammonium sulfate fractionation and two column chromatographies (Q-Sepharose FF and butyl-Sepharose FF). Fractions containing active transaminase showed yellow color throughout the column chromatography. The purified transaminase gave a single protein band on SDS-PAGE (Fig. 1). The following enzyme properties were examined with the purified transaminase.

Molecular mass and pI

The molecular mass of the native enzyme was estimated to be 100 kDa by gel filtration chromatography. The subunit molecular mass was determined by MALDI-TOF mass spectrometry, which gave a subunit mass of 50,130. These results suggest that the enzyme has a dimeric structure. The isoelectric point of the enzyme determined by IEF was estimated to be 5.4.

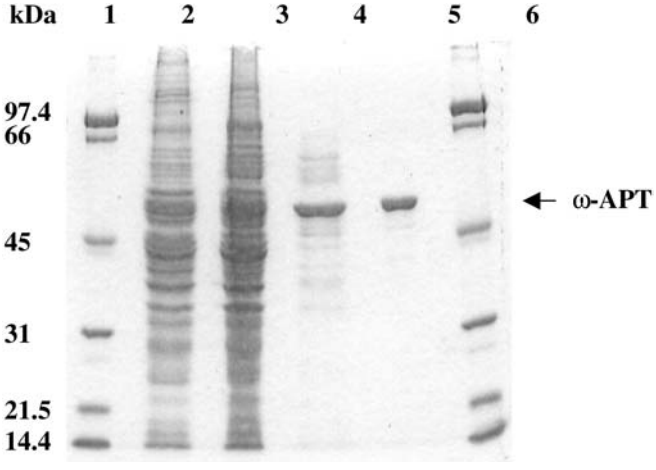

Fig. 1 SDS-PAGE of *Vibrio fluvialis* JS17 transaminase at different stages of purification. Proteins were separated on a 10% polyacrylamide gel in the presence of 1% SDS. Lanes: *1*, *6* Marker proteins (molecular mass 97.4, 66, 45, 31, 21.5, 14.4 kDa), *2* cell-free extract, *3* ammonium sulfate fraction (35–55%), *4* after Q-Sepharose, *5* after butyl-Sepharose

Table 1 Purification of the transaminase from *Vibrio fluvialis* JS17

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purifi-cation (fold)
Cell-free extract	1,240	1,248	1	100	1
Ammonium sulfate fractionation (35–55%)	740	842	1.14	67	1
Q-Sepharose FF	25.4	583	22.9	47	23
Butyl-Sepharose FF	10.8	528	48.9	42	49

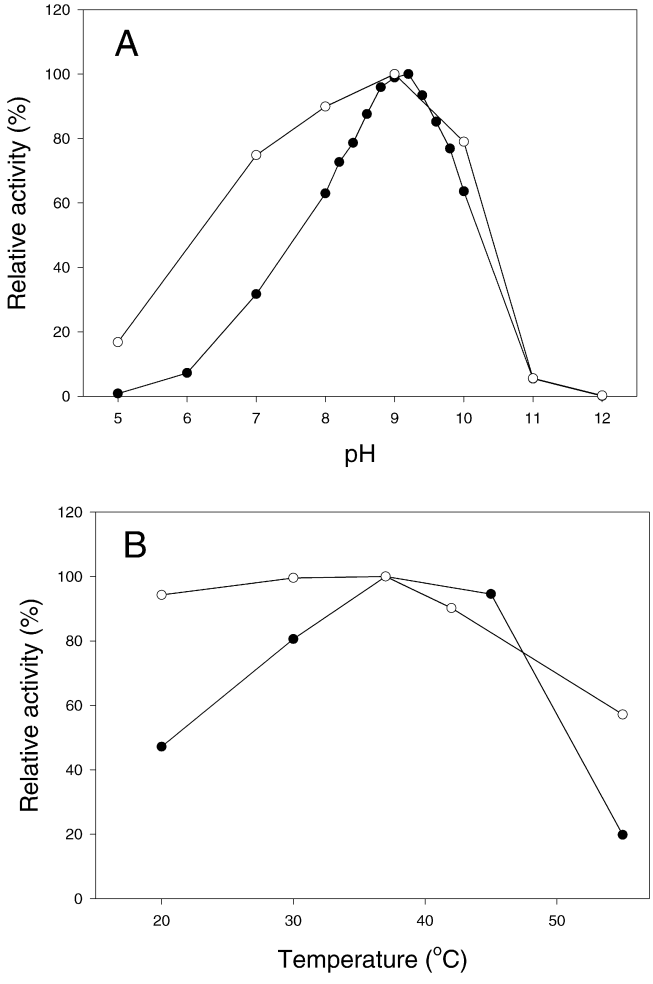

Fig. 2 Effect of pH (**A**) and temperature (**B**) on enzyme activity (●) and stability (○), as determined by measuring the residual activity after incubation at the pH indicated and 37°C for 1 day, or at different temperatures and pH 7.0 for 30 min, respectively. After incubation at each test condition, an aliquot (10 μl) of enzyme solution was added to a reaction solution (190 μl) consisting of 50 mM (S)-α-methylbenzylamine (MBA), 10 mM pyruvate, 200 mM potassium phosphate (pH 7.0). For pH stability experiments, the following buffers (200 mM) were used: pH 5–6 citrate, pH 7 potassium phosphate, pH 8–10 borate, pH 11–12 potassium phosphate

Optimal pH and temperature

As shown in Fig. 2a, the optimum pH for the enzyme activity was 9.2. The enzyme activity decreases more rapidly above pH 9 than at lower pH. Similarly, the enzyme shows the highest pH stability at pH 9. The optimal temperature for the enzyme activity is 37°C (Fig. 2b). The enzyme was prone to thermal inactivation above this temperature.

Absorption spectrum

The enzyme showed a typical transaminase absorption spectrum (Fig. 3). Absorption maxima were observed at

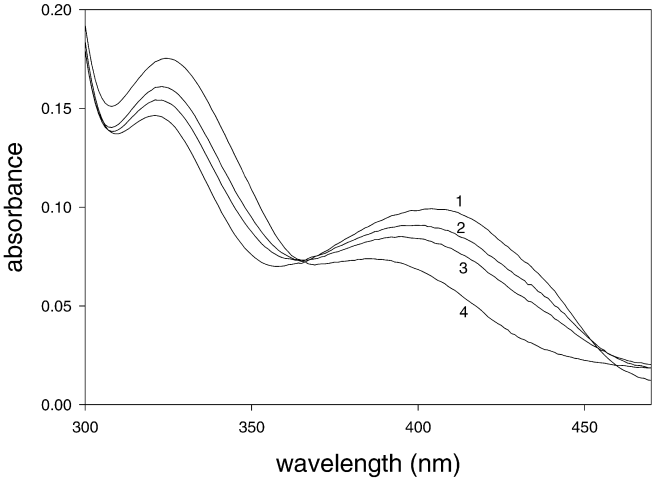

Fig. 3 Absorption spectra of the transaminase. The spectra were taken in 50 mM potassium phosphate buffer (pH 7.0) at room temperature. *1* Enzyme or enzyme with pyruvate (1 mM), *2* enzyme with (S)-1-methyl-3-phenylpropylamine (1 mM), *3* enzyme with (S)-α-MBA (1 mM), *4* enzyme with (S)-1-aminoindan (1 mM)

320 nm and 405 nm. As expected, addition of (S)-α-MBA caused an increase in absorbance at 320 nm and a decrease at 405 nm. However, addition of pyruvate did not affect the absorption spectrum of the enzyme because the enzyme was present as the PLP form. Interestingly, the changes in absorbance at both 320 and 405 nm increased as the reactivity of the (S)-amine increased (see also Table 2). This result suggests that more reactive (S)-amine induces a higher degree of conversion of enzyme-pyridoxal 5′-phosphate (E-PLP) to enzyme-pyridoxamine 5′-phosphate (E-PMP).

N-Terminal and internal peptide sequencing

The sequence of 13 amino acid residues (NKPQSWEARAETY) was obtained from N-terminal sequencing of the purified transaminase. A BLAST search showed no significant homology of this N-terminal sequence with known proteins. Out of 26 RP-HPLC-purified internal peptides, peptide 15 (FPGYHAFFGR) was chosen to design degenerate primers for PCR.

Substrate specificity and enantioselectivity

Table 2 shows the substrate specificity of the enzyme. The enzyme showed high activity toward arylic chiral amines such as (S)-α-MBA, (S)-1-methyl-3-phenylpropylamine, and (S)-1-aminoindan. However, among the various typical amino acid substrates for subgroup II ω-transaminases, such as β-alanine, γ-aminobutyric acid, ornithine, and 6-aminohexanoic acid, no amino acid was reactive. Polyamines such as spermidine and 1,3-diaminopropane were also inert. Of the twenty natural amino acids, only L-alanine showed reactivity. Regarding

Table 2 Substrate specificity of the transaminase from *V. fluvialis* JS17

Amino donor	Relative initial rate (%)[a]	Amino acceptor	Relative initial rate (%)[b]
(S)-α-Methylbenzylamine (MBA)	100	Pyruvate	100
(S)-1-Aminoindan	122	Oxalacetate	n.r.
(S)-1-Methyl-3-phenylpropylamine	61	α-Ketoglutarate	n.r.
β-Alanine[c]	n.r.[d]	Glyoxylate	60
γ-Aminobutyric acid	n.r.	Propionaldehyde	8
Ornithine	n.r.	Butyraldehyde	36
6-Aminohexanoic acid	n.r.	Benzaldehyde	17
spermidine	n.r.		
1,3-Diaminopropane	n.r.		
L-Alanine[e]	10		

[a] Each amino donor (20 mM) and pyruvate (20 mM) were used as substrates and initial reaction rate was measured by analyzing L-alanine produced
[b] (S)-α-MBA (20 mM) and each amino acceptor (20 mM) were used and initial reaction rate was measured by analyzing acetophenone produced
[c] Measurement of L-alanine concentration was difficult because β-alanine showed nearly identical retention time to L-alanine on HPLC. Therefore, 20 mM glyoxylate was used instead of pyruvate and glycine produced was analyzed
[d] Not reactive. Relative initial rate is lower than 1% of the reaction rate with 20 mM (S)-α-MBA and 20 mM pyruvate
[e] Reactivity of L-alanine was measured by using glyoxylate as an amino acceptor and compared with that of (S)-α-MBA with glyoxylate. Of the 20 natural amino acids, all amino acids except L-alanine were inert (i.e., relative initial rate of less than 1%)

amino acceptors, pyruvate was the most reactive. Among keto acids other than pyruvate, glyoxylate showed good reactivity whereas oxalacetate and α-ketoglutarate were inert. In addition to keto acids, aldehydes such as propionaldehyde, butyraldehyde, and benzaldehyde showed amino acceptor reactivity.

The enzyme showed (S)-enantioselectivity toward the arylic chiral amines in Table 2. Enantiomeric ratios for the three arylic amines were all above 140 (Shin et al. 2001). High enantioselectivity is beneficial for preparation of enantiopure amines via asymmetric synthesis as well as kinetic resolution (Yonaha and Yonaha 1980; Shin and Kim 1999; Shin et al. 2001).

Effect of additives on enzyme stability

Substrates for the enzyme, as well as PLP (prosthetic group of transaminase), were expected to affect enzyme stability. As shown in Fig. 4a, pyruvate showed a strong stabilizing effect whereas (S)-α-MBA severely inactivated the enzyme. The half-life of the enzyme increased a factor ~2 upon addition of 5 mM pyruvate to the enzyme solution; further increase in pyruvate concentration does not enhance the enzyme stability. Addition of 1 mM PLP enhances enzyme stability greatly but a further increase in the concentration reduced the stabilizing effect. In contrast, addition of only 5 mM (S)-α-MBA dramatically decreased the half-life from 115.2 h to 2.1 h. However, the destabilizing effect of (S)-α-MBA was cancelled out by PLP (Fig. 4b).

Effect of inhibitors

The presence of typical inhibitors of PLP-dependent enzymes, such as gabaculine, hydroxylamine, and aminooxyacetic acid, strongly inhibited enzyme activity. Most enzyme activity was lost at an inhibitor concentration of 1 mM. Residual activity in the presence of 0.1 mM gabaculine, hydroxylamine, or aminooxyacetic acid was 47, 2, and 87%, respectively.

Isolation and DNA sequencing of the transaminase gene

PCR was performed with degenerate primers designed on the basis of N-terminal (NKPQSWEARAETY) and one of the internal peptide sequences (FPGYHAFFGR) using genomic *V. fluvialis* JS17 DNA as a template. This resulted in the determination of a partial transaminase gene sequence of 259 bp. The amino acid sequence of the partial gene fragment corresponded to the peptide sequences of both the N-terminal and the internal peptide sequences. The partial gene fragment was used in colony hybridization to screen a *V. fluvialis* JS17 cosmid library. Several positive clones (8 out of 760 clones) were identified and one of these plasmids, named pHD1, was used for further characterization. Southern hybridization revealed that pHD1 contained a 3.7 kb *Pst*I fragment that hybridized to the oligonucleotide probe. Because the probe had an internal *Sma*I site (23 bp downstream of the N-terminal end of the probe), the *Pst*I fragment was digested with *Sma*I to determine whether the *Pst*I fragment contained the entire transaminase gene. *Sma*I digestion produced two fragments (1.9 and 1.8 kb), suggesting that the *Pst*I fragment encompassed the entire transaminase gene; the size of the gene was estimated to be, at most, 1.3 kb based on the subunit mass of the enzyme (50 kDa). The 3.7 kb *Pst*I fragment was cloned into pGEM-3 and subjected to DNA sequencing. The nucleotide sequence of the transaminase gene and its flanking regions is shown in Fig. 5 with the deduced amino acid sequence, putative −35 and −10 sequences, a

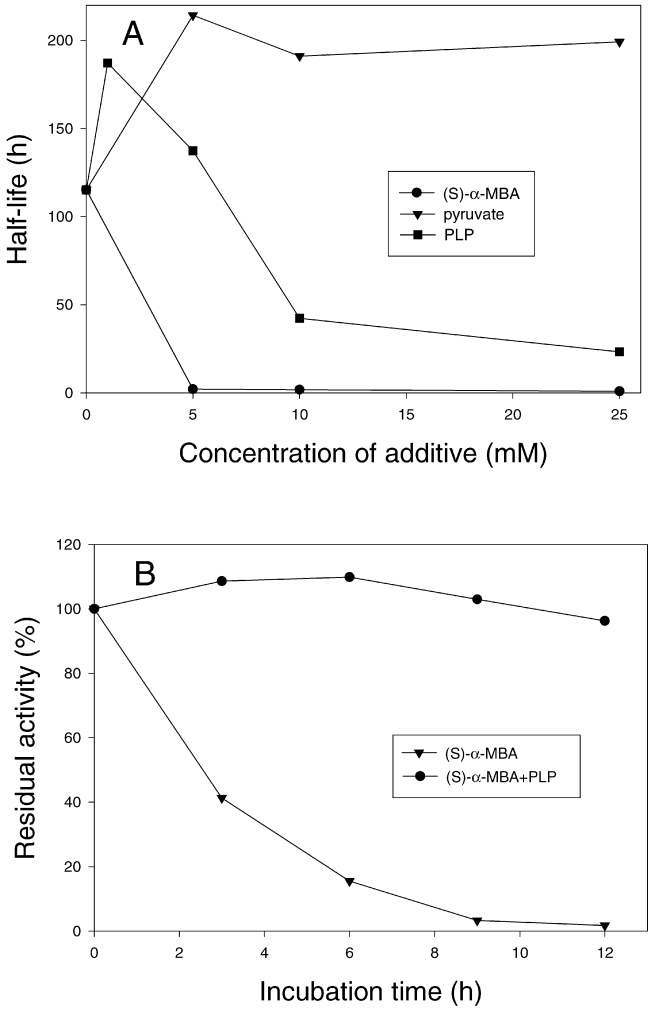

Fig. 4A, B Effect of additives on enzyme stability. Incubation was carried out at 37°C in 50 mM potassium phosphate (pH 7). Residual enzyme activity was measured as described in the legend to Fig. 2. **A** Effect of the additive concentration on enzyme stability. **B** Effect of pyridoxal 5′-phosphate (PLP) on enzyme inactivation by (S)-α-MBA. The additive concentration was 5 mM in both experiments [(S)-α-MBA + PLP (●) and (S)-α-MBA (▼) only]

ribosomal binding site, and a transcription terminator. The open reading frame of 1,359 bp was composed of 453 amino acid residues with a theoretical pI of 5.6 and a calculated molecular mass of 50,131 Da. A putative ribosomal binding site, GGAGG, was found 8 bp upstream of the presumptive start codon, ATG. The putative −35 and −10 promoter sequences were observed 58 bp upstream of the start codon. The stop codon (TGA) was followed by a sequence of dyad symmetry (10-nucleotide perfect repeat) that would be expected to function as a transcription terminator.

Comparison of amino acid sequence of the transaminase with other ω-APT

Homology analysis of the deduced amino acid sequence of the transaminase with the NCBI protein database using BLAST searches revealed the highest degree of identity to the ω-APT from *Mesorhizobium loti* (33%) (Kaneko et al. 2000), *Pseudomonas putida* (31%) (Yonaha et al. 1992), *Caulobacter crescentus* (31%) (Nierman et al. 2001), and *Pseudomonas aeruginosa* (31%) (Stover et al. 2000) among the proteins whose function was identified (Fig. 6). Of the 159 amino acid residues conserved in four ω-APTs, 80 were identical to those in the transaminase in this study (designated by plus signs in Fig. 6). However, despite the high similarity, the amino acid sequence of the transaminase showed distinct differences to those of the ω-APTs. Among the conserved residues in four ω-APTs, 79 amino acid residues were not conserved in the transaminase (designated by asterisks in Fig. 6).

Expression of the transaminase in *E. coli*

To overexpress the enzyme in *E. coli* BL21, the transaminase gene was cloned into an overexpression vector, pET24ma, under the control of the T7 promoter. The specific activity of cell-free extract of the expression strain after IPTG induction was 20 U/mg protein whereas no background activity was detected with *E. coli* BL21 harboring empty pET24ma plasmid. The expressed enzyme had the same molecular mass as the purified enzyme from *V. fluvialis* JS17 as determined by SDS-PAGE (data not shown). It also exhibited the same pH and temperature optima and the same substrate specificity.

Discussion

The transaminase family is divided into four subgroups on the basis of their mutual structural relatedness (Christen and Metzler 1985; Mehta et al. 1993). α-Transaminase consists of the transaminases in subgroups I, III, and IV. ω-Transaminase consists of those in subgroup II, which contains ω-APT (E.C. 2.6.1.18), ornithine transaminase (EC 2.6.1.11), γ-aminobutyrate transaminase (EC 2.6.1.19), diaminopelargonate transaminase (EC 2.6.1.62) and others (Mehta et al. 1993; Mehta and Christen 1994). To classify the transaminase in this study, we investigated the substrate specificity of the enzyme (Table 2). The distinct features of the substrate specificity are as follows: (1) L-alanine is the only reactive amino acid of the 20 natural amino acids; (2) the enzyme is inert toward amino acid substrates for the transaminases in subgroup II such as γ-aminobutyrate, ornithine, L-lysine, β-alanine, 6-aminohexanoic acid, spermidine, and 1,3-diaminopropane; (3) pyruvate is the only reactive amino acceptor among typical amino acceptors for transaminases such as oxalacetate, α-ketoglutarate, and pyruvate; (4) arylic

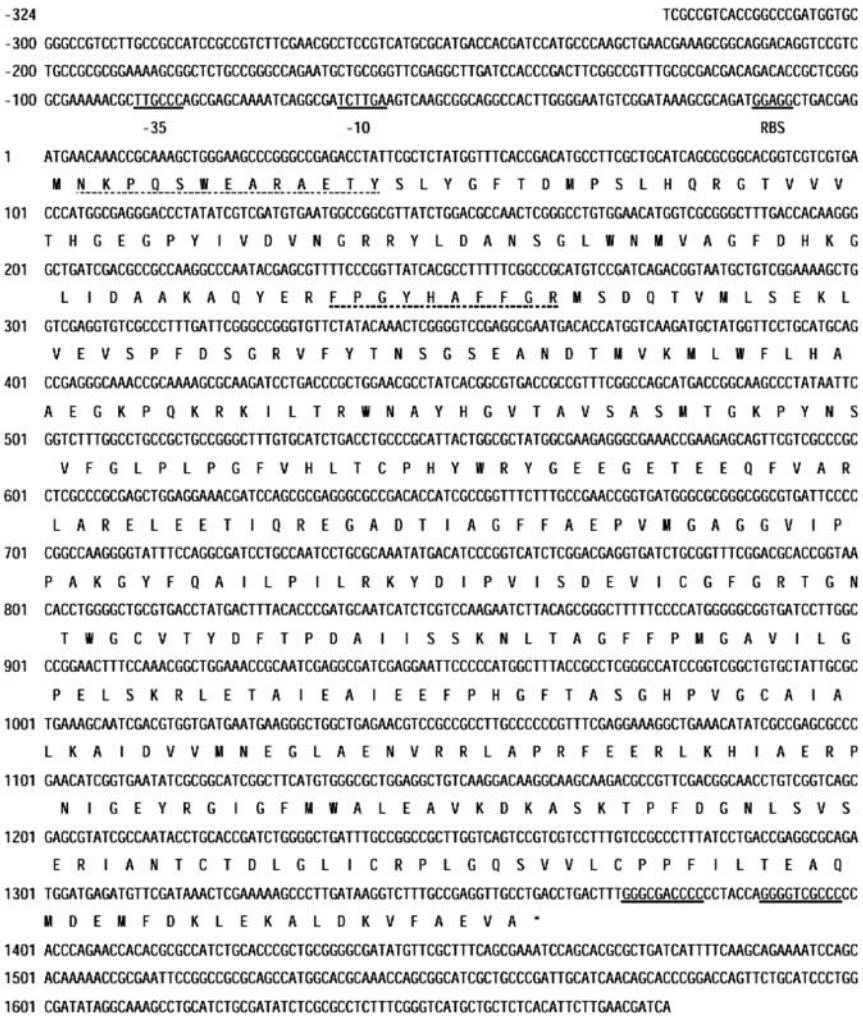

Fig. 5 Nucleotide and deduced amino acid sequences of the transaminase gene. The nucleotides are numbered with the first letter of the initiator Met codon as +1. The putative ribosomal binding site and −35 and −10 sequences are *underlined* and labeled. An *asterisk* indicates the stop codon. The N-terminal and internal peptide sequences used in primer design are *broken-underlined*. A possible transcription terminator downstream of the transaminase gene is *underlined*

chiral amines are the most reactive amino donors. Only ω-APT was reported to show catalytic activity towards primary amine compounds not bearing carboxyl groups (Yonaha and Yonaha 1980; Yonaha et al. 1983a, 1983b; Christen and Metzler 1985; Yonaha et al. 1987). Indeed, the substrate specificity of the transaminase in this study was similar to that of the ω-APT in many respects such as exclusive amino donor and acceptor specificity for L-alanine among natural amino acids and pyruvate among keto acids, respectively. Moreover, the homology analysis of the amino acid sequence showed the highest similarity to the ω-APT (Fig. 6). According to Shen et al. (1998), some essential residues of transaminase such as D259 (hydrogen bond with PLP), K288 (active site lysine), and R414 (salt bridge with α-carboxylate group of substrate) are conserved in the ω-APT from *P. putida* (Yonaha et al. 1992). These three residues are also well conserved in all transaminases listed in Fig. 6 (shaded residues). However, the most striking difference was the reactivity toward β-alanine. The transaminase in this study showed no activity toward β-alanine, whereas β-alanine was one of the most reactive amino donors for ω-APT. Taken together with this result, the presence of 79 residues that are conserved in four ω-APTs but not in the transaminase in this study strongly suggests that the enzyme is a novel one that has not been reported so far. We tentatively named the enzyme as an amine:pyruvate transaminase.

The enzyme was severely destabilized by (S)-α-MBA (Fig. 4a), which is reminiscent of the inactivation of D-amino acid transaminase by D-alanine (Manning 1998). The purified amine:pyruvate transaminase would exist as an E-PLP form, because the final step of enzyme purification was dialysis against buffer solution containing PLP. Addition of pyruvate or a low concentration of PLP to the enzyme solution would reinforce the formation of E-PLP, resulting in enhancement of enzyme stability. In contrast, the addition of (S)-α-MBA would convert E-PLP to E-PMP, resulting in a drastic decrease in enzyme stability. Therefore, the results of Fig. 4a suggest that E-PMP is much more unstable than E-PLP, which appears to result from different interactions between the prosthetic group and apoenzyme. PLP is covalently bound to the apoenzyme via internal aldimine linkage with the ε-amino group of an active-site lysine, whereas PMP interacts with apoenzyme via non-covalent interactions. Therefore, the dissociation constant of E-PMP is much higher than that

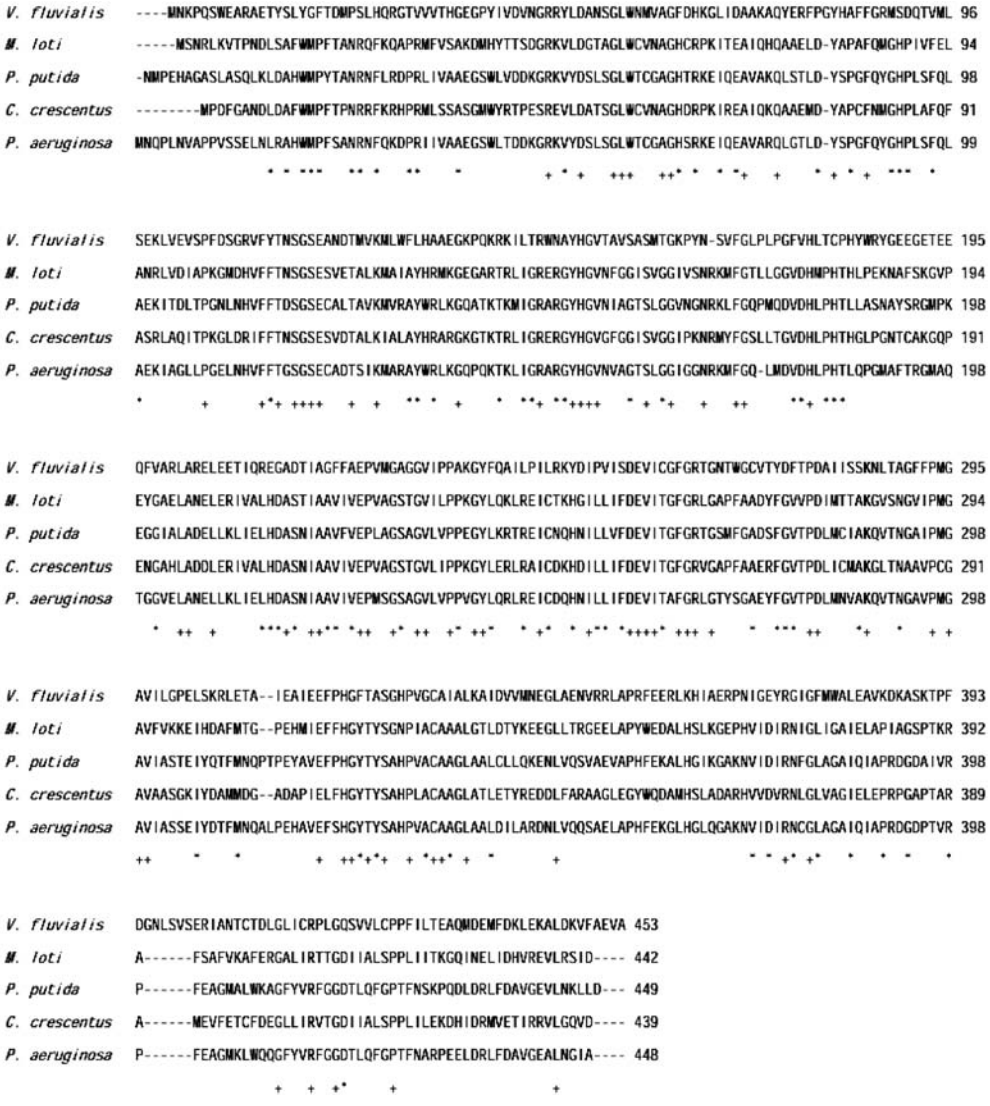

Fig. 6 Comparison of the amino acid sequence of the transaminase from *V. fluvialis* JS17 with those of the ω-APT from *Mesorhizobium loti*, *Pseudomonas putida*, *Caulobacter crescentus*, and *Pseudomonas aeruginosa*. *Plus signs* Amino acids conserved in the enzymes of all five bacteria, *asterisks* positions conserved only in all ω-APT, *shaded residues* essentially conserved among transaminases, *dashes* gaps introduced during alignment process. Numbers refer to the amino acid located at the end of each line

of E-PLP (Christen and Metzler 1985). The amino donor-assisted inactivation appears to be reversible because the destabilizing effect of (S)-α-MBA is completely cancelled out by the presence of PLP (Fig. 4b).

In this report, we have presented the purification, characterization, and molecular cloning of a novel amine:pyruvate transaminase capable of stereoselective transamination of arylic chiral amines. Highly strict enantioselectivity and broad amino donor specificity for chiral amines make the amine:pyruvate transaminase a highly suitable enzyme for the production of various chiral amines. To date, this is the first report on the biochemical investigation of a transaminase that is active toward arylic chiral amines.

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