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Alanine racemase from the green alga *Chlamydomonas reinhardtii*

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Summary. *Chlamydomonas reinhardtii*, a unicellular green microalga, could grow to a stationary phase having optical density of 2.0–2.5 at 750 nm in Tris-acetate-phosphate (TAP) medium containing 0.1% D-alanine. D-alanine has no inhibitory effect on growth and induced alanine racemase activity 130-fold more than without D-alanine in the green alga. Although *C. reinhardtii* cultured in the TAP medium showed alanine racemase activity, the content of free D-alanine was only 0.14%. The enzyme was partially purified by ammonium sulfate fractionation followed by three kinds of liquid chromatography using DEAE Toyopearl, Phenyl Sepharose, and TSK G3000 SWXL columns. The specific activity for L-alanine of the partially purified alanine racemase was 3.8 μmol/min/mg. The molecular weight of the enzyme was determined to be approximately 72,000 by gel filtration. The enzyme showed a maximum activity at 45 °C and pH 8.4 and requires pyridoxal 5′-phosphate as a coenzyme.

Keywords: Alanine racemase – D-Amino acid – D-Alanine, *Chlamydomonas reinhardtii* – Green alga

1. Introduction

Chlamydomonas is a genus within the unicellular green microalgae. This green microalga has recently been used as a model system in many fundamental studies in cell biology and molecular biology. In Phaeophyta, it was reported that the free D-alanine content was high in *Hizikia fusiformis*, *Heterochordaria abietina*, and *Sargassum nigrifolium* (Nagahisa et al., 1995). On the other hand D-aspartate was detected in both some fresh water microalgae and some marine diatoms, while D-alanine was only present in the latter (Yokoyama et al., 2003). There have been no studies, however, carried out regarding the presence of D-amino acids on *Chlamydomonas reinhardtii*. Studies of growth responses of *Arabidopsis thaliana* to D-alanine and D-serine show that these compounds inhibit growth even at quite low concentrations and are metabolized by recombinant D-amino acid oxidase into a non-toxic product (Erikson et al., 2004). Our preliminary experiment showed that *C. reinhardtii* could grow well in liquid medium containing 0.1% D-alanine, leading us to investigate enzymes that detoxify D-alanine.

In plants, only a few studies regarding the presence of an amino acid racemase have been conducted. Two studies on wheat and tomato tryptophan racemase were performed to examine characters such as activation by osmotic stress (Rekoslavskaya et al., 1997; Kopytina et al., 1998). Recently Yokoyama et al. reported alanine racemase activity in a diatom *Thalassiosira* sp. along with some of its properties (Yokoyama et al., 2005). In the present study, we report the partial purification and characterization of an alanine racemase from *C. reinhardtii*. We also demonstrate that D-alanine has no inhibitory effect on growth and induces alanine racemase activity in this green alga.

2. Materials and methods

2.1 Microorganism and cultivation

C. reinhardtii IAM C-540 (from the IAM Culture Collection, Tokyo, Japan) was grown at 30 °C in a Tris-acetate-phosphate (TAP) medium which contained 0.4 g of NH_4Cl, 51 mg of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.119 g of K_2HPO_4, 60.3 mg of KH_2PO_4, 2.42 g of Tris base, 1 ml of acetic acid, and 1 ml of Hunter's trace elements per liter of deionized water. Hunter's trace elements were composed of: Na_2EDTA, 50 g; $ZnSO_4 \cdot 7H_2O$, 22 g; H_3BO_3, 11.4 g; $MnCl_2 \cdot 4H_2O$, 5.06 g; $FeSO_4 \cdot 7H_2O$, 4.99 g; $CoCl_2 \cdot 6H_2O$, 1.61 g; $CuSO_4 \cdot 5H_2O$, 1.57 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.1 g; and KOH, 16 g, in 1,000 ml of deionized distilled water. TAP(+)D-alanine medium involved the addition of 1.0 g of D-alanine to the TAP medium, and TAP(−)NH_4Cl medium was prepared by omitting NH_4Cl from TAP(+)D-alanine medium. The lighting for the cultures was provided by three 21-W fluorescent lamps under a 16:8 light-dark cycle (h).

2.2 Determination of D- and L-amino acids

The determination of the enantiomeric concentrations of each amino acid was performed as described previously (Nagata et al., 1992). Free amino acids were treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Pierce, Rockford, IL, U.S.A.) to form diastereomers. The FDAA-derivatives were separated on a Silica Gel 60 plate (Merck, Darmstadt, Germany) by two-dimensional thin-layer chromatography. FDAA-amino acids recovered from the plate were analyzed by high-performance liquid chromatography (HPLC) for the resolution of D- and L-enantiomers, using a reversed-phase column, Nova-Pak C18 (150 mm × 3.9 mm i.d., Waters, Milford, MA, U.S.A.), and a PU-2089 plus quaternary gradient HPLC system (JASCO, Tokyo, Japan). The amounts of D- and L-enantiomers of amino acids were calculated based on the peak areas of the elution patterns obtained by a Chromato-Integrator (D-2500, Hitachi, Tokyo, Japan). As standard, known amounts of D- and L-enantiomers of the amino acids were derivatized with FDAA and purified by two-dimensional thin-layer chromatography as above, prior to HPLC.

2.3 Preparation of free amino acids from C. reinhardtii

Cells of *C. reinhardtii* cultured in TAP medium were harvested by centrifugation at 8000 *g* for 10 min. The cells (0.95 g) were suspended in deionized distilled water and disrupted by supersonic treatment, and unbroken cells were removed by centrifugation at 20,000 *g* for 20 min. To the supernatant solution, 10% trichloroacetic acid solution was added to yield a final concentration of 5% (w/v). After centrifugation, the supernatant was passed through a Dowex 50W × 8 (H^+ form) column, and eluted with 2 M NH_4OH after washing with deionized distilled water, to obtain purified free amino acids. The eluate was evaporated to dryness under vacuum in a centrifugal evaporator at 40 °C. The residue was dissolved in deionized distilled water and used for determination of free amino acids by FDAA treatment as described above.

2.4 Protein and enzyme assays

Protein concentrations were determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard. Enzyme activity was assayed by two methods. The standard reaction mixture was composed of 50 µg of enzyme sample, 25 mM L or D-amino acid, 50 µM pyridoxal 5′-phosphate (PLP), and 50 mM Tris–HCl buffer (pH 8.4) in a total volume of 100 µl. The mixture was incubated at 45 °C for 15 min, and the reaction was stopped by the addition of 11% (final) trichloroacetic acid. After centrifugation, D- and L-amino acids were purified and determined as described above. One enzyme unit was defined as the amount catalyzing the isomerization of 1 µmol of amino acid for 1 min. The other method was performed in the same manner as described previously (Nagata et al., 1988) except that Tris–HCl buffer was used instead of sodium pyrophosphate buffer in peroxidase solution. After the racemase reaction, 1 µl of D-amino acid oxidase (0.02 unit), 100 µl of phosphate buffered saline (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl), and 100 µl of peroxidase solution (167 mM Tris–HCl buffer, pH 8.5, 16.7 µg/ml flavinadeninedinucleotide, 167 µg/ml bovine serum albumin, 601 µg/ml horseradish peroxidase, 2.5 mg/ml sodium 3,5-dichloro-2-hydroxybenzensulfonic acid, 376 µg/ml 4-aminoantipyrine, and 25.0 µg/ml NaN_3) were added to the standard reaction mixture. The mixture was incubated at 25 °C for 30 min, and the reaction was terminated by adding 300 µl of 0.1 M sodium borate buffer (pH 10.0). After 2 min at room temperature, the resulting quinone-imine dye was detected by measuring the absorbance at 500 nm.

2.5 Purification of C. reinhardtii *alanine racemase*

Cells of *C. reinhardtii* cultured in TAP(+)D-alanine medium were harvested by centrifugation at 8000 *g* for 10 min. The cells (65 g) were suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM phenylmethanesulfonyl fluoride and 50 µM pyridoxal 5′-phosphate (PLP). The cells were disrupted by supersonic treatment for 40 min (Branson 450 Sonifire, Danbury, CT, U.S.A.), and unbroken cells were removed by centrifugation at 20,000 *g* for 20 min. Resultant supernatant solution was used as a cell-free extract. The cell-free extract was brought to 30% saturation with ammonium sulfate, and the resultant precipitate was removed by centrifugation. Similarly, ammonium sulfate fractionation was carried out with 60% ammonium sulfate. Precipitation of the 30–60% fraction was dissolved in buffer A (50 mM Tris–HCl, 50 µM PLP, pH 8.0) and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Toyopearl column (25 ml, TOHSO, Tokyo, Japan) equilibrated with modified buffer A by omitting PLP. A gradient elution was performed with buffer B (50 mM Tris–HCl, 300 mM NaCl, 50 µM PLP, pH 8.0) by increasing the concentration linearly from 0 to 100%. The active fractions were pooled, concentrated by ultrafiltration with 10,000 MWCO membrane (Vivascience AG, Hannover, Germany), and dialyzed against buffer A. The dialyzed solution was applied to 4 HiTrap DEAE-Sepharose columns (5 ml, Amersham Biosciences, Piscataway, NJ, U.S.A.) in series on an HPLC system (Shimadzu, Kyoto, Japan) equilibrated with buffer A. The enzyme was eluted and the active fractions were pooled and concentrated in the same manner as described above. The concentrated solution was further purified by gel-filtration with a TSK G3000 SWXL column (7.8 × 300 mm, TOHSO) on the Shimadzu HPLC system equilibrated with buffer C (50 mM sodium phosphate, 200 mM NaCl, 50 µM PLP, pH 7.2). An elution was performed with buffer C at a flow rate of 0.8 ml/min and molecular weight was estimated using five standards of a MW-Marker (Oriental Yeast, Tokyo, Japan). The active fractions were pooled and desalted by ultrafiltration through a 10,000 MWCO membrane.

3. Results and discussion

3.1 Cultivation of C. reinhardtii

C. reinhardtii could grow to a stationary phase having optical density of 2–2.5 at 750 nm in both TAP and TAP(+)D-alanine media, indicating that D-alanine has no inhibitory effect on growth of *C. reinhardtii*. However, *C. reinhardtii* could not grow in TAP(−)NH_4Cl medium, and thus cannot be used as a replacement nitrogen source for NH_4Cl. The specific activities for L-alanine of ammonium sulfate fractionation samples from TAP and TAP(+)D-alanine medium cultures were 1.6 and 210 (see Table 1) nmol/min/mg, respectively. This fact demonstrates that alanine racemase activity rose 130-fold as a

Table 1. Purification of alanine racemase from *C. reinhardtii*

	Total protein mg	Total activity µmol/min	Specific activity µmol/min/mg	Yield %
Cell free extract	2670	79.9	0.03	100
Ammonium sulfate fractionation	321	67.4	0.21	84
DEAE Toyopearl	62	41.5	0.67	52
DEAE Sepharose	3.7	4.6	1.2	5.8
TSK G3000 SWXL	0.5	1.9	3.8	2.4

L-Alanine was used as substrate

result of the induction of enzymatic activity in *C. reinhardtii* by D-alanine. Therefore, we used the TAP(+)D-alanine medium for cultivation of *C. reinhardtii* to purify alanine racemase.

D-Alanine and D-serine at less than 0.009% inhibit the growth of *A. thaliana* (Erikson et al., 2004). However, when these D-amino acids were converted into keto acids by recombinant D-amino acid oxidase, *Arabidopsis* plants could grow on media containing them. Whereas *C. reinhardtii* could grow in the TAP(+)D-alanine medium containing 0.1% D-alanine, a preliminary experiment that we conducted showed that *Volvox carteri*, a multicellular green alga, had no alanine racemase activity and could not grow in a standard *Volvox* medium containing 0.1% D-alanine (data not shown). These facts suggest that, unlike *C. reinhardtii* which may detoxify D-alanine by converting into L-alanine by alanine racemase, *V. carteri* cannot grow due to a lack of alanine racemase activity. Similar responses to D-alanine in culture media were observed in the yeasts *Saccharomyces cerevisiae* (lacking alanine racemase) and *Schizosaccharomyces pombe* (having alanine racemase) (Uo et al., 2001). Details of the function of alanine racemase in *C. reinhardtii* remain unknown.

3.2 Free D-amino acid content in C. reinhardtii

The occurrence of D-amino acids in *C. reinhardtii* is summarized in Table 2. In this green alga, free D-amino acids hardly exist. Although *C. reinhardtii* cultured in TAP medium had alanine racemase activity, the content of free D-alanine was only 0.14%. This finding may imply that the concentration of D-alanine may be kept low by a synergistic interaction of alanine racemase with some other detoxifying enzyme(s) because toxicity of D-alanine is very high in this green alga. Further studies are being performed on the growth of *C. reinhardtii* in culture media containing either D- or L-alanine.

Table 2. Free D-amino acid contents in *C. reinhardtii*

	L (nmol/g cells)	D (nmol/g cells)	D/(L + D) (%)
Ser	226 ± 26.1	0.00 ± 0.00	0.00 ± 0.00
Ala	2360 ± 416	3.46 ± 1.39	0.14 ± 0.03
Pro	265 ± 70.2	0.00 ± 0.00	0.00 ± 0.00
Asp	181 ± 8.39	0.00 ± 0.00	0.00 ± 0.00
Glu	1130 ± 173	1.35 ± 0.28	0.12 ± 0.03

Mean values and standard deviations of three independent experiments are shown

3.3 Purification and enzymatic properties of alanine racemase of C. reinhardtii

The purification magnification and recovery rate of the final active fraction were 127 and 2.4%, respectively, and the specific activity of the partially purified enzyme was 3.8 μmol/min/mg as shown in Table 1. The specific activity value of *C. reinhardtii* is a little higher than that of a diatom *Thalassiosira* sp. (Yokoyama et al., 2005), although both values are expected to be much higher after further purification steps. The molecular weight of the enzyme was determined to be approximately 72,000 by gel filtration.

Racemase activity was assayed with L-alanine, D-alanine, L-serine, L-proline, and L-aspartate as substrates, as shown in Fig. 1. The results indicate that alanine racemase was highly specific for alanine and did not catalyze racemization of L-proline and L-aspartate. The enzyme

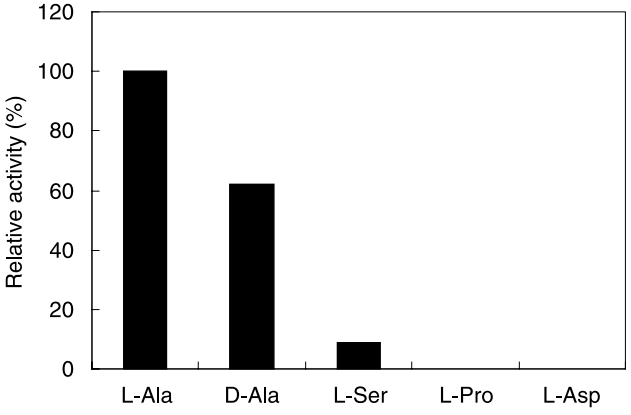

Fig. 1. Substrate specificity of alanine racemase of *C. reinhardtii*. The activity was expressed as a percentage of the activity for L-alanine

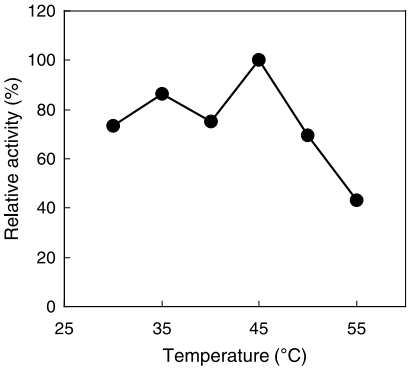

Fig. 2. Effect of temperature on alanine racemase activity of *C. reinhardtii*. The activity was expressed as a percentage of the activity at 45 °C

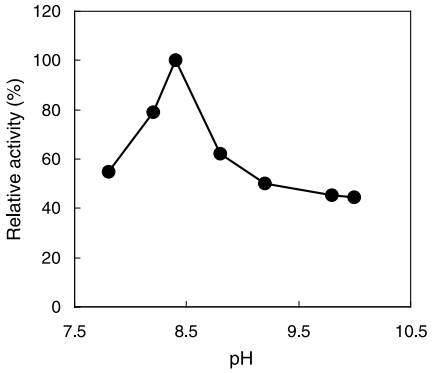

Fig. 3. Effect of pH on alanine racemase activity of *C. reinhardtii*. The activity was expressed as a percentage of the activity at pH 8.4. A citrate/KH_2PO_4/H_3BO_3 buffer was used

showed maximum activity at 45 °C (Fig. 2) and at pH 8.4 (Fig. 3).

In this study we examined some enzymatic properties of *C. reinhardtii* alanine racemase. Because the structure of this enzyme has not yet been investigated, except for molecular weight, the relationship between structure and properties cannot be considered. In the *C. reinhardtii* genome project, which is currently underway, it was found that a gene fragment encoding a part of protein has high homology with alanine racemase of *E. coli* (http://www.chlamy.org/cgi-bin/search.cgi). We are now attempting to clone *C. reinhardtii* alanine racemase by using this information in order to reveal the amino acid sequence and examine how the gene responds when stimulated by D-alanine.

Table 3. PLP dependency of alanine racemase of *C. reinhardtii*

	Relative activity (%)
Control	100
1 mM $NaBH_4$	0
Dialyzed against PLP*	53
1 mM NH_2OH	0
0 μM PLP	86

Enzyme sample was dialyzed against 50 mM Tris–HCl buffer (pH 7.5) and used for enzyme assay. Enzymatic reaction was performed in the standard reaction mixture containing 1 mM $NaBH_4$ or 1 mM NH_2OH, or omitting PLP using L-alanine as substrate. The activity was expressed as a percentage of the control activity and all values are the average of two separate experiments. * Enzyme sample was treated with 1 mM NH_2OH, dialyzed against 50 μM PLP, and used for enzymatic reaction

3.4 Pyridoxal 5′-phosphate (PLP) dependency of alanine racemase of C. reinhardtii

Table 3 shows PLP dependency of alanine racemase of *C. reinhardtii*. The enzymatic activity was absent in the presence of 1 mM hydroxylamine or 1 mM sodium borohydride. The activity lost with hydroxylamine was recovered to 53% by adding 0.05 mM PLP. These results indicate that the enzyme requires PLP as a coenzyme. The enzyme had kept 86% of activity after dialysis against a buffer without PLP, suggesting that the enzyme binds PLP tightly although some coenzymes are released.

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