Appl Microbiol Biotechnol (2000) 54: 44–51

ORIGINAL PAPER

J. Q. Liu · M. Odani · T. Yasuoka · T. Dairi
N. Itoh · M. Kataoka · S. Shimizu · H. Yamada

Gene cloning and overproduction of low-specificity D-threonine aldolase from *Alcaligenes xylosoxidans* and its application for production of a key intermediate for parkinsonism drug

Received: 9 September 1999 / Received revision: 1 November 1999 / Accepted: 12 November 1999

Abstract The *dtaAX* gene encoding a pyridoxal 5′-phosphate (pyridoxal-*P*)-dependent low-specificity D-threonine aldolase was cloned from the chromosomal DNA of *Alcaligenes xylosoxidans* IFO 12669. It contains an open reading frame consisting of 1,134 nucleotides corresponding to 377 amino acid residues. The predicted amino acid sequence displayed 54% identity with that of D-threonine aldolase from gram-positive bacteria *Arthrobacter* sp. DK-38, but showed no significant similarity with those of other known pyridoxal-*P* enzymes. This gram-negative bacterial enzyme was highly overproduced in recombinant *Escherichia coli* cells, and the specific activity of the enzyme in the cell extract was as high as 18 U/mg (purified enzyme 38.6 U/mg), which was 6,000 times higher than that from the wild-type *Alcaligenes* cell extract. The recombinant enzyme was thus feasibly purified to homogeneity by ammonium sulfate fractionation and DEAE-Toyopearl chromatography steps. The recombinant low-specificity D-threonine aldolase was shown to be an efficient biocatalyst for resolution of L-β-3,4-methylenedioxyphenylserine, an intermediate for production of a therapeutic drug for Parkinson's disease.

J. Q. Liu · M. Odani · T. Yasuoka · T. Dairi · N. Itoh (✉)
H. Yamada
Laboratory of Biocatalytic Chemistry,
Biotechnology Research Center,
Toyama Prefectural University,
Kurokawa 5180, Kosugi, Toyama 939-0398, Japan
e-mail: itoh@pu-toyama.ac.jp
Tel.: +81-766-567500
Fax: +81-766-562498

M. Kataoka · S. Shimizu
Division of Applied Life Sciences,
Graduate School of Agriculture,
Kyoto University, Kyoto 606-8502, Japan

Present address:
J. Q. Liu, Research and Development Division,
Analytical Technology and Research Department,
Procter and Gamble Far East, Inc.,
Higashinada, Kobe 658, Japan

Introduction

L-*threo*-β-(3,4-dihydroxyphenyl)serine (DOPS) has been recently used for treatment of Parkinson's disease in Japan (Maruyama et al. 1996). Industrial production of the drug consists principally of chemical synthesis of DL-*threo*-β-(3,4-methylenedioxyphenyl)serine (MDOPS) from glycine and piperonal, chemical resolution of L-*threo*-MDOPS, and cleavage of L-*threo*-MDOPS to L-*threo*-DOPS (Fig. 1) (Ohashi et al. 1984). This process has the following problems: (1) chemical resolution is lengthy and inefficient, requiring protection and deprotection of the amino group of the amino acid before and after the resolution operation; (2) additional steps are needed to recycle nonreactive counterpart, D-*threo*-MDOPS; (3) the process overuses organic solvents, resulting in an environmental problem. Accordingly, development of an efficient and simple enzymatic process is desirable.

Low-specificity D-threonine aldolase (D-TA), catalyzing the following reactions (cleavage of D-*threo*/D-*erythro*-β-hydroxy-α-amino acid to aldehyde and glycine)

$$RCHOHCHNH_2COO^- \rightarrow RCHO + CH_2NH_2COO^-$$

(where R is alkyl, phenyl, or a derivative of either), would be a better biocatalyst for the resolution of L-*threo*-MDOPS. The enzyme was previously purified from a gram-positive bacterium, *Arthrobacter* sp. DK-38 (Kataoka et al. 1997b), the gene was cloned and expressed in *Escherichia coli* cells (Liu et al. 1998a), and the recombinant enzyme was recently shown to be useful for the resolution of L-*threo*-3-[4-(methylthio)phenylserine] (Liu et al. 1999). Nevertheless, the established expression system for the *Arthrobacter* enzyme still could not supply enough aldolase for industrial purposes.

The present paper describes cloning and sequencing of the *dtaAX* gene encoding the low-specificity D-TA from a gram-negative bacterium *Alcaligenes xylosoxidans* IFO 12669, overexpression of the gene in *E. coli*

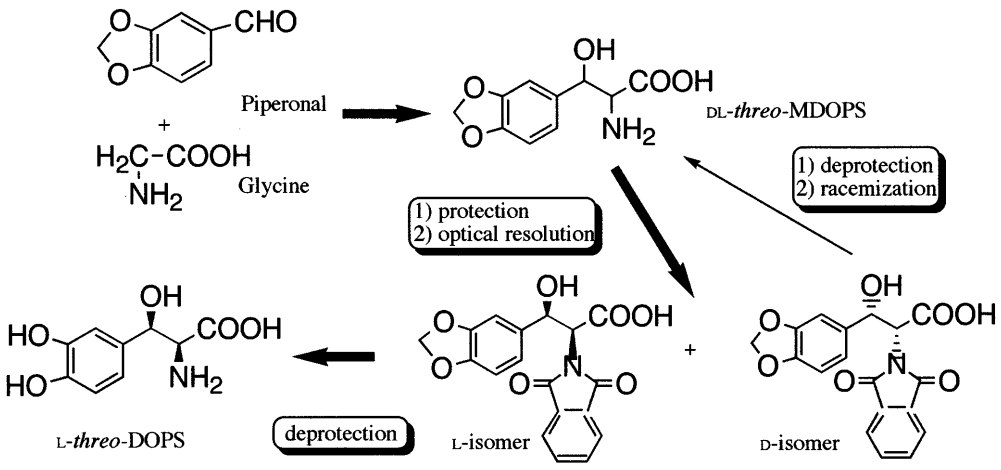

Fig. 1 An industrial process for production of L-*threo*-DOPS

cells, characterization of the recombinant enzyme, and its application for resolution of L-*threo*-MDOPS, an intermediate for production of a therapeutic drug for Parkinson's disease.

Materials and methods

Materials

DEAE-Toyopearl 650 M was purchased from Tosoh Corp. (Tokyo, Japan). DL-β-3,4-Methylenedioxyphenylserine was prepared according to the method of Ohashi et al. (1984). The other chemicals were all analytical-grade reagents.

Bacterial strains, plasmids, and culture conditions

Alcaligenes xylosoxidans IFO 12669 was used as the source of chromosomal DNA (Kataoka et al. 1997b). *E. coli* XL1-Blue MRF′ [*rec*A1 *thi end*A1 *sup*E44 *gyr*A46 *rel*A1 *hsd*R17 *lac*/F′ [pro-AB$^+$ *lac* I^q *lac*ZΔ M15::Tn *10* (*tet*^{r})] (Toyobo, Osaka, Japan) was used as a host for the gene cloning. Cosmid pWE15 (Stratagene, La Jolla, Calif., USA) was used as a vector for the construction of the gene library. Plasmid pUC119 (Takara Shuzo, Kyoto, Japan) was used as a vector for the gene subcloning. *Alcaligenes xylosoxidans* IFO 12669 was grown in a medium containing 1% peptone, 1% yeast extract, and 0.5% NaCl, pH 7.2. Recombinant *E. coli* cells were cultivated at 37 °C in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 1% NaCl, pH 7.2) containing 0.1 mg/ml ampicillin unless otherwise stated. For induction of the gene under the control of the *lac* promoter, 0.2 mM isopropylthio-β-D-galactoside (IPTG) was added to the LB medium.

General recombinant DNA technique

Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo and Toyobo, and used according to the manufacturers' protocols. Transformation of *E. coli* with plasmid DNA by electroporation was performed under the standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research Inc, San Diego, Calif., USA). Other general procedures were performed as described by Sambrook et al. (1989).

Cloning of the D-TA gene

Two oligonucleotide primers made to order were from Hokkaido System Science (Sapporo, Japan), each with additional restriction sites (underlined in the following sequences) added to the 5′ end to facilitate cloning of the amplified product: primer I, 5′-CCGAAGCTTATGTCNCARGARGTNAT-3′; and primer II, 5′-GCCGAATTCGGIGTRTCIACICGNGC-3′. Degenerate positions are indicated by "I" for deoxyinosine, "R" for A or G, and "N" for all bases. Primers I and II were based upon the NH_2-terminal amino acid sequence (Ser-Gln-Glu-Val-Ilu-Arg-Gly-Ile-Ala-Leu-Pro-Pro-Ala-Ala-Gln-Pro-Gly-Asp-Pro-Leu-Ala-Arg-Val-Asp-Thr-Pro-Ser) of the wild-type D-threonine aldolase from *Alcaligenes xylosoxidans* IFO 12669 (J.Q. Liu et al., unpublished data). Polymerase chain reaction (PCR) amplification was performed in 100 μl 10 mM TRIS/HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mM dNTP, 20 pmol of each primer, 1 μg of the genomic DNA, and 1 U *PWO* DNA polymerase (Boehringer Mannheim, Mannheim, Germany) at 94 °C for 1 min, 43 °C for 0.5 min, and 72 °C for 1 min in a total of 35 cycles. The amplified product was digested with *Eco*RI and *Hin*dIII, separated by agarose gel electrophoresis, and then purified with a GeneClean kit (Bio101 Inc., Vista, Calif.,USA). The amplified DNA of 90 bp was then cloned into pUC119.

Chromosomal DNA isolated from *Alcaligenes xylosoxidans* IFO 12669 cells was partially digested with *Sau*3AI and fractionated on a sucrose density gradient (10–40%) in a Beckman L-70 ultracentrifuge at 100,000*g* for 18 h. The fragments in the molecular weight range of 30–50 kb were collected and ligated into *Bam*HI-restricted pWE15. This concatemer was packaged in vitro to bacteriophage λ particles using an in vitro packaging kit (Gigapack III Gold Packaging Extract; Stratagene) and used to infect *E. coli* XL1-Blue MRF′. The genomic library was screened by colony hybridization with the [α-^{32}P]dATP-labeled 90-bp DNA fragment as a probe. The clone pWDTA carrying an approximately 32-kb DNA fragment was selected for further analysis.

Nucleotide sequence analysis

pWDTA was used as a sequencing template. The nucleotide sequence was determined by the dideoxy chain termination method with Cy5 AutoCycle sequencing kits and a Pharmacia LKB ALFred DNA sequencer. A homology search was performed by means of the sequence similarity searching programs Fasta (Pearson and Lipman 1988) and Blast (Altschul et al. 1994). The Clustal W method was used to align the sequences (Thompson et al. 1994).

Overexpression of the *dtaAX* gene in *E. coli*

To obtain the entire gene without excessive flanking parts, PCR amplification was carried out in 100 μl 10 mM TRIS/HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 mM dNTP, 20 pmol of each primer, 1 μg of the genomic DNA and 1 U *PWO*

DNA polymerase (Boehringer Mannheim) at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min in a total of 30 cycles. The 5′ primer containing a Shine-Dalgarno sequence (lower-case letters) and an ATG initiation codon (boldface), and the 3′ primer with the complement of the sequence as shown in Fig. 2, had the following sequences: 5′-GCCAAGCTTggagCGTCCCG**ATG**TCCCAGG-3′; and 3′ primer, 5′-CCGAAGCTTTCTAAAGCCTGCGTGTGAAGC-3′, which were designed on the basis of the nucleotide sequence of the *dtaAX* gene. To facilitate the cloning, an additional restriction site (underlined) was incorporated into both the 5′ primer and the 3′ primer. The amplified PCR product was digested with *Hin*dIII, separated by agarose gel electrophoresis, and then purified with a GeneClean kit. The amplified DNA of approximately 1.2 kb, which contained the complete coding sequences of the *dtaAX* gene, was inserted downstream of the *lac* promoter of pUC119, and then transformed *E. coli* XL1-Blue MRF′ cells. The constructed plasmid was designated pUDTA1.

Enzyme purification

All enzyme purification operations were carried out at 0–5 °C. 50 mM TRIS/HCl (pH 7.0) containing 10 μM pyridoxal-*P* was used as the buffer.

Step 1: Preparation of cell-free extract

Cells of the *E. coli* transformant harboring plasmid pUDTA1 were grown aerobically at 37 °C for 17 h in 2 l LB medium containing

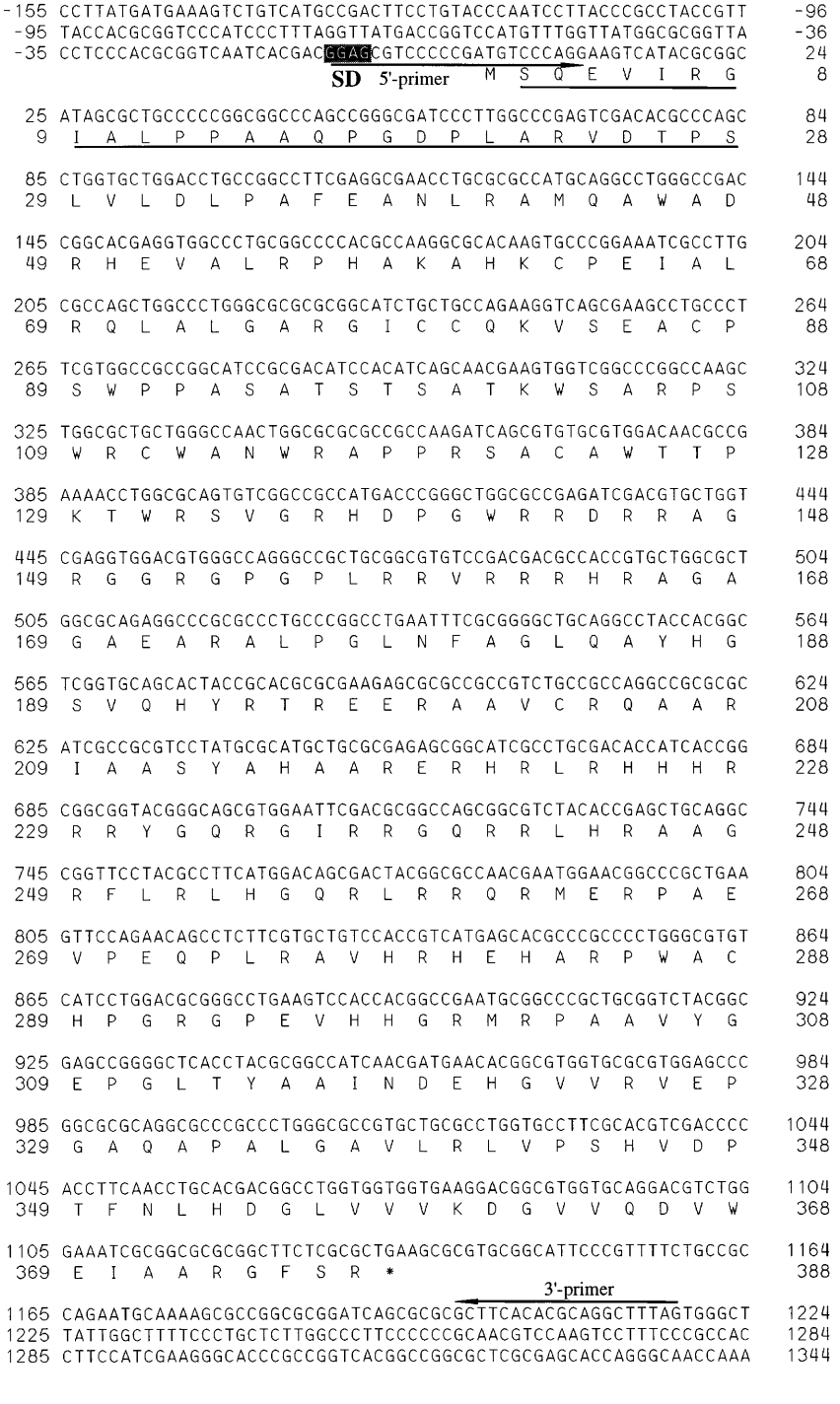

Fig. 2 Nucleotide and deduced amino acid sequences of the *dtaAX* gene and its flanking regions. A putative Shine-Dalgarno sequence is *shaded*. The underlined amino acid sequence is identical to that determined for the purified enzyme by Edman degradation. The *asterisk* denotes a translational stop codon. Two primers used for PCR amplification of the whole gene are indicated

0.1 mg/ml ampicillin and 0.2 mM IPTG. The cells were harvested and rinsed with the buffer twice. After being suspended in 200 ml of the buffer containing 100 μM $MnCl_2$, the cells were disrupted by ultrasonic oscillation at 4 °C for 20 min with a model 201 M ultrasonic oscillator (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation at 25,000*g* for 30 min.

Step 2: Ammonium sulfate fractionation

The supernatant solution was brought to 20% saturation with ammonium sulfate and centrifuged at 25,000*g* for 30 min. The precipitate collected by centrifugation was dissolved in the buffer and the enzyme solution was dialyzed against 1000 vol. buffer containing 100 μM $MnCl_2$.

Step 3: DEAE-Toyopearl column chromatography

The enzyme solution was applied to a DEAE-Toyopearl 650 M column (2.5 cm × 20 cm) equilibrated with the buffer. After the column had been washed thoroughly with the buffer, linear gradient elution was performed with the buffer supplemented with NaCl by increasing the concentrations from 0 mM to 300 mM. The flow rate was maintained at 4 ml/min. The fractions with high threonine aldolase activity were pooled and dialyzed against 1000 vol. buffer containing 100 μM $MnCl_2$ and 10 μM pyridoxal-*P*. The purified enzyme was stored at about 4 °C for at least 2 months without loss of enzyme activity.

Molecular mass determination

The molecular mass of the enzyme was determined by gel filtration on a TSK gel G3000 SW_{XL} column (7.5 × 600 mm) (Tosoh, Tokyo, Japan) with the following marker proteins: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa) (Wako Pure Chemicals, Osaka, Japan).

The subunit molecular mass of the enzyme was determined by SDS-PAGE with the following marker proteins: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Amersham Pharmacia Biotech, Uppsala, Sweden).

Enzyme assay

Threonine aldolase activity was measured spectrophotometrically at 340 nm by coupling the reduction of acetaldehyde (oxidation of NADH) with yeast alcohol dehydrogenase (Wako Pure Chemicals) as described previously (Liu et al. 1998a). The aldolase activities toward phenylserine, β-3,4-dihydroxyphenyphenylserine, and β-3,4-methylenedioxyphenylserine were measured spectrophotometrically at 279 nm, 350 nm, and 320 nm, respectively, employing molar extinction coefficients of 1.4×10^3 M^{-1} cm^{-1} for benzaldehyde, 8.9×10^3 M^{-1} cm^{-1} for protocatechualdehyde, and 17.6×10^3 M^{-1} cm^{-1} for piperonal.

Protein determination

Protein concentration was determined with a Bio-Rad protein assay kit.

Analysis of amino acid stereoisomer

The stereoisomers of various β-hydroxy-α-amino acids were analyzed by high-pressure liquid chromatography (HPLC) under the conditions described previously (Liu et al. 1998a). The retention times of L- and D-*threo*-MDOPS on Sumichiral OA-5000 column (4.6 × 150 mm) (Sumitomo Chemical, Osaka, Japan) with a solvent of 15% methanol solution containing 2 mM copper sulfate (flow rate, 1 ml/min, 30 °C) were 16.8 min for L-isomer and 18.2 min for D-isomer.

L-*threo*-MDOPS resolution

A reaction mixture, comprising 50 mM TRIS/HCl buffer (pH 8.0), 50 μM pyridoxal-*P*, 50 μM $MnCl_2$, and the indicated amount of substrate and enzyme at a final volume of 1 ml in a 2.5-ml microcentrifuge tube, was incubated at 30 °C. The preparative resolution was carried out with proportional scaling up in a final volume of 15 ml in a 50-ml flask with magnetic stirring at 30 °C.

The resolution reaction was terminated by heat treatment at 100 °C for 10 min. After centrifugation at 15,000*g* for 5 min, the supernatant was applied to a silica gel column (4.5 × 20 cm) (silica 60, Kanto Chemical Co., Tokyo, Japan) previously equilibrated with a solution comprising 69% (v/v) butanol, 17% ethanol, and 14% water. Elution was carried out with the same solvent. The fractions containing L-*threo*-MDOPS were pooled and concentrated in vacuo to dryness.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been deposited in the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide sequence databases under accession number AB026892.

Results

Cloning of the *dtaAX* gene

The primers used for cloning of the *dtaAX* gene by PCR were based on the NH_2-terminal amino acid sequence of the purified enzyme from *Alcaligenes xylosoxidans* IFO 12669, as described above. PCR with the primers and *Alcaligenes* chromosomal DNA as the template yielded an amplified band of 90 bp. The amplified DNA was then cloned into pUC119 in *E. coli*. Nucleotide sequencing of the 90-bp fragment showed the presence of an open reading frame (ORF) continuing over the entire sequence. The deduced amino acid sequence of the PCR fragment was in perfect agreement with the NH_2-terminal amino acid sequence determined from the purified D-TA of *Alcaligenes xylosoxidans* IFO 12669. Colony hybridization was then directly performed with the 90-bp fragment as a probe against the established *Alcaligenes xylosoxidans* IFO 12669 genomic library; eight positive recombinant *E. coli* clones were obtained from about 1,500 transfectants. One of the clones showing D-TA activity (0.07 U/mg toward D-threonine) was chosen for further characterization.

Sequence analysis of the *dtaAX* gene

The plasmid pWDTA extracted from the positive clone containing an approximately 32-kb insert was directly used as the template for sequencing the *dtaAX* gene by the gene-walking method, in which the first walking primer was designed on the basis of the nucleotide sequence of the 90-bp PCR product of the *dtaAX* N-terminus.

Sequence analysis of the double-strand DNA revealed that the ORF consists of 1134 bp starting with an initiation codon, ATG, and ending with a termination codon, TGA, at position 1132 (Fig. 2). A probable ribosome-binding sequence, GGAG, is present nine bases upstream of the putative translational start codon (Shine and Dalgarno 1975). However, sequences similar to the *E. coli* -35 sequence (TTGACA) and *E. coli* -10 sequence (TATAAT) were not found. The ORF encodes a protein of 377 amino acid residues. The predicted molecular weight is 42,110, which is in good agreement with the value determined for the purified enzyme from *Alcaligenes xylosoxidans* IFO 12669. The NH_2-terminal amino acid sequence coincided with that of the purified enzyme determined by Edman degradation (Fig. 2).

In a search of protein amino acid databases by means of the sequence similarity searching programs Fasta (Pearson and Lipman 1988) and Blast (Altschul et al. 1994), the predicted amino acid sequence was shown to have no significant similarity to those of the known pyridoxal-*P* enzymes, but the enzyme had 54% identity in primary structure to low-specificity D-TA from gram-positive bacterium *Arthrobacter* sp. DK-38 (Fig. 3). In addition, the enzyme was also found to have 17% and 22% identities in primary structure to D-serine deaminase (GenBank U41162) of *Burkholderia cepacia* and a hypothetical protein (GenBank U73935) of *Shewanella* sp. strain SCRC-2738, respectively. The amino acid sequence alignment of the four proteins is depicted in Fig. 3. Remarkably, Lys59 and Arg 157 of low-specificity D-TA were completely conserved in the four proteins (Fig. 3). In a previous work, we identified Lys59 of D-TA of *Arthrobacter* sp. DK-38 as the pyridoxal binding site of the enzyme by chemical modification with $NaBH_4$ (Liu et al. 1998a). The alignment result further supported that conclusion, and suggested that Lys59 should be the pyridoxal-*P* binding site of the aldolase from *Alcaligenes xylosoxidans*. Arg157, another solely

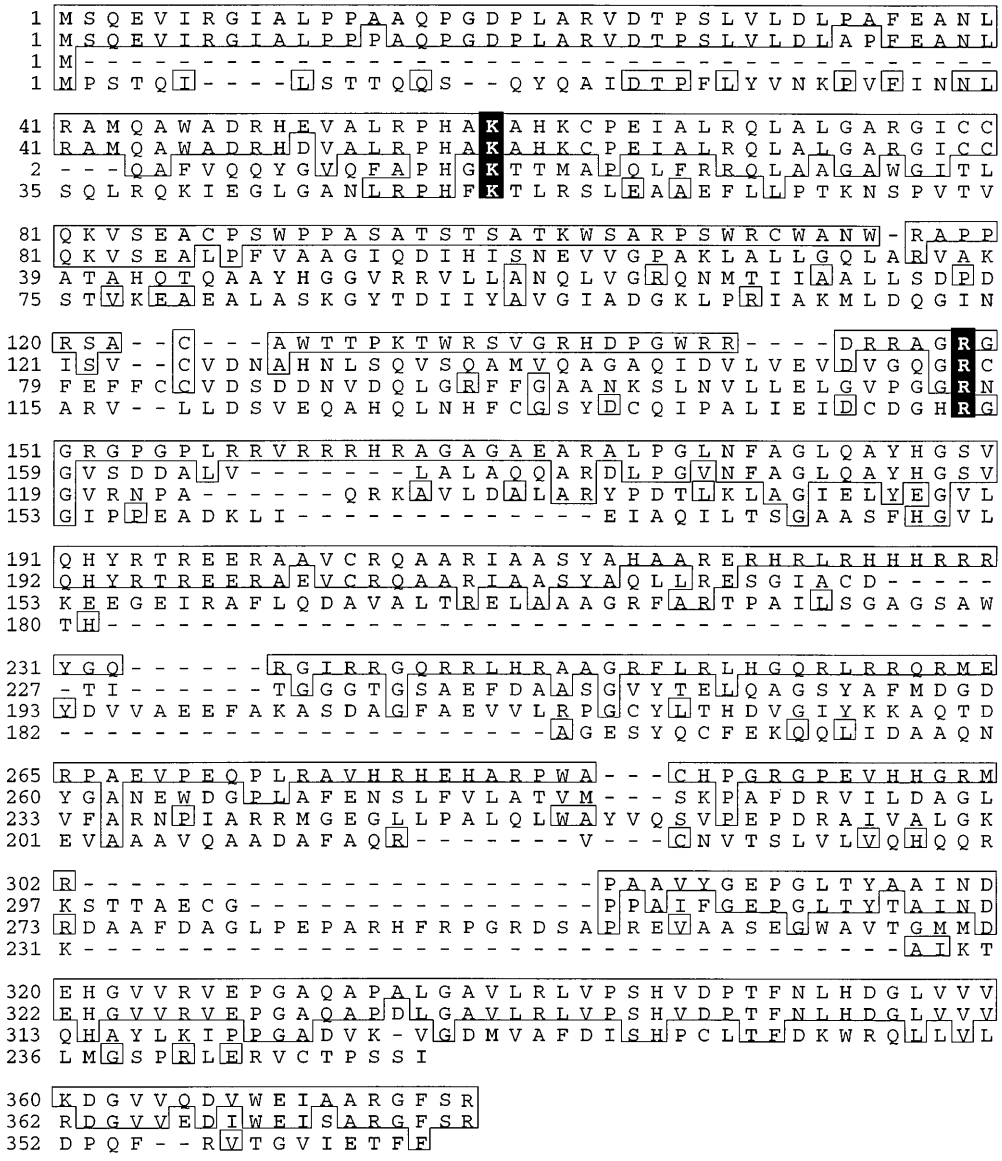

Fig. 3 Amino acid sequence alignment. From *top* to *bottom* in each set, the proteins are low-specificity D-TA from *Alcaligenes xylosoxidans* IFO 12669, low-specificity D-TA from *Arthrobacter* sp. DK-38, D-serine deaminase (GenBank U41162) of *Burkholderia cepacia,* and a hypothetical protein (GenBank U73935) of *Shewanella* sp. strain SCRC-2738. Identical residues are *boxed*. The numbers on the *left* are the residue numbers for each amino acid sequence

conserved amino acid residue with positive charge, might also be involved in the retro-aldol reaction, the role of which needs to be elucidated.

Overproduction and rapid purification of the enzyme

The whole *dtaAX* gene amplified by PCR directly from *Alcaligenes xylosoxidans* chromosomal DNA, with a putative Shine-Dalgarno sequence (GGAG) and an initiation codon (ATG), was inserted into the *Hin*dIII site of pUC119. The plasmid constructed, named pUDTA1, was introduced into *E. coli* XL1-Blue MRF′ cells. As shown in Fig. 4 and Table 1, the recombinant cells produced a large amount of low-specificity D-TA. Judging from the specific activity of the crude extract, the enzyme comprises about 40% of the total soluble protein. Low-specificity D-TA was feasibly purified by ammonium sulfate fractionation and DEAE-Toyopearl column chromatography steps, with a yield of 69% (Table 1).

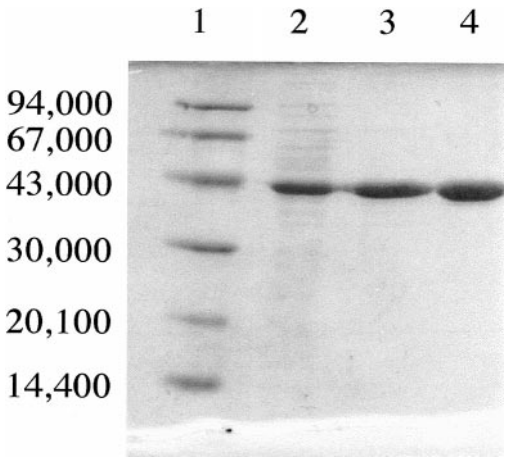

Fig. 4 Purification of the recombinant low-specificity D-TA of *Alcaligenes xylosoxidans* IFO 12669. The enzyme was loaded on a 10% SDS-polyacrylamide gel and stained with Coomassie blue after electrophoresis. *Lane 1* molecular mass standards; *lane 2* cell extracts; *lane 3* ammonium sulfate fractionation; *lane 4* DEAE-Toyopearl. The numbers on the *left* indicate the molecular masses of the marker proteins

Enzyme properties

The purified enzyme showed a single protein band on SDS-PAGE with a molecular mass of about 42 kDa (Fig. 4). The native molecular mass of purified low-specificity D-TA was determined to be 48 kDa by gel filtration. These results suggest that the recombinant low-specificity D-TA from *Alcaligenes xylosoxidans* IFO 12669 exists in a monomer, being the same as the *Arthrobacter* aldolase.

The recombinant enzyme exhibited absorption maxima at 278 and 417 nm, with an A_{279}/A_{417} ratio of 5.1 (data not shown). As has been demonstrated with other pyridoxal-*P* enzymes, the absorption peak around 417 nm is characteristic of an azomethine linkage between the coenzyme and a protein amino group. Like low-specificity D-TA from *Arthrobacter*, the enzyme also required divalent cations for its highest activity: the enzyme lost 90% of its activity by treatment with 10 mM EDTA, but the activity was completely recovered by addition of 1 mM $MnCl_2$ to a 0.2 mg/ml enzyme solution after the elimination of EDTA by extensive dialysis.

The aldolase of *Alcaligenes xylosoxidans* IFO 12669, like the *Arthrobacter* D-TA, was also active toward a broad range of β-hydroxy-α-amino acids, suggesting its potential application for resolution of these amino acids (Table 2). Among the compounds examined, DL-threonine hydroxamate, DL-threonine methyl ester and DL-threoninamide were not the substrates of the aldolase, indicating that the carboxyl group is essential for the enzyme activity.

To examine the effect of pH on the enzyme activity, the initial reaction velocity was measured by the standard assay method using D-threonine as substrate with the following buffers of various pHs: 2-(*N*-morpholino)ethanesulfonic acid (pH 5–6.5), HEPES-NaOH (pH 7.0–8.0), and 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 7.0–9.5). The maximum activity of the D-TA was found to be at pH 8.5. The enzyme retained 90% activity upon treatment in buffers between pH 5.5 and 8.5 for 25 min at 30 °C. The effect of temperature was also examined. The optimum temperature for D-TA of *Alcaligenes xylosoxidans* IFO 12669 was 50–55 °C, and the enzyme retained 85% activity after heating at 50 °C for 30 min.

Resolution of L-*threo*-MDOPS

The resolution reaction, monitored by HPLC, was carried out under the conditions described above. As shown in Fig. 5, the recombinant low-specificity D-TA dem-

Table 1 Purification of the recombinant low-specificity D-threonine aldolase of *Alcaligenes xylosoxidans* IFO 12669[a]

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Cell-free extract	13,900	774	18.0	1.0	100
Ammonium sulfate	11,300	464	24.3	1.4	81.3
DEAE-Toyopearl	9,600	294	38.6	2.1	69.1

[a] Threonine aldolase activity was determined with D-threonine as substrate, as described in the materials and methods section

Table 2 Relative activities and steady-state kinetic constants of the recombinant low-specificity D-threonine aldolase of *Alcaligenes xylosoxidans* IFO 12669

Compound	Relative activity[a] (%)	V_{max} (U mg^{-1})	K_m (mM)	V_{max}/K_m (U mg^{-1} mM^{-1})
D-Threonine	100	38.6	1.0	38.6
D-*allo*-Threonine	75.1	29.0	1.12	25.9
L-Threonine	0	–	–	–
L-*allo*-Threonine	0	–	–	–
threo-Phenylserine				
D-*threo* form	119	46.1	1.02	45.1
L-*threo* form	0	–	–	–
erythro-Phenylserine				
D-*erythro* form	50.0	19.3	0.58	33.5
L-*erythro* form	0	–	–	–
β-3,4-Dihydroxyphenylserine				
D-*threo* form	14.3	5.53	2.28	2.5
L-*threo* form	0	–	–	–
threo-β-3,4-Methylenedioxyphenylserine				
D-*threo* form	214	82.5	1.16	82.1
L-*threo* form	0	–	–	–
erythro-β-3,4-Methylenedioxyphenylserine				
D-*erythro* form	85.8	33.1	0.58	57.1
L-*erythro* form	0	–	–	–
DL-Threonine hydroxamate	0	–	–	–
DL-Threonine methyl ester	0	–	–	–
DL-Threoninamide	0	–	–	–

[a] Relative values as to the activity of the enzyme toward D-threonine

onstrated a time-dependent retro-aldol reaction pattern. D-*threo*-MDOPS disappeared after 20 min, with a molar yield of 50% for L-*threo*-MDOPS and an optical purity of almost 100% ee (Fig. 5, inset). The resolution was also carried out with more concentrated substrate at 50 °C, and complete resolution was observed up to 600 mM DL-*threo*-MDOPS. The resultant L-*threo*-MDOPS, purified subsequently with silica gel column chromatography as described above was identified by both nuclear magnetic resonance (NMR) and HPLC analysis. ^{1}H-NMR (D_2O/TFA-d): δ 4.14 (d, $J = 3.9$ Hz,1H), 4.04(brs, 1H), 5.22(d, $J = 4.1$ Hz, 1H), 5.84 (s, 2H) 6.77–6.84 (m, 3H); ^{13}C-NMR (D_2O/TFA-d): δ 170.4, 148.4, 148.1, 133.7, 120.5, 109.5, 107.2, 102.2, 71.8, 60.2; HPLC: ee > 99.9%.

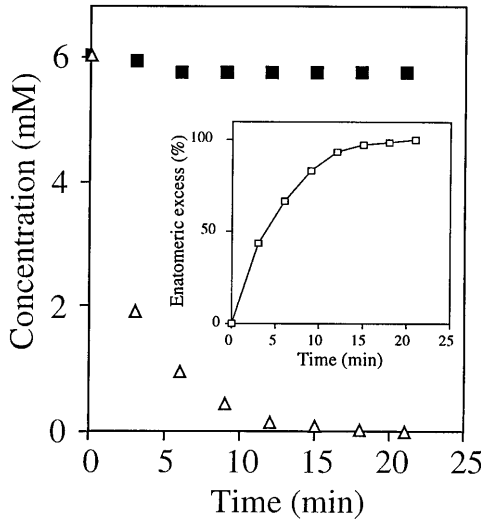

Fig. 5 Resolution of L-*threo*-MDOPS with recombinant low-specificity D-TA of *Alcaligenes xylosoxidans* IFO 12669. MDOPS stereoisomers were measured by HPLC with a Sumichiral OA-500 column. The reaction mixture, comprising 50 mM TRIS/HCl (pH 8.0), 50 μM pyridoxal-*P*, 50 μM manganese chloride, 12 mM DL-*threo*-MDOPS, and 2 U of the purified enzyme, was incubated at 30 °C. D-*threo*- and L-*threo*-MDOPS are indicated as △ and ■, respectively. The optical purity of L-*threo*-MDOPS in the reaction mixture has been replotted (□)

Discussion

A novel D-threonine aldolase occurred in both gram-positive and gram-negative bacteria (Kataoka et al. 1997b). Isolation of the enzyme from these wild-type bacteria was tedious and time-consuming owing to the low expression level (Kataoka et al. 1997b). We previously succeeded in expressing the gene encoding low-specificity D-TA from *Arthrobacter* sp. DK-38, and the specific activity of the enzyme in the recombinant cell extract was increased to 1.8 U/mg, compared to 0.01 U/mg in *Arthrobacter* cell extract (Liu et al. 1998a). To further reinforce the enzyme expression, in this study we cloned the *dtaAX* gene encoding the enzyme from the genomic DNA of *Alcaligenes xylosoxidans*, determined its primary structure, and constructed an overexpresion system.

The *dtaAX* gene has a putative Shine-Dalgarno sequence, but not an apparent σ^{70} type promoter (Fig. 2). However, the enzyme was produced efficiently by *E. coli* recombinant cells under regulation of the *lac* promoter in the presence of IPTG; the specific activity of the enzyme in the cell extract was as high as 18 U/mg (purified enzyme 38.6 U/mg), which was ca. 6,000 times higher than that from the *Alcaligenes* cell extract. This overexpression system led to feasible purification of the

enzyme by ammonium sulfate fractionation and DEAE-Toyopearl chromatography steps, providing us with sufficient D-TA to study the structural and functional relationships of the enzyme and its application.

In this study, we established a batch bioprocess for resolution of L-*threo*-MDOPS, an intermediate for production of the Parkinson's disease therapeutic drug L-*threo*-DOPS. This bioprocess has several merits over the running chemical process: (1) The process does not need any protection and deprotection operations. (2) The by-products formed, glycine and piperonal, could be recycled as synthetic materials for DL-*threo*-MDOPS. (3) The process becomes a "green process" without using organic solvent. If there is any disadvantage, it is that this enzymatic process needs an additional step for the bacterial cultivation. We are now trying to establish an immobilized enzyme/cell system for multiple cycling of the biocatalyst.

Synthesis of optically pure β-hydroxy-α-amino acids has been attracting a great deal of attention, not only because of their key roles as intermediates for the production of important drugs, but also because of their potential application as chiral building blocks for the synthesis of biologically active molecules such as thiamphenicol and florfenicol (Herbert et al. 1993; Vassilev et al. 1995a, b; Shibata et al. 1996; Laib et al. 1998; Liu et al. 1999). The process established for L-*threo*-MDOPS in this study might be extended as a universal method for the synthesis of various optically pure β-hydroxy-α-amino acids by using either D-TA or L-TA (Kataoka et al. 1997a, b; Liu et al 1997a, b, 1998b, c). D-TA would give pure L-*threo*-amino acid from DL-*threo*-amino acid, and L-*erythro*-amino acid from DL-*erythro*-amino acid; similarly, L-TA would produce pure D-*threo*-amino acid from DL-*threo*-amino acid, and D-*erythro*-amino acid from DL-*erythro*-amino acid. The by-products aldehyde and glycine could be recycled as materials for chemical synthesis.

Acknowledgements This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sport of Japan and for RFTF from JSPS.

References

Altschul SF, Boguski MS, Gish W, Wootton JC (1994) Issues in searching molecular sequence data bases. Nat Genet 6: 119–129

Herbert RB, Wilkinson B, Ellames GJ, Kunec EK (1993) Stereospecific lysis of a range of β-hydroxy-α-amino acids catalysed by a novel aldolase from *Streptomyces amakusaensis*. J Chem Soc Chem Commun pp 205–206

Kataoka M, Wada M, Nishi K, Yamada H, Shimizu S (1997a) Purification and characterization of L-*allo*-threonine aldolase of *Aeromonas jandaei*. FEMS Microbiol Lett 151: 245–248

Kataoka M, Ikemi, Morikawa T, Miyoshi T, Nishi K, Wada M, Yamada H, Shimizu S (1997b) Isolation and characterization of D-threonine aldolase, a pyridoxal-5′-phosphate-dependent enzyme from *Arthrobacter* sp. DK-38. Eur J Biochem 248: 385–393

Laib T, Chastanet J, Zhu J (1998) Diastereoselective synthesis of γ-hydroxy-β-amino alcohols and (2S, 3S)-β-hydroxyleucine from chiral D-(*N*,*N*-dibenzylamino)serine (TBDMS) aldehyde. J Org Chem 63: 1709–1713

Liu JQ, Dairi T, Kataoka M, Shimizu S, Yamada H (1997a) L-*allo*-Threonine aldolase from *Aeromonas jandaei* DK-39: gene cloning, nucleotide sequencing, and identification of the pyridoxal-5′-phosphate-binding lysine residue by site-directed mutagenesis. J Bacteriol 179: 3555–3560

Liu JQ, Nakata S, Dairi T, Misono H, Shimizu S, Yamada H (1997b) *GLY1* gene of *Saccharomyces cerevisiae* encodes a low-specificity L-threonine aldolase that catalyzes cleavage of L-*allo*-threonine and L-threonine to glycine: expression of the gene in *Escherichia coli* and purification and characterization of the enzyme. Eur J Biochem 245: 289–293

Liu JQ, Dairi T, Itoh N, Kataoka M, Shimizu S, Yamada H (1998a) A novel metal-activated pyridoxal enzyme with a unique primary structure, low-specificity D-threonine aldolase from *Arthrobacter* sp. strain DK-38: molecular cloning and cofactor characterization. J Biol Chem 273: 16678–16685

Liu JQ, Dairi T, Itoh N, Kataoka M, Shimizu S, Yamada H (1998b) Gene cloning, biochemical characterization and physiological role of a thermostable low-specificity L-threonine aldolase from *Escherichia coli*. Eur J Biochem 254: 220–226

Liu JQ, Dairi T, Itoh N, Kataoka M, Shimizu S, Yamada H (1998c) Low-specificity L-threonine aldolase of *Pseudomonas* sp. NCIMB 10558: purification, characterization and its application to β-hydroxy-α-amino acids syntheses. Appl Microbiol Biotechnol 49: 702–708

Liu JQ, Odani T, Dairi T, Itoh N, Kataoka M, Shimizu S, Yamada H (1999) A new route to L-*threo*–3-[4-(methylthio)phenylserine], a key intermediate for the synthesis of antibiotics: recombinant low-specificity D-threonine aldolase-catalyzed stereospecific resolution. Appl Microbiol Biotechnol 51: 586–591

Maruyama W, Naoi M, Narabayashi H (1996) The metabolism of L-DOPA and L-*threo*-3,4-dihydroxyphenylserine and their effects on monoamines in the human brain: analysis of the intraventricular fluid from Parkinsonian patients. J Neurol Sci 139: 141–148

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 2444–2448

Ohashi N, Nagata S, Ishizumi K, Maeshima K (1984) Process for producing threo-3(3,4-dihydroxyphenyl)serine. European patent 0084928

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Shibata K, Shingu K, Vassilev VP, Nishide K, Fujita T, Node M, Kajimoto T, Wong CH (1996) Kinetic and thermodynamic control of L-threonine aldolase catalyzed reaction and its application to the synthesis of mycestericin D. Tetrahedron Lett 37: 2791–2794

Shine J, Dalgarno L (1975) Determinant of cistron specificity in bacterial ribosomes. Nature 154: 34–38

Thompson JD, Higgins DJ, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Vassilev VP, Uchiyama T, Kajimoto T, Wong CH (1995a) L-threonine aldolase in organic synthesis: preparation of novel β-hydroxy-α-amino acids. Tetrahedron Lett 36: 4081–4084

Vassilev VP, Uchiyama T, Kajimoto T, Wong CH (1995b) An efficient chemo-enzymatic synthesis of α-amino-β-hydroxy-γ-butyrolactone. Tetrahedron Lett 36: 5063–5064