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Cloning and Expression of D-Threonine Aldolase from *Ensifer arboris* NBRC100383

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***Abstract*—**D-erythro-cyclohexylserine (D-erythro-CHS) is a chiral unnatural β-hydroxy amino acid expected for the synthesis of drug for AIDS treatment. To develop a continuous bioconversion system with whole cell biocatalyst of D-threonine aldolase (D-TA) genes for the D-erythro-CHS production, D-threonine aldolase gene was amplified from *Ensifer arboris* 100383 by direct PCR amplication using two degenerated oligonucleotide primers designed based on genomic sequence of *Shinorhizobium meliloti* 1021 (SMb21134). Sequence analysis of the cloned DNA fragment revealed one open-reading frame of 1059 bp and 386 amino acids. This putative D-TA gene was cloned into NdeI and EcoRI (pEnsi-DTA[1]) without His-tag sequence or BamHI (pEnsi-DTA[2]) site with His-tag sequence of the pET21(a) vector. The expression level of the cloned gene was extremely overexpressed by *E. coli* BL21(DE3) transformed with pEnsi-DTA[1] compared to *E. coli* BL21(DE3) transformed with pEnsi-DTA[2]. When the cells expressing the wild-type enzyme were used for D-TA enzyme activity, 12 mM glycine was successfully detected in HPLC analysis. Moreover, the whole cells harbouring the recombinant D-TA was able to synthesize D-erythro-CHS with a yield of 0.6 mg/ml in a batch reaction.

***Keywords*—**About four key words or phrases in alphabetical order, separated by commas.

I. Introduction

WE have been interested in developing an enzymatic synthesis of D-erythro-cyclohexylserine (D-erythro-CHS), a chiral unnatural β-hydroxy amino acid expected for the synthesis of drug for AIDS treatment, since the

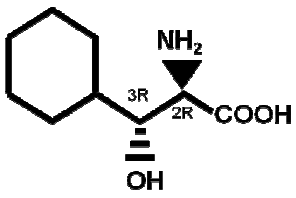

Fig. 1 Structure of D-erythro-cyclohexylserine

commercial production of this drug so far has been accomplished only by a chemical synthesis that consists of very complex multi-step reactions including several protection- and deprotection reactions. On the other hand, low-specificity D-threonine aldolase (D-TA, E.C. 4.1.2.5), which catalyzes the

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TABLE I
ABSOLUTE CONFIGURATION OF CYCLOHEXYLSERINE

No.	Compound	Absolute configuration
1	L-threo-type	2S,3R
2	D-threo-type	2R,3S
3	L-erythro-type	2S,3S
4	D-erythro-type	2R,3R

cleavage of various D-β-hydroxy amino acids into glycine and corresponding acetaldehydes, can also catalyze D-erythro-CHS as substrate resulting glycine and cyclohexylserine. Since this aldol reaction is reversible, it also can directly synthesize D-erythro-CHS by using the equilibrium approach, namely by supplying excess amounts of glycine and 2,3-hydroxybenzaldehyde in the enzyme reaction [1]. Although lots of D-TA that has been known to be exist in a wide range of microorganisms, but very few studies on their properties has been known until now.

One of the main purposes of our work has been to develop enhanced diastereoselective D-TA, suitable for an enzymatic synthesis of D-erythro-CHS. To develop a continuous bioconversion system with whole cell biocatalyst of D-TA genes for the D-erythro-CHS production, D-TA gene was cloned from *Ensifer arboris* NBRC100383 genomic DNA. Here, we report that cloning of this genes and expression in *E. coli* strain.

II. Experiments

A. Strain, plasmid and medium

Ensifer arboris NBRC100383 was obtained from NBRC (National Bioresource Center, National Institute of Technology and Evaluation, Japan). Basic medium containing 1% peptone, 0.5% yeast extract, 1% NaCl and 0.5% D-erythro-CHS was used for a routine cultivation and preservation of the strain. Oligonucleotide primers used in this study are described in Table 1. All the restriction endonucleases were purchased from New England Biolabs.

B. Genetic manipulation and nucleotide sequencing analysis

General DNA manipulation such as plasmid preparation and subcloning was performed by following the method of Sambrook et al. unless otherwise stated [2]. The nucleotide sequences were analyzed *via* a dye terminator cycle-sequencing reaction by following the supplier's instructions (Applied Biosystems). The sequencing products were detected with a

377 DNA sequencer (Applied Biosystems), and the resulting sequence data was analyzed by the WinGene 2.31 software.

C. Cloning of DNA fragment encoding putative D-TA

The genes encoding for putative D-TA were amplified directly from *E. arboris* NBRC100383 genomic DNA by means of polymerase chain reaction (PCR). Two oligonucleotide primers: forward primer, sinoDN and reverse primer: sinoDC were prepared to anneal N-terminal sequence or C-termianl sequence of putative D-TA on genomic DNA of *E. arboris* NBRC100383. PCR was done by LA-taq polymerase kit (TAKARA) for 25cycle of: 94 °C for 120 s, 55 °C for 30 s and 72 °C for 1 min. The PCR amplified approximately 1-kbp fragment was directly inserted into TA cloning vector (pTA-DTAEnsi).

D. Subcloning of putatative D-TA gene for high level expression in E. coli

The obtained pTA-DTAEnsi clone contained a putatative D-TA gene was subcloned into pET expression system under T7 promoter in order to reconstruct high level expression vector. PCR was done to amplify putatative D-TA gene on pTA-DTAEnsi by using 3 oligonucleotide primers (Table 2). PCR was done by KOD plus PCR kit (Toyobo) for 25cycle of: 94 °C for 60 s, 55 °C for 30 s and 68 °C for 1 min. The PCR amplified approximately 1-kbp fragment was directly inserted into NdeI or EcoRI and BamHI of pET21(a) expression vector (pEnsi-DTA[1] and pEnsi-DTA[2]) and transformed *E. coli* BL21(DE3) competent cell. The recombinant proteins were incubated until OD_{612} was reached 0.85 (approximately 3 h). Then cultivation was continued until 9 hrs at 25°C with reciprocal shaking. The cells were harvested by centrifugation (10,000 × g, 10 min, 4°C), washed twice with 0.85% NaCl solution, and suspended to Tris-HCl buffer (10 mM, pH7.0).

E. High level expression of putatative D-threonine aldolase gene

The constructed expression vector, pEnsi-DTA[1] (NdeI and BamHI) and pEnsi-DTA[2] (EcoRI and BamHI) was transformed *E. coli* BL21(DE3) by using heat shock procedure and was grown in LB medium containing 100 ug/ml ampicillin. A recombinant D-TA protein was induced by addition of 1 mM IPTG when OD600 was reached 0.6 (approximately 3 h). Then cultivation was continued until 9 h at 30°C with reciprocal shaking. The cells were harvested by centrifugation (10,000× g, 10 min, 4°C), washed twice with 0.85% NaCl solution, and suspended to Tris-HCl buffer (10 mM, pH7.0). Expression of the recombinant D-TA in *E. coli* was confirmed by SDS-PAGE on 12% polyacrylamide gel (Nielsen and Reynolds 1978) .

F. Analysis of D-erythro-CHS

For quantitive analysis of D-erythro-CHS, 10 µL of the reaction solution was applied onto HITACHI L-2200 HPLC equipped with a COSMOSIL 5C18-MS column (4.6m × 150mm) using a mobile phase consisting 0.1% (w/v) 1-heptanesulfonic acid sodium salt in 10% MeOH. The column temperature maintained at 20°C. A linear relationship was achieved on the peak area ratio of D-erythro-CHS standard. The protein concentration was determined by Bio-Rad Protein Assay kit with bovine serum albumin as a standard.

TABLE II
OLIGONUCLEOTIDES USED IN THIS STUDY

Name	Olignucleotide sequence
sinoDN	5`-ATGACACTGCCGATCGAAACCCCCGCCGTG-3`
sinoDC	5`-TCAGAGGACACGTCCGCGGGCGACGACCGG-3`
DTA-Nde	5`-AAAAAAACATATGACACTGCCGATCGAAAC-3`
DTA-Eco	5`-TATACCATGGAATTCACACTGCCGATCGAAAC-3
DTA-Bam	`
	5`-ATCTAGAGGATCCGAGGACACGTCCGCG-3`

III. Results and Discussions

A. Screening

We screened soil samples obtained Chiba area in Japan to find strains for threonine aldolase activity since an enzymatic synthesis approach using low-specific D-TA (E.C. 4.1.2.42) has a considerable merit to synthesis useful β-hydroxy amino acids like D-erythro-CHS directly by one step aldol condensation reaction. Thus, D-TA producing strain was screened from various soils by using enrichment culture technique. Among the obtained approximately 200 strains, a strain was isolated and characterized. The 16S rDNA sequence analysis suggested the obtained strains, NK-121 as Ensifer sp. Furthermore, to obtain better strain with enhanced D-TA activity, we screened 10 Ensifer sp. strains founded in culture collection at NBRC in Japan, resulting best strain with D-TA activity, *Ensifer arboris* NBRC100383.

B. Gene cloning and expression

We tried to find D-TA gene which might be responsible for the hydrolysis of D-erythro-CHS. However, even though we did not find any homologus sequence for D-TA from already known Ensifer sequences through internet databases, we found that most similar strain, *Sinorhizobium meliloti* 1021 that is already obtained genomic sequencing. From the database of genome database of NCBI, a putative D-TA gene sequence (SMB21134) in S. *meliloti* 1021 was identified. Thus, the oligonucleotide primer was designed to amplify D-TA from E. arboris NBRC100383 by PCR, resulting amplification product of approximately 1 kb as shown in Fig. 2(a, lane3). Sequencing analysis showed that the obtained D-TA gene has a single open-reading frame of 1059 base pairs coding 386 amino acids. The molecular weight estimated by SDS-PAGE was 36kDa. The open reading frame showed a G+C content of 57.7 mol%, and an isoelectric point (pI) of 5.87. Comparison of the deduced amino acid sequence with other published sequences showed very low similarity with already published as low-specific D-threonine aldolase sequence of Pseudomonas sp. (40%) and E. coli (16.2%).

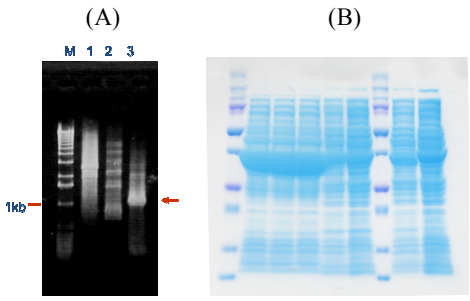

Fig. 2 Cloning and Expression of D-threonine aldolase gene from E. arboris NBRC100383 (A) PCR amplication
Lane 1: sense primer only, Lane 2: antisense primer only, Lane 3: sense and antisense primer. (B) SDS-PAGE analysis of expressed D-TA in E. coli. Lane 1 and 7:marker, Lane 2-4: cell-free extract of E. coli BL21(DE3), transformed pEnsi-DTA[1] after 12 h IPTG induction, Lane 8 and 9: E. coli BL21(DE3), transformed pEnsi-DTA[2]

However, it showed higher similarity with Sinorhzobium species (98%) and Mesorhizobium species (97%).The recombinant E. coli BL21(DE3), transformed pEnsi-DTA[1] under the control of pT7 promoter was overproduced soluble active recombinant D-TA after 12 h induction as shown in Fig.2 lane2-4. Over induction of *E. coli* cells over 13 h was inhibitory with dramatic decreasing soluble active recombinant D-TA as shown in Fig.2 lane5 even though target D-TA still remained in a high level. Compared to E. coli BL21(DE3), transformed pEnsi-DTA[1], *E. coli* BL21(DE3), transformed pEnsi-DTA[2] did not show enhanced expression level compared to E. coli BL21(DE3), transformed pEnsi-DTA[1]. It seems that the addition of his-tag sequence at C-terminal did not effective for high-level expression and for simple purification (Fig. 2 lane 8 and 9). When the cells expressing the wild-type enzyme were examined on plate containing D-erythro-CHS, the recombinant *E. coli* strain showed clear halo around the recombinant E. coli strain harboring pEnsi-DTA[1] compared to negative control E. coli strain which did not containe D-TA gene, indicating strong hydrolysis activity for D-erythro-CHS. When the obtained cell-free extract after sonication and used for D-TA enzyme activity, 12 mM glycine was successfully detected in HPLC analysis. Moreover, the whole cells harbouring the recombinant D-TA was able to synthesize D-erythro-CHS with a yield of 0.6 mg/ml in a batch reaction as shown in Fig. 3(B).

IV. Conclusion

D-threonine aldolase gene was amplified from *Ensifer arboris* NBRC100383 by direct PCR amplication using two oligonucleotide primers designed based on genomic sequence of Shinorhizobium meliloti 1021 (SMb21134). Sequence analysis of the cloned DNA fragment revealed one open-reading frame of 1059 bp and 386 amino acids.

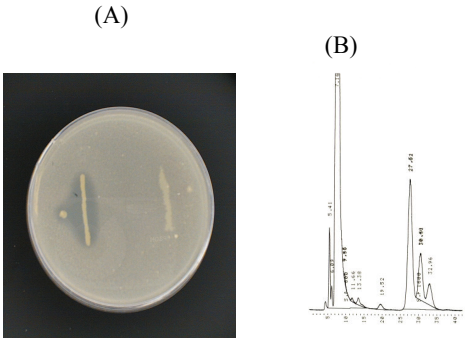

Fig. 3 Hydrolysis and synthesis activity of D-threonine aldoase from *E. arboris* NBRC100383 high level expressed in E. coli
(A) Plate assay for hydrolysis activity of D-erythro-CHS. Left strain: E. coli BL21(DE3), transformed pEnsi-DTA[1], Right strain: E. coli BL21(DE3), transformed pET vector without D-TA gene. (B) HPLC analysis for D-erythro-CHS synthesis activity.

This putative D-TA gene was cloned into NdeI and EcoRI site of the pET21(a) vector. The expression level of the cloned gene was extremely overexpressed. When the cells expressing the wild-type enzyme were used for D-TA enzyme activity, 12 mM glycine was successfully detected in HPLC analysis. Moreover, the whole cells harboring the recombinant D-TA was able to synthesize D-erythro-CHS with a yield of 0.6 mg/ml in a batch reaction.

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