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Review article

Diversity of microbial threonine aldolases and their application

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Abstract

Threonine aldolase catalyzes the reversible interconversion of certain β-hydroxy-α-amino acids and glycine plus the corresponding aldehydes. Various microbial threonine aldolases with different stereospecificities were found on extensive screening, and the genes encoding the proteins were cloned and heterogeneously overexpressed in *Escherichia coli*. By using recombinant threonine aldolases, an enzymatic resolution process was established for the production of optically pure β-hydroxy-α-amino acids. In addition, the threonine aldolase-catalyzed direct synthesis of β-hydroxy-α-amino acid from aldehyde and glycine is discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Threonine aldolase; β-Hydroxy-α-amino acid; Enzymatic resolution; Stereoselective synthesis

1. Introduction

The synthesis of β-hydroxy-α-amino acids has been attracting a great deal of attention because of their roles as medicines in their own right, and as intermediates or chiral building blocks for the production of drugs. L-*threo*-β-(3,4-Dihydroxyphenyl)serine, for example, is a special remedy for Parkinson's disease [1], L-*threo*-β-(4-methylthiophenyl)serine is an intermediate for the production of antibiotics, florfenicol and thiamphenicol [2], 4-hydroxy-L-threonine is a precursor of rizobitoxine, and 3,4,5-trihydroxy-L-aminopentanoic acid is a key component of polyoxins [3]. The D-isomers are also biologically significant, because they not only exist in mature mammals [4] but are also constituents of a range of antibiotics, for example, Fusaricidin [5] and Viscosin [6].

Except for L-threonine and L-serine, which are produced via microbial fermentation, β-hydroxy-α-amino acids are mainly produced through chemical synthesis processes, which consist principally of: (i) chemical synthesis of a racemic amino acid mixture from glycine and aldehydes; (ii) protection of the amino groups of amino acids and chemical resolution of the L-

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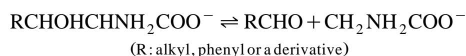
form and D-form of protected amino acids; (iii) deprotection of amino groups to form free L- or D-amino acids; and (iv) recycling of the non-reactive counterparts [7]. These processes have the following problems: (i) chemical resolution is lengthy and inefficient, needing protection and deprotection of the amino groups of the amino acids before and after the resolution process; (ii) the process needs additional steps to recycle the non-reactive counterparts; and (iii) the overuse of organic solvents results in environmental problems. Accordingly, the development of an efficient and clean enzymatic process is desirable.

Threonine aldolase (TA) (EC 4.1.2.5), which catalyzes the reversible interconversion of certain β-hydroxy-α-amino acids and glycine plus the corresponding aldehydes,



$$RCHOHCHNH_2COO^- \rightleftharpoons RCHO + CH_2NH_2COO^-$$
(R: alkyl, phenyl or a derivative)

should be a useful biocatalyst for the synthesis of β-hydroxy-α-amino acids. In this mini-review, we discuss the discovery of various microbial threonine aldolases, gene cloning and overproduction of the proteins, and establishment of an enzymatic resolution process for the production of optically pure β-hydroxy-α-amino acids. In addition, the research progress in the development of a means of threonine aldolase-catalyzed direct synthesis of β-hydroxy-α-amino acids from aldehydes and glycine is discussed.

2. Enzyme occurrence

Threonine has four stereoisomers owing to its two chiral centers. The enzyme appears to fall into two types: L-type and D-type, on the basis of the stereospecificity of the cleavage reaction as to the α-carbon of threonine. L-type TA, acting on L- and/or L-*allo*-threonine, is further divided into three types based on its stereospecificity toward the β-carbon of threonine: (i) L-*allo*-TA is specific to L-*allo*-threonine; (ii) L-TA only acts on L-threonine; and (iii) low-specificity L-TA can use both L-threonine and L-*allo*-threonine as substrates. All the three L-type enzymes have been found to exist in nature. Likewise, D-type TA, acting on D-threonine and/or D-*allo*-threonine, might include D-*allo*-TA, D-TA and low-specificity D-TA.

Low-specificity L-TA activity is widespread in various microorganisms, e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *Arthrobacter aerogenes*, *A. simplex*, *A. globiforms*, *A. pascens*, *A. ureafaciens*, *Proteus vulgaris*, *Bacillus mesentericus*, *Bacterium cadaveris*, *B. succinicum*, *Staphylococcus aureus*, *Xanthomonas campestris*, *Candida utilis*, *C. humicola* and *C. rugosa* [8]; *Pseudomonas putida*, *P. fragi* and *Pseudomonas* sp. [9,10], and *Streptomyces amakusaensis* [11]. L-TA was reported to occur in *Lactobacillus bulgaricus* [12,13] and *Clostridium pasteurianum* [14]. L-*allo*-TA was found in *Aeromonas jandaei*, *Flavobacterium meningosepticum*, *Bacillus* sp., *Alcaligenes piechaudii* and Coryneform bacteria [10]. Unlike L-type TA, D-type TA activity was only detected in *Arthrobacter* sp. and *Alc. xylosoxidans* in a search of more than 1000 threonine-using bacterial strains isolated from soil and preserved type strains, and the enzyme was identified as a low-specifity D-TA [15]. In fact, D-*allo*-TA and D-TA have not been found yet.

3. Properties of various threonine aldolases

All of the known threonine aldolases are constitutive and intracellular enzymes. So far, three kinds of threonine aldolases with different stereospecificities have been purified to homogeneity. L-*allo*-TA was purified from *Aer. jandaei* [16]; low-specificity L-TAs were purified from *Candida humicola* [17], *Sacchromyces cerevisiae* [19], *Escherchia coli* [19] and *Pseudomonas* sp. [20,21]; and low-specificity D-TA was purified from *Arthrobacter* sp. [15]. The purified L-*allo*-TA acts stereospecifically on L-

allo-threonine out of the four stereoisomers, but the enzyme uses both L-*threo*- and L-*erythro*-phenylserine, and their derivatives as substrates. The reason why L-*allo*-TA has lost its stereospecificity towards β-hydroxy-α-amino acids with a phenyl group is unknown, so we are trying to elucidate the three-dimensional structure of the enzyme to solve this puzzle. From the application point of view, low-specificity L-TA and low-specificity D-TA, being specific to the α-position and non-specific towards the β-position of a substrate, can decompose a broad range of β-hydroxy-α-amino acids to aldehydes and glycine, indicating that these enzymes might be useful biocatalysts for the resolution of certain β-hydroxy-α-amino acids, such as phenylserine, β-(3,4-methylenedioxyphenyl)serine, β-(3,4-dihydroxyphenyl)serine, β-(4-methylthiophenyl)serine, β-hydroxyleucine, β-hydroxyvaline, etc.

A summary of the properties of various threonine aldolases is given in Table 1. All these proteins need pyridoxal 5'-phosphate (PLP) as a cofactor for their catalytic activity, which might account for the slightly alkaline optimal pH values of these aldolases (Table 1). Remarkably, in addition to PLP, low-specificity D-TA from *Arthrobacter* sp. DK-38 requires divalent ions such as Mn^{2+}, Mg^{2+}, Co^{2+}, etc. as activators to express its highest aldolase activity. Equilibrium dialysis and atomic absorption studies have revealed that low-specificity D-TA can bind to 1 mol of Mn^{2+} ion per mol of subunit [22]. This is the first example showing that a PLP-dependent enzyme needs divalent ions as cofactors. The role of a divalent ion bound to the enzyme has not been elucidated yet, but it seems to be involved in the formation of an aldimine between the active site lysine residue (Lys 59) and the substrate β-hydroxy-α-amino acid, as judged from the results of spectroscopic studies (Liu et al., unpublished data). On the other hand, a divalent ion has also been supposed to play an important role in the thermostability of the enzyme [21]. As shown in Table 1, the subunit molecular masses of these aldolases are similar, and both L-*allo*-TA and low specificity L-TAs have a tertrameric structure, while low-specificity D-TA from *Arthrobacter* sp. DK-38 is a monomeric protein.

Table 1
Comparison of various threonine aldolases

Enzyme	L-*allo*-TA	Low-specificity L-TA		Low-specificity D-TA
Microorganism	*Aer. jandaei* DK-39	*E. coli* K-12	*Pseudomonas* sp. NCIMB10558	*Arthrobacter* sp. DK-38
Molecular mass (Da)	152,000	140,000	145,000	51,000
Subunit molecular mass (Da)	36,000	36,500	38,000	40,000
Cofactor	PLP	PLP	PLP	PLP, divalent cation
Optimum temperature (°C)	35–45	65–70	25	65
Thermostability (treatment conditions/remaining activity)	50°C, 15 min/ 15%	60°C, 60 min/ 100%	50°C, 15 min/ 10%	50°C, 15 min/ 100%
Optimum pH	8.5–10.5	8.5–9.0	8.0–8.5	8.0–9.0
pH stability	6.0–9.0	6.0–9.5	5.5–9.0	7.0–8.5
V_{max} (μmol/min per mg)				
L-threonine	–	1.7	44.2	–
L-*allo*-threonine	45.2	5.5	31.1	–
D-threonine	–	–	–	32.1
D-*allo*-threonine	–	–	–	34.9
K_m (mM)				
L-threonine	–	2.85	14.7	–
L-*allo*-threonine	1.45	0.22	14.6	–
D-threonine	–	–	–	8.4
D-*allo*-threonine	–	–	–	4.4

4. Gene cloning and heterogeneous expression

The wild-type strains produced too low amounts of threonine aldolase to be directly used as biocatalysts for industrial application. We thus attempted to clone threonine aldolase genes and to construct expression systems for the enzymes. Based on the N-terminal sequence of the purified L-*allo*-TA from *Aer. jandaei* DK-39, a set of primers was designed and synthesized. PCR with these primers and *Aer. jandaei* DK-39 chromosomal DNA as the template yielded an amplified band corresponding to 100-bp. The amplified DNA was then cloned into pUC118 in *E. coli*. The deduced amino acid sequence of the PCR fragment perfectly agreed with the NH_2-terminal amino acid sequence determined for the purified L-*allo*-TA from *Aer. jandaei* DK-39 [16]. Colony hybridization with the 100-bp fragment as a probe against the *Aer. jandaei* DK-39 genomic library resulted in the cloning of the entire L-*allo*-TA gene [23]. This was the first paper describing the gene cloning of threonine aldolase. Through a similar approach, low-specificity D-TA threonine aldolase genes have also been successfully isolated from *Arthrobacter* sp. DK-38 and *Alc. xylosoxidans* IFO 12669, respectively [22,24]. A complementary cloning technique was used to isolate low-specificity L-TA from *Pseudomonas* sp. NCIMB 10558, using a glycine-auxotrophic *E. coli* strain GS245 as a host [25]. Low-specificity L-TA genes have also been

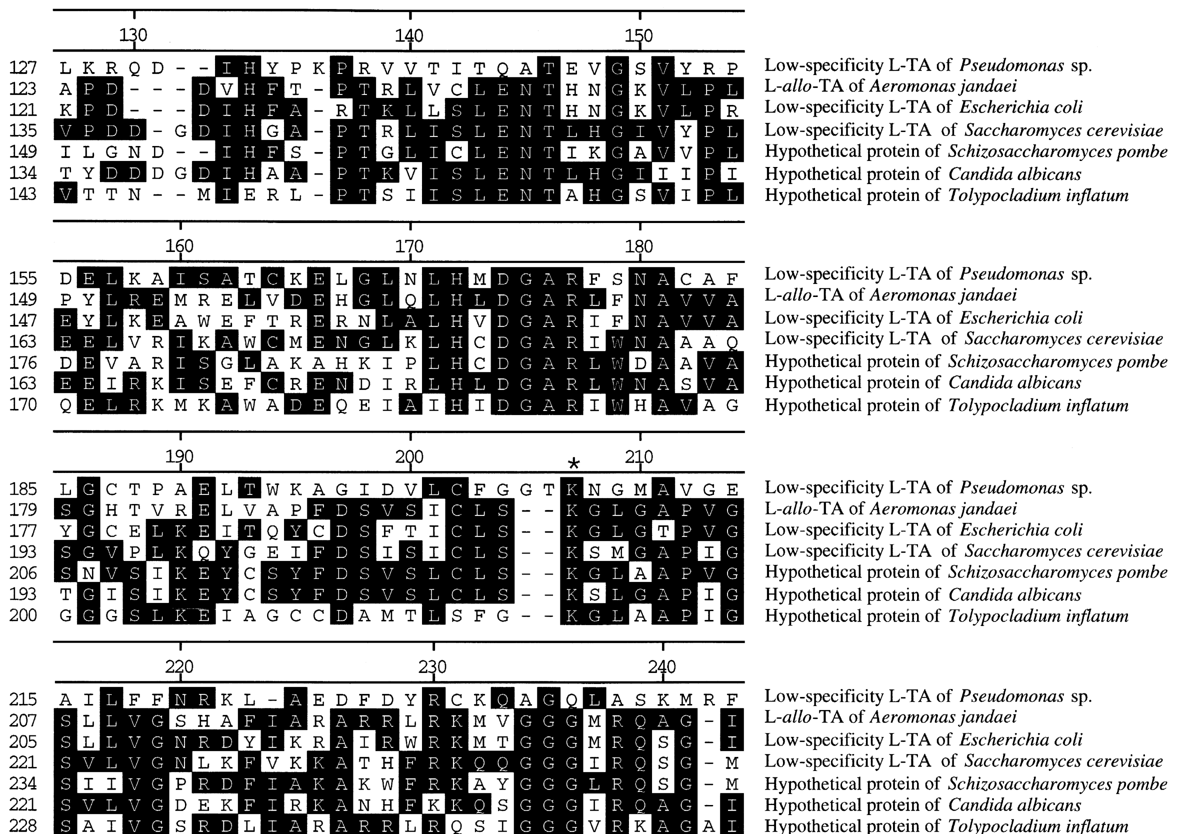


Fig. 1. Segmental sequence alignment of various L-type TAs. The identical residues are boxed. The putative active site lysine residue is indicated by an asterisk. The numbers on the left are the residue numbers of each amino acid sequence, and those at the top are the residue numbers of the *Pseudomonas* aldolase.

cloned from *Saccharomyces cerevisiae* [18] and *E. coli* [19] by means of a PCR approach.

In a search of a nucleotide and amino acid database, we found that L-*allo*-TA and low-specificity L-TAs are not only similar in primary structure, but also show significant amino acid sequence similarity with numerous hypothetical proteins from other microorganisms, such as *C. albicans*, *Schizosaccharomyces pombe*, *Tolypocladium inflatum*, *Rhodobacter capsulatus*, *Cellulomonas fimi* and *Eremothecium gossypii*. Partial alignment of some of these proteins is shown in Fig. 1. We believe that these hypothetical proteins are threonine aldolases. Noticeably, Lys 199 of L-*allo*-TA of *Aer. jandaei* DK-39 and Lys 207 of low-specificity L-TA of *Pseudomonas* sp. NCIMB 10558, which are completely conserved in these hypothetical proteins and threonine aldolases, were identified as the PLP-binding sites of the enzymes in site-directed mutagenesis experiments [23,25].

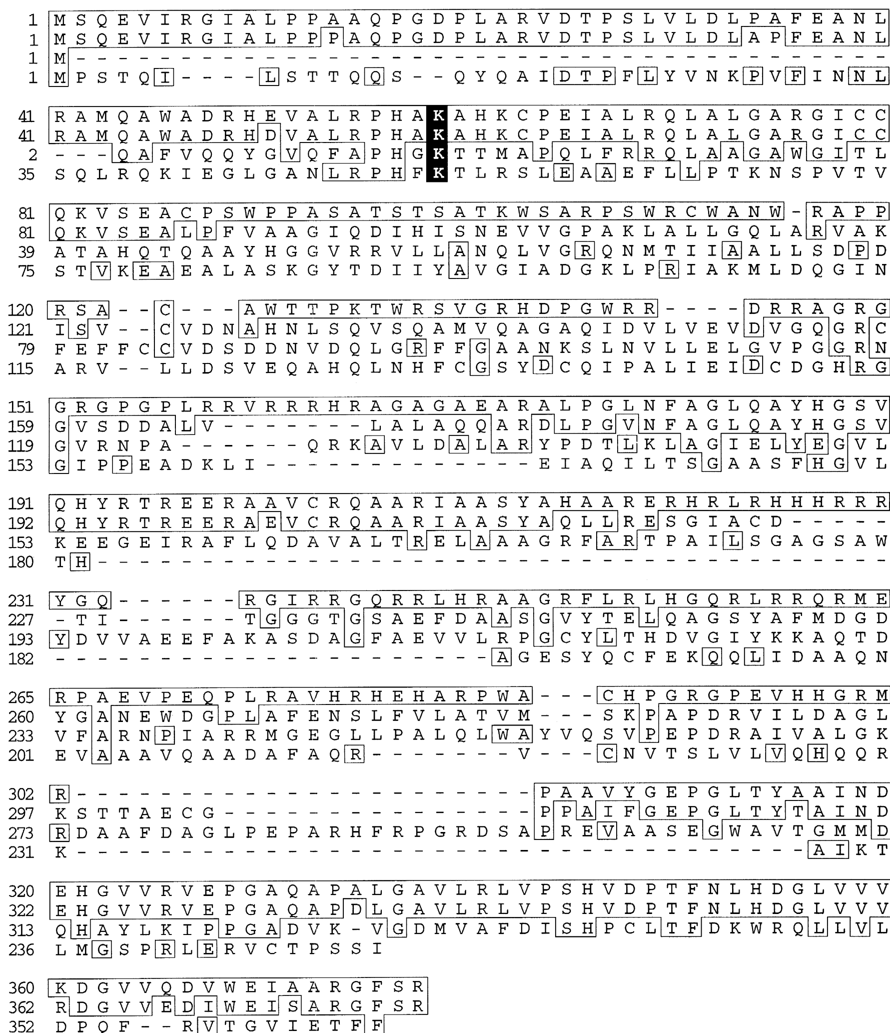


Fig. 2. Amino acid sequence alignment of D-type TAs. From top to bottom in each set, the proteins are low-specificity D-TA from *Alc. xylosoxidans* IFO 12669, low-specificity D-TA from *Arthrobacter* sp. DK-38, D-serine deaminase (GenBank U41162) of *B. cepacia*, and a hypothetical protein (GenBank U73935) of *Shewanella* sp. strain SCRC-2738. The identical residues are boxed. The numbers on the left are the residue numbers for each amino acid sequence. The putative active site lysine residues are boxed in black.

The primary structures of low-specificity D-TAs of *Arthrobacter* sp. DK-38 and *Alc. xylosoxidans* IFO 12669, which are similar to each other, showed no significant similarity to those of the currently known pyridoxal enzymes. However, in a search of protein and nucleotide sequence databases (GenBank, EMBL, DDBJ, and PDB) using the sequence similarity searching program Blast [26], D-serine deaminase (GenBank, U41162) of *Burkholderia cepacia* and a hypothetical protein (GenBank, U73935) of *Shewanella* sp. strain SCRC-2738 were found to be significantly similar in primary structure to low-specificity D-TAs. Amino acid sequence alignment of these proteins is shown in Fig. 2. Lys59 of low-specificity D-TA of *Arthrobacter* sp. DK-38 (Fig. 2), the sole conserved lysine residue in these proteins, was determined to be the PLP-binding site by chemical modification with $NaBH_4$ [22]. A site-directed mutagenesis study further showed that replacement of Lys59 by Arg resulted in the complete loss of the aldolase activity of the enzyme (unpublished data). These results strongly suggested that Lys 59 is an active site residue, playing an important role in the enzymatic reaction. Low-specificity D-TA, requiring divalent ions for its aldolase activity, with a unique primary structure, might represent a novel metal-activated pyridoxal enzyme family.

To overproduce threonine aldolases, the entire threonine aldolase genes were amplified by PCR and then inserted into downstream of promoter pT7 of pT7Blue, *tac* of pKK223-3, or *lac* of pUC118/pUC119. Upon induction with isopropylthio-β-D-galactoside, the host cells, *E. coli* BL21 or XL1-blue carrying the constructed plasmids, produced a large amount of enzymes. The threonine aldolase-specific activity of cell extracts increased by at least 200 times in all cases, compared to in the cases of the wild-type strains [18,19,21–25,27]. The established over-expression systems can supply us with enough of various threonine aldolases for applications.

5. Production of optically pure β-hydroxy-α-amino acids by enzymatic resolution

A universal process for the production of optically pure β-hydroxy-α-amino acids by means of threonine aldolases is shown in Fig. 3. Low-specificity D-TA should give pure L-*threo*-amino acid from DL-*threo*-amino acid, and L-*erythro*-amino acid from DL-*erythro*-amino acid; similarly, low-specificity L-TA should pro-

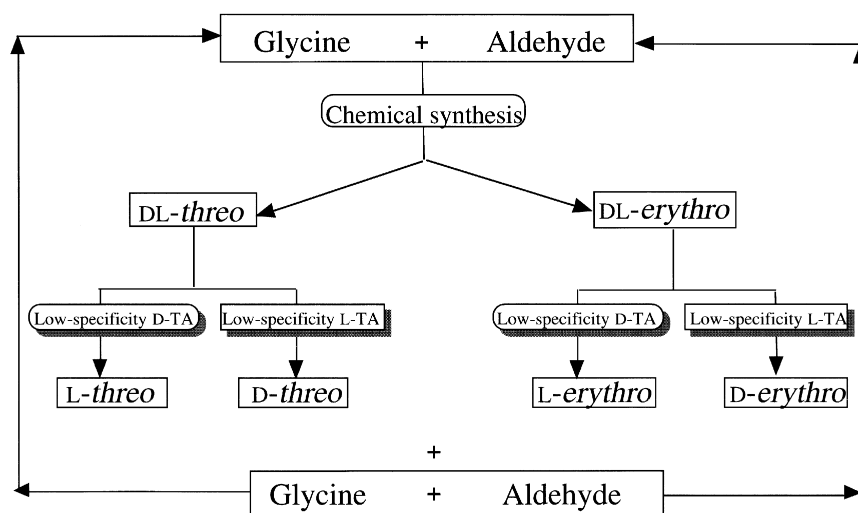


Fig. 3. Proposed universal chemo-enzymatic process for the production of optically pure β-hydroxy-α-amino acids.

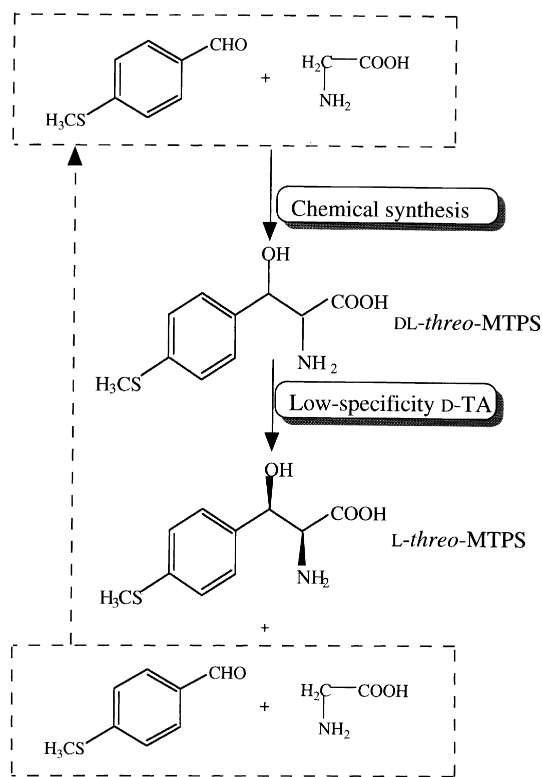


Fig.4. Chemo-enzymatic process proposed for the production of L-*threo*-β-(4-methylthiophenyl)serine, an intermediate for antibiotics florfenicol and thiamphenicol. MTPS, β-(4-methylthiophenyl)serine.

duce pure D-*threo*-amino acid from DL-*threo*-amino acid, and D-*erythro*-amino acid from DL-*erythro*-amino acid. The by-products, aldehydes and glycine, can be recycled as starting materials for chemical synthesis. As a model system, we succeeded in synthesizing the four stereoisomers of phenylserine by using low-specificity L-TA of *Pseudomonas* sp. NCIMB 10558 and low-specificity D-TA of *Arthrobacter* sp. DK-38 [28]. Recently, this approach was applied to the production of L-*threo*-β-(4-methylthiophenyl)serine (Fig. 4), an intermediate for the synthesis of florfenicol and thiamphenicol [27], and L-β-(3,4-methylenedioxyphenyl)serine, an intermediate for the production of a Parkinson's disease therapeutical drug [24]. The *E. coli* recombinant cells harbouring the low-specificity D-TA gene were shown to be as efficient as the purified enzyme for use as resolving agents (Fig. 5) [27]. Under the experimental conditions used, 200 mM DL-*threo*-β-(4-methylthiophenyl)serine and 600 mM DL-*threo*-β-(3,4-methylenedioxyphenyl)serine could be completely resolved, with a molar yield of no less than 48% and an optical purity of higher than 99% ee for L-*threo*-isomers [24,27].

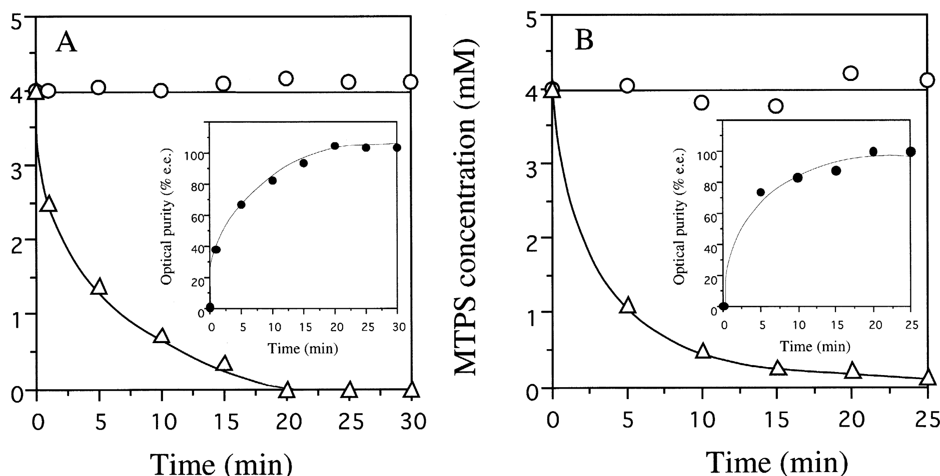


Fig. 5. Resolution of L-*threo*-β-(4-methylthiophenyl)serine with recombinant low-specificity D-TA of *Arthrobacter* sp. DK-38. The reaction mixture, comprising 50 mM Tris–HCl (pH 8.0), 50 μM pyridoxal 5'-phosphate, 50 μM manganese chloride, 8 mM DL-*threo*-β-(4-methylthiophenyl)serine, and 2 U of the purified enzyme (A) or 0.4 mg of wet *E. coli* cells containing plasmid pTDTA (B), was incubated at 50°C. D-*threo*-and L-*threo*-β-(4-methylthiophenyl)serine are denoted as (Δ) and (○), respectively. The insets are for an enantiomeric excess of L-*threo*-β-(4-methylthiophenyl)serine in the reation mixture. MTPS, β-(4-methylthiophenyl)serine.

6. TA-catalyzed direct synthesis of β-hydroxy-α-amino acids from glycine and aldehydes

Undoubtedly, a direct enzymatic synthesis method would theoretically be more efficient and promising, compared with an enzymatic resolution process, whose maximal molar yield is 50%. To investigate the synthetic utilities of threonine aldolases, we selected the synthesis, from benzaldehyde and glycine, of phenylserine stereoisomers as a model system by using various threonine aldolases with different stereospecificities, because not only are phenylserine stereoisomers easy to analyze, but phenylserine derivatives are important intermediates for the production of drugs. As a consequence, low-specificity D-TA of *Arthrobacter* sp. DK-38 catalyzed the formation of D-*threo*-phenylserine with an optical purity of 95% ee and a molar yield of 48% from 40 mM benzaldehyde and 1 M glycine [28]. When L-*allo*-TA of *Aer. jandaei* DK-39 and low-specificity L-TA of *Pseudomonas* sp. NCIMB 10558 were used as catalysts, L-*threo*-phenylserine was produced with optical purities of 80% ee and 78% ee, respectively, and molar yields of 80% and 75%, respectively, from 25 mM benzaldehyde and 2 M glycine [29]. It is quite interesting that the stereospecificity at β-carbon of substrate with L-*allo*-TA in the synthetic reaction of phenylserine (*threo*-specific) was opposite to that in the degradation reaction of threonine (*erythro*-specific). However, cause of the stereospecificity inversion of L-*allo*-TA dependent on substrates is not clarified yet. Recently, Kimura et al. successfully synthesized various β-hydroxy-α-amino acids on a preparative scale by using low-specificity L-TA and low-specificity D-TA [30]. Their elegant work showed that a reaction under kinetically controlled conditions provided L-*erythro*-β-hydroxyleucine with 92% ee and a 17% yield with low-specificity L-TA, and D-*threo*-β-hydroxyleucine with 86% ee and a 49% yield with low-specificity D-TA [30]. These authors also found that low-specificity L-TA catalyzes the aldol condensation of glycine and an aldehyde to give a β-hydroxy-α-amino acid with a high diastereoisomeric *erythro/threo* ratio when an oxygen exists at the β-position of the substrate aldehyde. L-*erythro*-γ-(Benzyloxy)-threonine, an intermediate for the monobactam antibiotic Carumoman, was synthesized from benzyloacetaldehyde and glycine with a yield of 78% and optical purity of 92% ee [31].

In spite of these achievements in the development of a direct threonine aldolase-catalyzed synthesis process for β-hydroxy-α-amino acids, extension of this method to industrial application is still far away, and several problems, such as the equilibrium of the aldol and retro-aldol reactions, the solubility of aldehydes, the inhibitory effect of aldehydes on threonine aldolases, etc., should be solved in future works.

7. Prospects

As described above, three different types of microbial threonine aldolases were found on extensive screening. These enzymes were purified and characterized, and the genes encoding them were cloned and overexpressed. By using low-specificity L-TA and low-specificity D-TA as catalysts, a universal resolution process was established for the production of industrially important β-hydroxy-α-amino acids.

From a scientific point of view, it would be interesting to elucidate the physiological roles of threonine aldolase and serine hydroxymethyltransferase, both of which catalyze the interconversion of threonine and glycine plus aldehydes. In spite of this common property, the two enzymes differ from each other in the following: (1) different primary structures; and (2) serine hydroxymethyltransferase, requiring H_4 folate as a cofactor, cleaves serine to yield glycine, which threonine aldolase cannot do.

The threonine aldolase-catalyzed one-step synthesis process would be a better method for the production of optically pure β-hydroxy-α-amino acids. To make it industrially applicable,

there are several possible approaches, such as the following. (1) Screening of a new enzyme with both a preferable stereospecificity and predominant synthetic activity over cleavage activity. (2) Converting currently available threonine aldolases to proteins with improved functions by means of protein engineering, such as random mutation by means of PCR, chemical modification and/or DNA shuffling techniques. (3) Reaction engineering approaches: using immobilized enzymes, immobilized cells, or surface-modified enzymes to make the enzymes more stable; establishing a two-phase reaction and/or a continuous reaction system to solubilize aldehydes and lessen the inhibition effect of concentrated aldehydes on the enzymes.

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