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Cloning and characterization of D-threonine aldolase from the green alga *Chlamydomonas reinhardtii*

Yuki Hirato [a], Mayumi Tokuhisa [a], Minoru Tanigawa [a], Hiroyuki Ashida [b], Hiroyuki Tanaka [c], Katsushi Nishimura [a, d, *]

[a] *Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, 1-8-14 Kanda-Surugadai, Chiyoda-Ku, Tokyo, 101-8308, Japan*
[b] *Department of Molecular and Functional Genomics, Interdisciplinary Center for Science Research, Shimane University, Nishikawatsu 1060, Matsue, Shimane, 690-8504, Japan*
[c] *Department of Biochemistry and Molecular Biology, Shiga University of Medical Science, Seta, Shiga, 520-2192, Japan*
[d] *Department of Biotechnology and Materials Chemistry, Junior College, Nihon University, 7-24-1Narashinodai, Funabashi, Chiba, 274-8501, Japan*

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ABSTRACT

D-Threonine aldolase (DTA) catalyzes the pyridoxal 5'-phosphate (PLP)-dependent interconversion of D-threonine and glycine plus acetaldehyde. The enzyme is a powerful tool for the stereospecific synthesis of various β-hydroxy amino acids in synthetic organic chemistry. In this study, DTA from the green alga *Chlamydomonas reinhardtii* was discovered and characterized, representing the first report to describe the existence of eukaryotic DTA. DTA was overexpressed in recombinant *Escherichia coli* BL21 (DE3) cells; the specific activity of the enzyme in the cell-free extract was 0.8 U/mg. The recombinant enzyme was purified to homogeneity by ammonium sulfate fractionation, DEAE-Sepharose, and Mono Q column chromatographies (purified enzyme 7.0 U/mg). For the cleavage reaction, the optimal temperature and pH were 70 °C and pH 8.4, respectively. The enzyme demonstrated 90% of residual activity at 50 °C for 1 h. The enzyme catalyzed the synthesis of D- and D-*allo* threonine from a mixture of glycine and acetaldehyde (the diastereomer excess of D-threonine was 18%). DTA was activated by several divalent metal ions, including manganese, and was inhibited by PLP enzyme inhibitors and metalloenzyme inhibitors.

1. Introduction

D-Threonine aldolase (DTA, EC 4.1.2.42) catalyzes the interconversion of β-hydroxy-D-amino acids (e.g., D-threonine) and glycine plus the corresponding aldehyde. It is pyridoxal 5′-phosphate (PLP)-dependent and classified as a fold-type III enzyme, which depends on several divalent metal ions (Liu et al., 2000a). DTA demonstrates high selectivity for α-carbon configurations but low selectivity for β-carbon configurations (Franz and Stewart, 2014).

DTA is a powerful tool for catalyzing C—C bond cleavage and formation in synthetic organic chemistry (Dückers et al., 2010). The cleavage reaction is important for the chiral resolution of β-hydroxy-amino acids such as intermediates in the production of antibiotics and therapeutic drugs for Parkinson's disease (Liu et al., 1999, 2000b). The synthesis reaction leads to the production of various useful β-hydroxy-α-amino acids such as active pharmaceutical ingredients of drug development candidates (Goldberg et al., 2015). While glycine is frequently used as the donor molecule, DTA presented in several bacteria, such as *Pseudomonas* sp., also accepts D-alanine and D-serine (Fesko et al., 2010, 2015).

The physiological role of the corresponding L-threonine aldolase (LTA, EC 4.1.2.5) is believed to be related to glycine biosynthesis (Liu et al., 2000a; Franz and Stewart, 2014; Dückers et al., 2010) and LTA has been found to be necessary in glycine auxotrophic yeast (McNeil et al., 1994). However, it has been reported that DTA and LTA are two phylogenetically unique families from different origins, and the physiological role of DTA is not well understood (Liu et al., 2015).

The enzymatic properties of DTA in several bacteria, such as *Arthrobacter* sp. DK-38, have been studied in detail (Liu et al.,

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* Corresponding author. Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, 1-8-14 Kanda-Surugadai, Chiyoda-Ku, Tokyo, 101-8308, Japan.
E-mail address: nishimura.katsushi@nihon-u.ac.jp (K. Nishimura).

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1998; Kataoka et al., 1997). In addition, a mutant alanine racemase from *Geobacillus stearothermophilus*, which shows DTA activity, has been developed (Fesko et al., 2008; Seebeck and Hilvert, 2003). Furthermore, the crystal structure of bacterial DTA was recently reported, and the enantio-complementarity of DTA and LTA was explained by the approximate mirror symmetry of crucial active site residues (Uhl et al., 2015). However, to date, the existence of eukaryotic DTA has not been reported. In this study, we report the discovery and characterization of eukaryotic DTA from the green alga *Chlamydomonas reinhardtii*. To our knowledge, this is the first study to report the existence of eukaryotic DTA.

2. Results and discussion

2.1. Cloning and sequencing of the gene encoding D-threonine aldolase

The sequence of pasa_Sanger_mRNA23075 of *Chlamydomonas reinhardtii*, defined as the alanine racemase N-terminal domain, was obtained from the database at the Chlamydomonas resource center. The gene encoding DTA from *Chlamydomonas reinhardtii* was isolated and sequenced. The open reading frame of 1287 bp (accession number LC185459) encoded a protein of 428 amino acids with a calculated molecular mass of 44,999 Da (Fig. 1). Sequence comparison of the open reading frame with pasa_Sanger_mRNA23075 revealed that there was a deletion of 913–948 in the database.

The expression plasmid was constructed from the gene coding DTA (*dta*) and pET41b(+) and the plasmid was transferred into *E. coli* BL21 (DE3) cells. However, protein expression was not detected. As *dta* contains many rare codons of *E. coli*, pCrDTA (*dta*' + pET41b(+)) was used to eliminate the rare codons and was transferred into *E. coli* BL21 (DE3) cells. When the recombinant *E. coli* was cultured under conditions of addition of 0.2 mM IPTG at 37 °C, the protein was expressed as insoluble inclusion bodies. We investigated culture conditions (temperature, IPTG concentration, culture time) that the enzyme was overexpressed as soluble protein. The cells were grown at 25 °C for 16 h without IPTG, resulting in the overexpression of a protein possessing DTA activity. The specific activity of cell-free extract was 0.8 U/mg.

Alanine racemase activity has been detected in *Chlamydomonas reinhardtii*, but the gene encoding alanine racemase has not been determined (Nishimura et al., 2007). We predicted that pasa_Sanger_mRNA23075 was the gene of *Chlamydomonas reinhardtii* alanine racemase because it was annotated as alanine racemase N-terminal domain. However, the product did not show alanine racemase activity, but, rather, DTA activity. That was because CrDTA was similar to alanine racemase as shown below.

We performed amino acid homology search with the primary sequence of eukaryotic DTA from *Chlamydomonas reinhardtii* (CrDTA). CrDTA belongs to the alanine racemase family of PLP enzymes (fold-type III). CrDTA was found to be more similar to bacterial DTA than to other PLP enzymes (*e.g*., bacterial alanine racemase and eukaryotic D-serine dehydratase). The amino acid sequence of CrDTA had low sequence identity with bacterial DTAs (39% and 40% sequence identity with *Arthrobacter* sp. and *Alcaligenes xylosoxidans*, respectively), although bacterial DTA from *Arthrobacter* sp. showed high sequence identity (91%) to another bacterial DTA from *A. xylosoxidans*. However, the active site residues were conserved between CrDTA and bacterial DTA from *A. xylosoxidans*. Therefore, the physiological role of CrDTA should be characterized. Our preliminary data indicated that *Chlamydomonas reinhardtii* exhibited D-threonine aldolase activity, and we are presently attempting to characterize effects of D-threonine on growth of *Chlamydomonas reinhardtii* and on the activity of CrDTA in the green algal cells.

2.2. Purification of recombinant CrDTA

CrDTA was purified to homogeneity from *E. coli* BL21 (DE3) cells carrying pCrDTA, with a yield of 3.5% by ammonium sulfate fractionation, and DEAE-Sepharose and Mono Q column chromatographies (Table 1). The purified enzyme gave a single band with a molecular mass of 45 kDa on SDS-PAGE (Fig. 2). The N-terminal amino acid sequence of the protein was determined to be MRALVSKARLAH. The analyzed N-terminal amino acid sequence was consistent with that of the deduced sequence from *dta*. The molecular mass of the purified enzyme was determined by gel filtration to be 60 kDa.

2.3. Effect of pH and temperature on CrDTA

The optimum pH of CrDTA was determined in 0.1 M MES-NaOH buffer (pH 4.7–7.0), 0.1 M HEPES-NaOH buffer (pH 6.8–8.1), 0.1 M HEPES-NaOH–NaCl (pH 8.4–8.6), and 0.1 M Bis-Tris-propane-HCl buffer (pH 8.3–9.0) at 50 °C. The enzyme showed a maximum activity at pH 8.4 in 0.1 M HEPES-NaOH buffer (Fig. 3).

The optimum temperature of the enzyme was determined by measuring its activity in the standard reaction mixture and varying the temperature from 20 °C to 70 °C. The enzyme showed its maximum activity at 70 °C (Fig. 4a); however, high temperatures lager than 70 °C could not be assayed due to the thermal denaturation of alcohol dehydrogenase, which was used in the assay. Seventy percent of its maximum activity was retained between 50 °C and 70 °C.

After incubation of the enzyme at various temperatures for 0, 5, 10, 30, 60, 120, and 180 min, enzyme activity was measured in the standard reaction mixture at 50 °C. The enzyme showed 90% of residual activity after 180 min at 50 °C (Fig. 4b). The enzyme began to be inactivated within 5 min at temperatures greater than 70 °C, and lost its activity at both 70 °C for 60 min and 80 °C for 5 min.

2.4. Substrate specificity and kinetic parameters of CrDTA

The substrate specificity and kinetic parameters of CrDTA were examined with various amino acids (Table 2). The enzyme acted on β-hydroxy-D-amino acids, such as D-threonine, D-*allo*-threonine, and D-*threo*-phenylserine. D-*threo*-Phenylserine was the best substrate among the amino acids tested and CrDTA exhibited $35 \times 10^4\ M^{-1}s^{-1}$ with it. However, inhibition was observed when the concentration of D-*threo*-phenylserine exceeded 1 mM. In all cases of using 1, 3, and 5 mM D-*threo*-phenylserine, absorbance at 340 nm decreased linearly. These results, strongly suggested that inhibition was caused by not product benzaldehyde but substrate D-*threo*-phenylserine. While the enzyme acted on D-forms of β-hydroxy amino acids, the L-forms of serine and threonine were inert, suggesting the enzyme could stereochemically distinguish the α-carbon of a substrate but could not distinguish the β-carbon.

2.5. Synthesis of D-threonine and D-allo-threonine by CrDTA

CrDTA catalyzed the synthesis of D-threonine and D-*allo*-threonine from glycine and acetaldehyde. The enzymatic product comprised a mixture of D-threonine and D-*allo*-threonine. After 1 h incubation, conversion rates of D-threonine and D-*allo*-threonine were 6.7 and 4.6%, respectively, and the diastereomer excess of D-threonine was determined to be 18%. Specific activities of synthesis for D-threonine and D-*allo*-threonine were 50.9 and 36.8 μmol/min/mg, respectively. The ratio of conversion rate of D-threonine to that

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