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

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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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0031-9422/

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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Original          1      ATGCGGGCGCTGGTTTCCAAAGCGCGGTTGGCCCACTCGGTGGGCGGGCGGGCCTCGCAG
Optimized         1      ATGCGTGCTCTGGTTTCCAAAGCTCGTCTGGCTCACTCAGTCGGTGGCCGTGCAAGTCAG
Translated        1       M  R  A  L  V  S  K  A  R  L  A  H  S  V  G  G  R  A  S  Q

Original         61      GCCACAAGATGTGCAGCCACCATCTCTGCTAGCCGGGCACCCGCACACCTCGGCGACGCC
Optimized        61      GCTACCCGCTGTGCTGCTACCATCTCCGCAAGTCGTGCCCCGGCACATCTGGGTGATGCC
Translated       21       A  T  R  C  A  A  T  I  S  A  S  R  A  P  A  H  L  G  D  A

Original        121      CTTCACGATGTGGACACGCCTGCCCTCATCTTGGACTTGGATGCTTTTGACAGAAACTGT
Optimized       121      CTGCATGATGTGGACACCCCGGCGCTGATTCTGGATCTGGACGCCTTTGATCGTAACTGC
Translated       41       L  H  D  V  D  T  P  A  L  I  L  D  L  D  A  F  D  R  N  C

Original        181      GAGAAGCTAAAGGGCGTCATGGCGGGCTTCCCGGGCGTGGCAGTGCGCCCCCACGCCAAG
Optimized       181      GAAAAACTGAAAGGCGTGATGGCAGGTTTCCCGGGTGTGGCTGTTCGCCCGCATGCTAAA
Translated       61       E  K  L  K  G  V  M  A  G  F  P  G  V  A  V  R  P  H  A  K

Original        241      GCCCACAAGTGTGCGGAGGTGGCGCGGCGGCAGCTGCAACTGCTGGGGGCGAAGGGCGTG
Optimized       241      GCGCACAAATGTGCGGAAGTTGCGCGTCGCCAACTGCAGCTGCTGGGTGCCAAAGGCGTT
Translated       81       A  H  K  C  A  E  V  A  R  R  Q  L  Q  L  L  G  A  K  G  V

Original        301      TGCTGCCAAAAGGTCATCGAGGCTGAGGCGATGGCGGAGGGCGGGGTGTCCGATCTGCTG
Optimized       301      TGCTGTCAGAAAGTCATCGAAGCCGAAGCAATGGCTGAAGGCGGTGTTTCAGATCTGCTG
Translated      101       C  C  Q  K  V  I  E  A  E  A  M  A  E  G  G  V  S  D  L  L

Original        361      CTGTCCAATGAGGTCATTGCGCCCAGGAAAATTGACCGCTTGGTTGGCCTTGCGGCCGCG
Optimized       361      CTGTCGAATGAAGTCATTGCCCCGCGTAAAATTGACCGCCTGGTGGGTCTGGCCGCGGCA
Translated      121       L  S  N  E  V  I  A  P  R  K  I  D  R  L  V  G  L  A  A  A

Original        421      GGGGCGCGTGTGGGCGTGTGCTACGAGCGGGAGGACAACCTGCGGCAGCTGAATGCTGCG
Optimized       421      GGTGCACGCGTCGGCGTGTGCTATGAACGTGAAGATAATCTGCGCCAGCTGAATGCGGCC
Translated      141       G  A  R  V  G  V  C  Y  E  R  E  D  N  L  R  Q  L  N  A  A

Original        481      GCGGCGGCACGTGGCACGCATCTGGATGTGCTGGTGGAGCTGAACGTGGGGCAAGACAGG
Optimized       481      GCGGCAGCTCGTGGCACCCATCTGGATGTTCTGGTGGAACTGAACGTGGGTCAAGATCGC
Translated      161       A  A  A  R  G  T  H  L  D  V  L  V  E  L  N  V  G  Q  D  R

Original        541      TGTGGCGTGAACTCGGCGGATGAGGTAGTGCAGCTGGCGCGCGCGGCGGCGGGGCTGGAC
Optimized       541      TGTGGCGTTAATTCAGCAGATGAAGTGGTTCAGCTGGCGCGTGCGGCAGCAGGTCTGGAT
Translated      181       C  G  V  N  S  A  D  E  V  V  Q  L  A  R  A  A  A  G  L  D

Original        601      AATGTGCGGTTTGCCGGCATTCAGGCCTACCACGGCGGGCTACAACATGTTCGTGATCCC
Optimized       601      AACGTGCGCTTTGCGGGCATTCAAGCCTACCATGGCGGTCTGCAGCACGTCCGTGATCCG
Translated      201       N  V  R  F  A  G  I  Q  A  Y  H  G  G  L  Q  H  V  R  D  P

Original        661      CGCGACCGCGCGCGCAGCGAGTGGGGCAGGTAGTGGGGCGGGCCCGGGCGGCGGTGGATGCC
Optimized       661      CGTGACCGTGCCCAACGTGTGGGTCAGGTTGTGGGTCGTGCCCGCGCTGCGGTTGATGCA
Translated      221       R  D  R  A  Q  R  V  G  Q  V  V  G  R  A  R  A  A  V  D  A

Original        721      CTGAAGGCGGCTGGACTGCCCTGCGACACGGTCACGGGGGGCGGCACTGGCACCTACAGG
Optimized       721      CTGAAAGCCGCAGGTCTGCCGTGTGACACCGTTACGGGCGGTGGCACCGGTACCTATCGT
Translated      241       L  K  A  A  G  L  P  C  D  T  V  T  G  G  G  T  G  T  Y  R

Original        781      GTTGAGGCGGCCAGCGGTGTGTTCACGGAGGTGCAGCCGGGCTCGTTCGCCTTCAGCGAC
Optimized       781      GTCGAAGCTGCGTCGGGTGTCTTCACGGAAGTGCAGCCGGGCAGTTTTGCCTTCTCCGAT
Translated      261       V  E  A  A  S  G  V  F  T  E  V  Q  P  G  S  F  A  F  S  D

Original        841      GCAGACTACGCACGCAACCTGCAGGAGGACGGCGGCGTTGGCGAGTGGGAGCAGAGCCTG
Optimized       841      GCAGACTACGCTCGCAATCTGCAGGAAGATGGTGGCGTGGGTGAATGGGAACAAAGTCTG
Translated      281       A  D  Y  A  R  N  L  Q  E  D  G  G  V  G  E  W  E  Q  S  L

Original        901      TGGGTGCTCACGCAGGTTATGAGCGTCACCCCAGCTCGGGGCCTGGCGGTGGTGGACGCC
Optimized       901      TGGGTTCTGACCCAGGTGATGTCCGTTACGCCGGCACGTGGTCTGGCTGTTGTCGATGCG
Translated      301       W  V  L  T  Q  V  M  S  V  T  P  A  R  G  L  A  V  V  D  A

Original        961      GGCACCAAGGCGGTGTCATTGGACAGTGGGCCGCCGCGACTGCCGCCCGCCTTCGAGGCG
Optimized       961      GGCACCAAAGCCGTGAGCCTGGACTCTGGCCCGCCGCGTCTGCCGCCGGCGTTTGAAGCC
Translated      321       G  T  K  A  V  S  L  D  S  G  P  P  R  L  P  P  A  F  E  A

Original       1021      GCGTACGGCACAATGATGGAGTACGGCAGCGGCGGCGACGAGCACGGCAAGCTCATGTGG
Optimized      1021      GCGTATGGCACGATGATGGAATACGGCTCTGGTGGCGATGAACATGGTAAACTGATGTGG
Translated      341       A  Y  G  T  M  M  E  Y  G  S  G  G  D  E  H  G  K  L  M  W

Original       1081      CCGCAGGGCGCCTACCAGCTGCCCATGTCGCTGCCGGAGGTGGGCAGCCTGCTGCTGCTA
Optimized      1081      CCGCAAGGCGCCTATCAGCTGCCGATGAGCCTGCCGGAAGTTGGTTCTCTGCTGCTGCTG
Translated      361       P  Q  G  A  Y  Q  L  P  M  S  L  P  E  V  G  S  L  L  L  L

Original       1141      CAGCCGGGCCACTGCGACCCCACCGTCAACCTGTATGACTGGCTGGTGGCGGCGCGGCGG
Optimized      1141      CAGCCGGGCCACTGTGATCCGACCGTCAACCTGTATGATTGGCTGGTTGCGGCCCGTCGC
Translated      381       Q  P  G  H  C  D  P  T  V  N  L  Y  D  W  L  V  A  A  R  R

Original       1201      CAGCAGGGGGGGCAGCAGCAGGGGGGCGTGGACGGGTGGCGAGTGGAGGCGGTGTGGCCC
Optimized      1201      CAGCAAGGTGGCCAGCAACAGGGTGGCGTGGATGGTTGGCGTGTGGAAGCCGTGTGGCCG
Translated      401       Q  Q  G  G  Q  Q  Q  G  G  V  D  G  W  R  V  E  A  V  W  P

Original       1261      ATCCGGGGGCGGGGGCCAGGGCAATGA
Optimized      1261      ATTCGTGGTCGTGGTCCGGGTCAATAA
Translated      421       I  R  G  R  G  P  G  Q  *
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Fig. 1. Nucleotide and deduced amino acid sequences of *dta* and *dta*'. The asterisk denotes a translational stop codon. The underlined amino acid sequence is identical to that was determined by Edman degradation for the purified enzyme.

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Table 1
Purification of CrDTA from *E. coli* BL21 (DE3) harboring pCrDTA.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	4520	5490	0.823	1.00	100
Ammonium sulfate	2750	3280	0.840	1.02	60.9
DEAE-Sepharose	482	115	4.20	5.11	10.7
Mono Q	159	22.5	7.05	8.56	3.51

Threonine aldolase activity was determined using D-threonine as substrate.

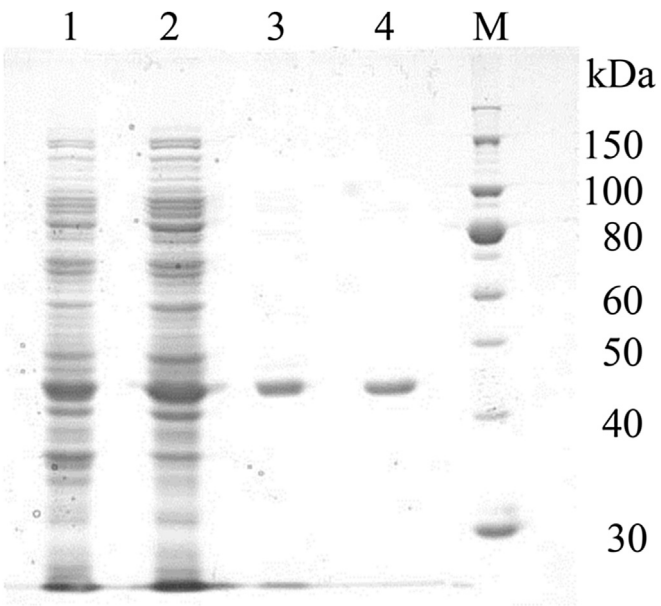

Fig. 2. Analysis of the expression and purification of CrDTA by SDS-PAGE. Cell-free extract (lane 1), active fractions from ammonium sulfate fractionation (lane 2), DEAE-Sepharose (lane 3), and Mono Q (lane 4), and protein marker (lane M) for calibration. Protein bands were detected by Coomassie Brilliant Blue staining.

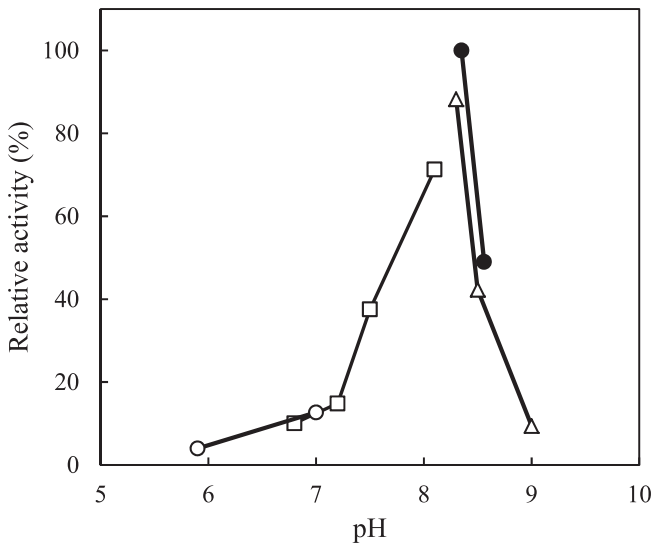

Fig. 3. Effect of pH on CrDTA activity using 0.1 M MES-NaOH buffer (pH 4.7–7.0, open circle), 0.1 M HEPES-NaOH buffer (pH 6.8–8.1, open square), 0.1 M Bis-Tris-propane-HCl buffer (pH 8.3–9.0, open triangle), 0.1 M HEPES-NaOH–NaCl buffer (pH 8.4–8.6, closed circle). Activity was expressed relative to that determined at pH 8.4 (100%).

of D-*allo*-threonine was 1.46, and the ratio of specific activity with D-threonine to that with D-*allo*-threonine was 1.38. Therefore, the difference between specific activities with D-threonine and D-*allo*-threonine was a cause of diastereomer excess in the enzymatic synthesis.

2.6. Effect of various compounds on CrDTA

CrDTA was activated by $MnCl_2$, $CoCl_2$, $NiCl_2$, and $MgCl_2$, but was strongly inhibited by $CaCl_2$, $CuCl_2$, and $ZnCl_2$ (all 100 μM) (Table 3). In addition, the metalloenzyme inhibitors (EDTA and sodium cyanide) strongly inhibited the enzyme activity. These results demonstrate that CrDTA requires particular divalent metals as cofactors. The enzyme reaction was also strongly inhibited by hydroxylamine (Table 3). Moreover, at pH 8.0, the enzyme showed maximum absorptions at 280 and 416 nm (data not shown). This inhibition and the absorption spectrum indicate that the enzyme requires PLP as a coenzyme. Thiol reagents, iodoacetate and *p*-chloromercuribenzoic acid, and the chelating agent 8-hydroxyquinoline had no effect on the enzyme reaction.

These results indicating the metal dependency of CrDTA agreed well with bacterial DTA from *Arthrobacter* sp. DK-38 (Kataoka et al., 1997). The zinc ion in eukaryotic D-serine dehydratase is a five coordination (Ito et al., 2008), and the copper ion is a skewed octahedron. In this study, the enzyme was activated in the presence of divalent metal ions which are regular octahedrons. We considered that the divalent metal ion activating CrDTA was stringently controlled by the coordinate bond of CrDTA active site residues. Then, invasion of 8-hydroxyquinoline might be prevented by the stringently controlled metal binding site, resulting in no effect as mentioned above. The crystal structure of bacterial DTA from *A. xylosoxidans* identified metal-binding residues in the active site (Uhl et al., 2015). The observation was almost consistent with our thought about metal coordination for CrDTA. X-ray crystallography of CrDTA-substrate complex is currently being examined to clarify the residues that interact with substrate, coenzyme, and metal ion.

Bacterial DTA has been shown to be strongly inhibited by thiol reagents (Kataoka et al., 1997); however, these agents had no effect on CrDTA. Amino acid sequence comparison between CrDTA and bacterial DTA indicated that the cause of inhibition was likely the cysteine 303 of bacterial DTA in the vicinity of the active site. In the case of CrDTA, this position is occupied by a serine residue at 329.

3. Materials and methods

3.1. Materials

Microalgal strain *Chlamydomonas reinhardtii* NIES-2237 was obtained from the National Institute for Environmental Studies (Ibaraki, Japan). D-*threo*-Phenylserine was kindly provided by Associate Professor Hisashi Muramatsu (Kochi University). NADH, yeast alcohol dehydrogenase, and molecular marker proteins for gel filtration were obtained from Oriental Yeast (Tokyo, Japan). Restriction enzymes for genetic manipulation were obtained from Takara Bio (Siga, Japan). Plasmids were obtained from Merck Millipore (Massachusetts, USA) and GenScript (New Jersey, USA). Genetic manipulation kits were obtained from Qiagen (Hilden,

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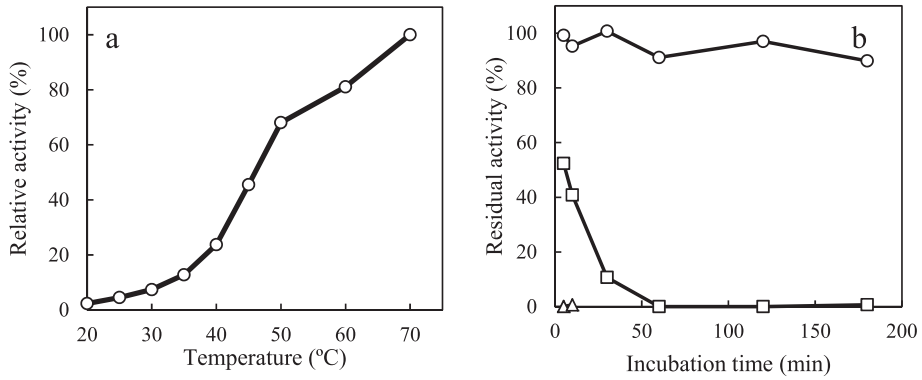

Fig. 4. (a) Effect of temperature on CrDTA activity. Activity was expressed relative to that determined at 70 °C (100%). (b) Thermostability of DTA at 50 °C (open circle), 70 °C (open square), and 80 °C (open triangle). Activity was expressed as residual activity remaining when compared to using enzyme before incubation (100%).

Table 2
Substrate specificity and kinetic parameters of CrDTA.

Substrate	Relative activity (%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
D-Threonine	100	0.31	4.2	1.3×10^4
D-*allo*-Threonine	406	2.4	25	1.1×10^4
L-Threonine	ND	–	–	–
L-*allo*-Threonine	ND	–	–	–
D-*threo*-Phenylserine	1230	0.81	280	35×10^4
D-Serine	ND	–	–	–
L-Serine	ND	–	–	–

ND, not detectable.

Germany), Takara Bio, and Toyobo (Osaka, Japan). Oligonucleotide primers were synthesized by Fasmac (Kanagawa, Japan). All other reagents of analytical grade were obtained from Sigma (St. Louis, USA), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan). HiTrap DEAE FF, Mono Q 5/50 GL, and Superose 12 10/300 GL were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). The Cosmosil $5C_{18}$-AR-300 packed column was purchased from Nacalai Tesque.

3.2. Enzyme assays

Threonine aldolase activity was spectrophotometrically measured at 340 nm by coupling the reduction of acetaldehyde (oxidation of NADH) with yeast alcohol dehydrogenase. We modified the coupling method as described previously (Liu et al., 1998).

Table 3
Effect of various compounds on the activity of CrDTA.

Compounds	Concentration (mM)	Relative activity (%)
None[a]		40.8
$MnCl_2$[a]	0.1	100
$CoCl_2$[a]	0.1	87.5
$NiCl_2$[a]	0.1	83.6
$MgCl_2$[a]	0.1	75.7
$CaCl_2$[a]	0.1	13.3
$CuCl_2$[a]	0.1	0
$ZnCl_2$[a]	0.1	0
Hydroxylamine[b]	1	5.2
Iodoacetate[b]	1	89.2
p-Chloromercuribenzoic acid[b]	1	93.2
8-Hydroxyquinoline[b]	1	105
EDTA[b]	1	0
Sodium cyanide[b]	1	12.7

[a] Effect of metal salts on the activity was assayed using standard reaction mixture except for $MnCl_2$.
[b] Effect of inhibitors was tested with reaction mixture containing $MnCl_2$.

Briefly, the assay mixture comprised 100 mM HEPES-NaOH (pH 8.1), 5 mM D-threonine, 50 μM PLP, 100 μM $MnCl_2$, 200 μM NADH, 26 U/mL yeast alcohol dehydrogenase, and appropriate amounts of CrDTA solution in a final volume of 0.5 mL. One unit of the enzyme was considered the amount of enzyme that catalyzed the formation of 1 μmol of acetaldehyde (1 μmol of NAD^+) per min at 50 °C; the molar extinction coefficient of NADH is 6.2×10^3 M^{-1} cm^{-1}. Aldolase activity toward phenylserine was measured spectrophotometrically at 280 nm; the molar extinction coefficient of produced benzaldehyde is 1.4×10^3 M^{-1} cm^{-1}. Control experiments were carried out under the same conditions without the addition of enzyme solution.

The ability of CrDTA to synthesize D-threonine and D-*allo*-threonine was assayed by high performance liquid chromatography (HPLC). We modified the synthetic activity analysis method as described previously (Kataoka et al., 1997). Briefly, the reaction mixture, composed of 100 mM HEPES-NaOH (pH 8.1), 100 mM glycine, 350 mM acetaldehyde, 10 μM PLP, 1 mM $MnCl_2$, and appropriate amounts of the enzyme solution in a final volume of 0.5 mL, was incubated at 50 °C for 0, 15, 30, or 60 min, and terminated with 0.5 mL of 1 M HCl. After appropriate dilution and filtration, the synthesized amino acids were derivatized with derivatization reagent. To prepare the derivatization reagent, 15 mg of *o*-phthaldialdehyde was dissolved in 0.5 mL ethanol and mixed with 11 mL of 0.1 M sodium borate buffer (pH 10) and 30 mg *N*-acetyl-L-cysteine. Three-hundred-microliters of this derivatization reagent was added to 100 μL of the sample solution. A portion of the derivatized sample (20 μL) was used to determine the amount of produced D-threonine and D-*allo*-threonine. The concentrations and optical purities of D-threonine and D-*allo*-threonine were analyzed using an HPLC system equipped with a Cosmosil $5C_{18}$-AR-300 packed column (4.6 × 250 mm) maintained at 30 °C. Derivatized samples were applied and eluted with a linear gradient of 22–25% methanol in 50 mM sodium acetate buffer (pH 6.5). The flow rate was 0.7 mL/min, and elution times for D-threonine and D-*allo*-threonine were 8.5 and 18.5 min, respectively.

3.3. Isolation and sequencing of the gene encoding D-threonine aldolase from Chlamydomonas reinhardtii

Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN) with *Chlamydomonas reinhardtii* NIES-2237. Based on the cording sequence in the database of the Chlamydomonas resource center (Chlre4, http://www.chlamycollection.org/), the oligonucleotide primers, 5′-ATGCGGGCGCTGGTTTCC-3′ as the sense primer and 5′-TCATTGCCCTGGCCCCCGCCC-3′ as the antisense primer, were designed. Reverse transcription was performed with

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ReverTra Ace (Toyobo), the antisense primer, and total RNA of *Chlamydomonas reinhardtii* NIES-2237. Amplification of nucleotides between the two primers was performed using PCR with KOD-plus- Ver. 2 (Toyobo) and the reverse transcription product as the template. The amplified product was purified using SUPREC-PCR (Takara Bio). Nucleotide sequencing was analyzed using a genetic analyzer 3100xl equipped with a Big Dye Terminator ver. 3.1 (Applied Biosystems). We named the gene encoding DTA *dta.*

3.4. Expression and purification of D-threonine aldolase in E. coli BL21 (DE3)

Based on genetic information revealed from mRNA, the expression plasmid pCrDTA was constructed from the gene encoding DTA with codons optimized for expression in *E. coli* (*dta'*, Fig. 1) and pET-41b(+). The pCrDTA was transferred into chemically competent *E. coli* BL21 (DE3) cells using the heat shock method to produce recombinant DTA. Twenty-four-milliliters of the transformant, which was precultured at 37 °C overnight, was inoculated into 1.2 L of LB medium (in a 2 L Marineflask, Furukawa riko, Tokyo, Japan) containing 25 μg/mL kanamycin. Cells were grown at 25 °C for 16 h and harvested by centrifugation. *E. coli* cells harboring pCrDTA were suspended in 100 mM Tris-HCl buffer (pH 8.0) containing 50 μM PLP and 1 mM phenylmethylsulfonyl fluoride, and disrupted by ultrasonication on ice. After centrifugation, the cell-free extract was fractionated by ammonium sulfate precipitation. The active fraction (25–60% saturation) was resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 20 μM PLP and dialyzed against the same buffer. The enzyme solution was centrifuged and filtered to remove particulates, applied to ten tandemly connected Hitrap DEAE FF columns equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 20 μM PLP, and eluted with a linear gradient of 0–500 mM NaCl in the same buffer at a flow rate of 1 mL/min. Active fractions were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 20 μM PLP. After filtration, the enzyme solution was then applied to a Mono Q 5/50 GL column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 20 μM PLP and eluted with a linear gradient of 0–500 mM NaCl in the same buffer at a flow rate of 0.5 mL/min. The active fractions were pooled and stored at −80 °C until use.

3.5. Other analytical methods

Estimation of the molecular mass was performed using Superose 12 column chromatography with the following protein markers: glutamate dehydrogenase (290 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome *c* (12.4 kDa). The protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The purity and molecular mass of each subunit of the enzyme were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the CLEARLY Protein Ladder (Takara Bio). Gels were stained with Quick Blue Staining Solution (BioDynamics Laboratory Inc., Tokyo, Japan). The N-terminal amino acid sequence of the enzyme was determined by the Edman degradation procedure with a Procise 492 cLC protein sequencer (Applied Biosystems, CA, USA) and a PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan). Nucleotide and amino acid sequences were obtained from the database at the Chlamydomonas resource center and NCBI (www.ncbi.nlm.nih.gov/). A homology search was performed with the BLAST program (Altschul et al., 1990) at NCBI. Multiple alignments were performed with DNAMAN 6.0 (Lynnon LLC; CA, USA). Kinetic parameters (k_{cat} and K_m) were calculated according to Lineweaver-Burk plots.

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