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Alanine racemase of alfalfa seedlings (*Medicago sativa* L.): First evidence for the presence of an amino acid racemase in plants

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Abstract

We demonstrated several kinds of D-amino acids in plant seedlings, and moreover alanine racemase (E.C.5.1.1.1) in alfalfa (*Medicago sativa* L.) seedlings. This is the first evidence for the presence of amino acid racemase in plant. The enzyme was effectively induced by the addition of L- or D-alanine, and we highly purified the enzyme to show enzymological properties. The enzyme exclusively catalyzed racemization of L- and D-alanine. The K_m and V_{max} values of enzyme for L-alanine were 29.6×10^{-3} M and 1.02 mol/s/kg, and those for D-alanine are 12.0×10^{-3} M and 0.44 mol/s/kg, respectively. The K_{eq} value was estimated to be about 1 and indicated that the enzyme catalyzes a typical racemization of both enantiomers of alanine. The enzyme was inactivated by hydroxylamine, phenylhydrazine and some other pyridoxal 5′-phosphate enzyme inhibitors. Accordingly, the enzyme required pyridoxal 5′-phosphate as a coenzyme, and enzymologically resembled bacterial alanine racemases studied so far.

Keywords: Alfalfa; *Medicago*; Alanine racemase; Pyridoxal 5′-phosphate; D-Amino acid

1. Introduction

Various D-amino acids occur in both free and bound states not only in bacteria (Walsh, 1989), but in yeast (Uo et al., 2001), plants (Ogawa et al., 1973a; Zenk and Scherf, 1963) insects (Corrigan, 1969), mammals (Hashimoto and Oka, 1997; Hashimoto et al., 1993), microalgae (Yokoyama et al., 2003), hyperthermophiles (Nagata et al., 1999), shell (Watanable et al., 1998), and fish (Sarower et al., 2003). For example, D-alanine and D-glutamate are almost ubiquitously found as essential constituents of bacterial cell wall peptidoglycans, though D-glutamine or D-aspartate is substituted for D-glutamate in a few kinds of bacteria (Osborn, 1969; Schleifer and Kandler, 1972). Bacterial enzymes participating in D-amino acid metabolism, in particular amino acid racemases (Soda and Esaki, 1994; Yoshimura et al., 1992) and D-amino acid aminotransferase (Soda et al., 2001; Yoshimura et al., 1996) have been studied in detail. The recent research has focused on D-amino acids and amino acid racemases in animals and mammalian tissues.

D-Alanine (**1a**) was shown to be involved in the osmotic stress response in marine and euryhaline invertebrates (Abe et al., 2005; Fujimori and Abe, 2002). D-Serine acts as a neuromodulator in mammals (Fadda et al., 1988; Schell et al., 1995; Wolosker et al., 1999), and D-aspartate plays various neuronal and endocrine roles (Hashimoto and Oka, 1997; Hashimoto et al., 1993). In spite of the occurrence of D-amino acids such as D-alanine **1a** and D-glutamate in pea seedlings (Ogawa et al., 1973a; Zenk and Scherf, 1963) and other plants, their metabolism, in particular their

Abbreviations: OPA, *o*-phthalaldehyde; NAC, *N*-acetyl-L-cysteine; PLP, pyridoxal 5′-phosphate; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid.

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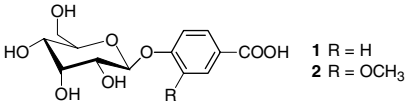
origin is substantially unknown, though D-amino acid aminotransferase activity was demonstrated in pea seedlings (Ogawa et al., 1973b).

We successfully detected alanine racemase (E.C.5.1.1.1) activity in the seedlings of alfalfa (*Medicago sativa* L.) and some other kinds of plants, and isolated the enzyme. In fact, several D-amino acids including D-alanine **1a** were determined in these plants by an accurate enantioselective method. The alfalfa enzyme was effectively induced by incubation with the induction medium containing L-alanine **1b**, glucose and several inorganic compounds, and highly purified the enzyme, which requires pyridoxal 5′-phosphate (PLP) as a cofactor, and racemized alanine (**1a/b**) as a sole substrate. This is the first example of the occurrence and characterization of a plant amino acid racemase.



2. Results and discussion

We found the activity of alanine racemase in the seedling extracts of *M. sativa* (specific enzyme activity (kat/kg of protein); 1.42×10^{-5}), *Raphanus sativas* (7.67×10^{-6}), *Glycine max* (1.40×10^{-6}) and *Brassica oleracea* (1.06×10^{-5}). The enzyme activity, which was assayed by determination of L- and D-alanine (**1b** and **1a**) from their enantiomers, was confirmed by determination of both enantiomers by means of enantioselective column chromatography and D-amino acid oxidase reaction. The highest activity was shown in the extract of alfalfa (*M. sativa* L.) seedlings. The enzyme was inducibly formed and the activity, which was assayed in the whole homogenate, was enhanced about 1500-fold (data not shown) by the induction medium that contains both D- or L-alanine (**1a** and **1b**) (20 mM) and D-glucose (0.5% (w/v)). However, addition of neither pyridoxine hydrochloride nor other vitamin B_6 compounds affected the induction. The time-dependent induction pattern was shown in Fig. 1. The activity was rapidly induced for 8 h on the induction and then gradually decreased thereafter.

When the enzyme was extracted from alfalfa seedlings by homogenization with buffer A, about 70% of the enzyme activity was found in the 1000–5000*g* sedimented fractions and about 95% of the activity was collected in the fractions sedimented below 10,000*g*. This shows that the enzyme would be localized in cellular compartments, though we have not examined the enzyme localization in more detail. We highly purified the enzyme by Ether-Toyopearl, Phenyl-Toyopearl and DEAE-Toyopearl column chromatographic steps (Table 1), but all attempts to purify the enzyme to apparent homogeneity were without success owing to enzyme instability. The racemization of D- and L-alanine (**1a** and **1b**) proceeded in proportion to the amount of enzyme and the incubation time. The time courses of enzyme reactions with D- and L-alanine (**1a** and **1b**) were characteristic of racemization; they show symmetry (Fig. 2). The enzyme was specific for alanine **1**, whereas D- and L-aspartate, glutamate, serine, arginine and several other amino acids were inert as a substrate. Apparent K_m and V_{max} values for L- and D-alanine (**1b** and **1a**) were estimated to be 29.6×10^{-3} M and 1.02 mol/s/kg, 12.0×10^{-3} M and 0.44 mol/s/kg, respectively. The V_{max}/K_m values for the racemization of L- and D-alanine (**1b** and **1a**) were closely similar to each other: 34.5 and 36.7 mol/s/kg/M, respectively. The K_{eq} value was estimated as about 1. This value was substantially consistent with the theory of racemization reactions shown by Briggs and Haldane (1925).

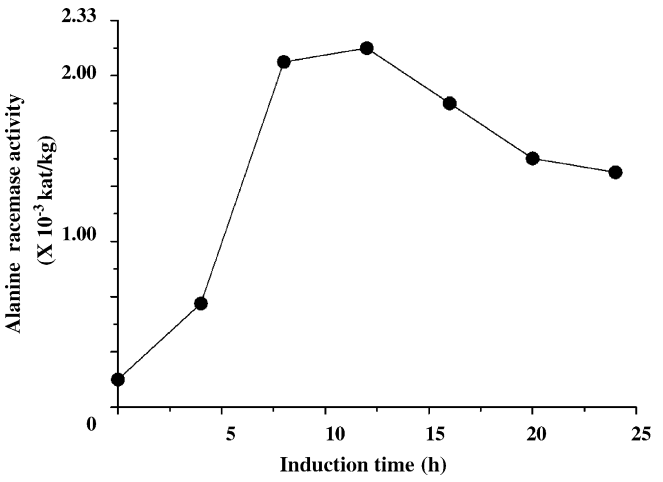


Fig. 1. Time course of induction pattern of alanine racemase in alfalfa seedlings. About 800 g of alfalfa seedlings were subjected to enzyme induction for 24 h, other conditions were the same as described in Section 4. Seedlings were collected every 4 h and homogenized with buffer B. After centrifugation, the enzyme activity of supernatant solution was assayed as described in Section 4.

Table 1
Purification of alanine racemase from alfalfa seedlings

Step	Total activity ($\times 10^{-6}$ kat)[a]	Total protein ($\times 10^{-6}$ kg)[b]	Specific activity (kat/kg)	Purification (fold)	Yield (%)
Crude extract	9.95	849	0.012	1.0	100
Ether-Toyopearl	1.95	7.91	0.247	21	20
Phenyl-Toyopearl	0.90	3.44	0.262	22	9
DEAE-Toyopearl	0.20	0.0165	12.121	1010	2

[a] One Katal is defined as the amount of enzyme that catalyzes the production of 1 mol of D-alanine from L-alanine per second.
[b] About 6 kg of alfalfa seedlings were used.

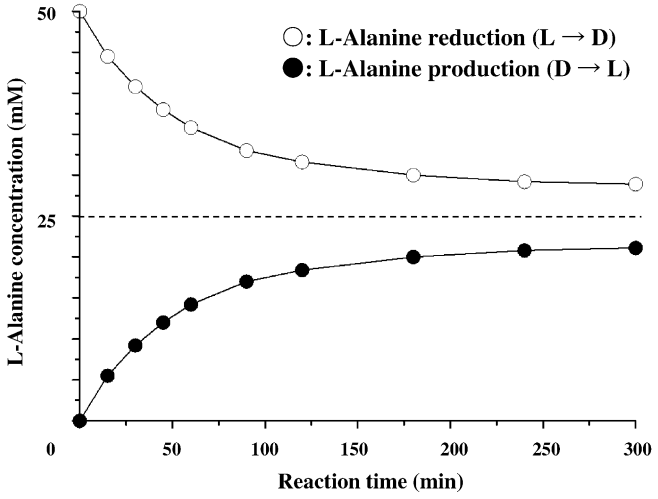


Fig. 2. Racemization of D-alanine (**1a**) and L-alanine (**1b**) with alanine racemase of alfalfa seedlings. The reaction mixture containing 100 mM CHES (pH 9.0), 50 mM D- or L-alanine, 30 μM PLP and enzyme was incubated at 37 °C. D- and L-Alanine (**1a** and **1b**) were determined as described in Section 4.

Amino acid racemases are categorized into two groups; (I) The PLP-dependent enzymes, and (II) the PLP-independent ones, which require neither cofactors nor metals (Soda and Esaki, 1994). All alanine racemases studied so far required PLP as a coenzyme. The enzyme from alfalfa seedlings was strongly inhibited by 1 mM PLP-enzyme inhibitors such as hydroxylamine (inhibition; 97%), phenylhydrazine (59%), aminooxyacetate (100%) and sodium borohydride (97%). The alfalfa seedlings enzyme could be almost fully inactivated by dialysis against a buffer containing 1 mM hydroxylamine for 18 h, whereas it could be reactivated by about 55% on subsequent dialysis against the buffer containing 30 μM PLP. When the enzyme was dialyzed against PLP-free buffer, about 95% of the activity was lost, but about 80% of the enzyme activity was recovered by a second dialysis against buffer containing PLP (Table 2). Accordingly, PLP is loosely bound to the enzyme protein, and the apo form of enzyme is less stable than the holoenzyme.

Table 2
Effect of PLP on the activity of alanine racemase from alfalfa seedlings

First dialysis	Second dialysis	Specific activity (kat/kg)	Relative activity (%)
Not done	Not done	0.278	100
− Hydroxylamine	− PLP	0.0145	5
− Hydroxylamine	+ PLP	0.242	87
+ Hydroxylamine	− PLP	0	0
+ Hydroxylamine	+ PLP	0.154	55

The enzyme was dialyzed against 2500 vol of 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM DTT, 1 mM EDTA and either 50 mM or no hydroxylamine for 18 h at 4 °C (first dialysis), followed by dialysis against 2500 vol of 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM DTT, 1 mM EDTA and either 30 μM PLP or H_2O for 12 h (second dialysis). The enzyme assay is described in Section 4.

Over 30 years ago, several D-amino acids in leguminous plants (Ogawa et al., 1973a), and a D-amino acid aminotransferase activity in pea seedlings (Ogawa et al., 1973b) were determined. However, the biosynthetic pathway of D-amino acids in plants has not yet been elucidated. In this study, we confirmed the occurrence of D-amino acids in the seedlings by means of a more accurate procedure. Considerably high molar ratios of D-alanine (**1a**) to the amount of DL-alanine (**1a**/**1b**) were found in the seedlings extracts. To compare D-alanine (**1a**) contents of the seedlings extracts, the relative contents (%) of D-alanine (**1b**) against total amount of D- and L-alanine (**1a** and **1b**) in various seedlings were calculated, and 100% means all alanine existed in the seedlings as the D-enantiomer: *M. sativa* (19.7%), *R. sativas* (14.6%), *Glycine max* (11.6%), *B. oleracea* (5.32%). And a few other kinds of D-amino acids, for example, such as D-glutamate, D-aspartate, and D-threonine were found in *M. sativa* seedlings. The concentrations of D-alanine (**1a**), D-glutamate, D-aspartate, and D-threonine were 10.8, 0.11, 0.20, and 3.14 μM in the 70% ethanol extract of *M. sativa* seedlings, respectively. A variety of D-amino acids are found in conjugated forms such as *N*-acyl-D-amino acids (Gamburg and Rekoslavskaya, 1991) in plants, but neither the purified enzyme nor the seedling homogenate acted on several kinds of *N*-acyl-amino acids such as *N*-acetyl-D-alanine, *N*-acetyl-L-alanine and *N*-acetyl-L-α-aminobutyrate. The results obtained show that D-alanine (**1a**) in alfalfa seedlings is produced by alanine racemase, but other amino acids are not enzymatically racemized, and other D-amino acids other than D-alanine (**1a**) found in seedlings are probably formed from D-alanine (**1a**) and corresponding α-keto acids by a D-amino acid aminotransferase (Ogawa et al., 1973b).

3. Concluding remarks

D-Alanine (**1a**) and alanine racemase probably play a role in the metabolism and growth of plant seedlings, though we currently have no evidence for this. Direct evidence has not been found even for the role of a bacterial amino acid racemase, such as a glutamate racemase of *Pediococcus pentosaceus* (Choi et al., 1992) and an arginine racemase (Yorifuji and Ogata, 1971; Yorifuji et al., 1971) which was studied enzymologically in detail. The metabolic functions of only glutamate racemase isozyme (Glr and Yrpc) of *Bacillus subtilis* IFO3336, which are involved in the poly-γ-glutamate synthesis and peptidoglycan synthesis, respectively (Ashiuchi et al., 1999), and alanine racemase of *Escherichia coli*, which plays a part in the peptidoglycan synthesis (Neidhart et al., 1987) were shown.

This is the first report of presence of an amino acid racemase in plant, and we are currently studying further purification, characterization, and also the physiological function of the enzyme in alfalfa seedlings.

4. Experimental

D-Amino-acid oxidase (EC.1.4.3.3) from pig kidney, horseradish peroxidase, *o*-phthalaldehyde (OPA), *N*-acetyl-L-cysteine (NAC), D- and L-alanine, pyridoxal 5′-phosphate (PLP) were purchased from Wako (Osaka, Japan); 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES), *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) from Dojindo (Kumamoto, Japan), and DEAE-Toyopearl and Toyopearl HW-55 from Tosoh (Tokyo, Japan). Seedlings of alfalfa (*M. sativa*. L.) and some other plants were purchased from a local store at Suita, Japan.

4.1. Purification of alanine racemase

All the procedures were carried out at 4 °C unless otherwise stated. Alfalfa seedlings (about 800 g, wet weight) were soaked in five volumes of the induction medium (pH 6.5) consisting of 20 mM L-alanine (**1b**), 0.5% (w/v) D-glucose, 2 mM potassium phosphate, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 50 μM $FeSO_4$, 70 μM H_3BO_4, 14 μM $MnCl_2$, 10 μM NaCl, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.2 μM $NaMoO_4$, 0.2 μM $CoCl_2$, and 100 μg/ml ampicillin under aeration in the dark at room temperature (about 20 °C) for 12 h. The induction medium was changed four times every 4 h.

After enzyme induction, the seedlings were homogenized with two volumes of buffer A, which consists of 0.5 M sucrose, 100 mM potassium phosphate buffer (pH 8.0), 1 mM dithiothreitol (DTT), 2 mM ethylenediamine-tetraacetic acid (EDTA) and 50 μM PLP. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 8000*g* for 30 min. The precipitate was re-suspended in buffer B consisting of 100 mM potassium phosphate buffer (pH 7.2), 1 mM DTT, 1 mM EDTA, 20 μM PLP, 10 μM (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride, 1 μg/ml leupeptin and 0.5 μg/ml pepstatin A. The suspension was ultrasonicated, and was centrifuged at 230,000*g* for 1 h. The supernatant solution was dialyzed overnight against buffer C composed of 10 mM potassium phosphate buffer (pH 7.2), 1 mM DTT, 1 mM EDTA and 20 μM PLP containing 1.5 M $(NH_4)_2SO_4$ for 12 h, and centrifuged at 17,000*g* for 30 min. The supernatant solution was loaded onto an Ether-Toyopearl column (∅ 3.0 × 12.5 cm) equilibrated with buffer C supplemented with 1.5 M $(NH_4)_2SO_4$. The column was washed with the same buffer, and the enzyme was eluted with the buffer containing 1.1 M $(NH_4)_2SO_4$. The enzyme was placed onto a Phenyl-Toyopearl column (∅ 2.0 × 9.0 cm) equilibrated with buffer C supplemented with 1.1 M $(NH_4)_2SO_4$. After washing the column with same buffer, the enzyme was eluted with buffer containing 0.6 M $(NH_4)_2SO_4$. This active fractions were filtered through a USY-5 ultrafilter unit (ADVANTEC, Japan) to remove $(NH_4)_2SO_4$ from the buffer. This enzyme solution was loaded onto a DEAE-Toyopearl column (∅ 2.0 × 5.0 cm) equilibrated with buffer C, which was washed with same buffer. The enzyme was eluted with the buffer supplemented with 20 mM KCl, and concentrated to one-tenth of the original volume with a USY-5 ultrafilter unit (ADVANTEC, Japan). The enzyme was stored at −20 °C until use.

4.2. Protein and enzyme assay

Alanine racemase activity during enzyme purification was assayed as follows. D-Alanine (**1a**) enzymatically formed from the L-enantiomer (**1b**) was determined with D-amino acid oxidase (Yamauchi et al., 1992). Hydrogen peroxide produced was determined spectrophotometrically in the form of a quinoneimine dye. The amount of quinoneimine dye was measured at 555 nm. The assay mixture containing 100 mM CHES–NaOH buffer (pH 9.0), 50 mM L-alanine (**1b**), 30 μM PLP, 0.1 mg/ml bovine serum albumin and enzyme was incubated at 37 °C for an appropriate time, and the reaction was terminated by addition of 0.1 M HCl (a final concentration, 0.05 M). The precipitated protein was removed by centrifugation, and the supernatant solution was mixed with a reaction mixture consisting of 40 μl of 0.5 M CHES–NaOH buffer (pH 9.0), 5 μl of 0.1 M TOOS, 5 μl of 0.1 M 4-aminoantipyrine, 5 μl of 2 mg/ml D-amino acid oxidase, 5 μl of horseradish peroxidase (0.2 mg/ml) and distilled water in a final volume of 200 μl, followed by incubation at 37 °C for 30 min. The absorption of quinoneimine dye formed was measured at 555 nm, and the amount of D-alanine (**1a**) was estimated.

To characterize the enzyme, the reaction mixture containing 100 mM CHES–NaOH buffer (pH 9.0), 50 mM D- or L-alanine (**1a** or **1b**) (or other amino acids), 30 μM PLP, and enzyme in a final volume of 100 μl was incubated for an appropriate time. Then, 100 μl of 0.1 M HCl was added to the mixture to stop the enzyme reaction, and the mixture was centrifuged at 10,000*g* for 20 min. The supernatant solution was used for derivatization. The derivatization of amino acids with OPA and NAC was performed by the method of Aswad (1984). The HPLC analysis was carried out by means of dual LC-10AS pumps, an RF-$10A_{XL}$ fluorescence detector, an SIL-$10A_{XL}$ auto injector, a CTO-10A column oven, an SCL-10A system controller, a DGU-14A degasser, a C-R7A plus chromatopack (Simadzu, Kyoto, Japan), and a CAPCELL PAK C18 column (∅ 6.0 × 250 mm) (Shiseido Fine Chemicals, Tokyo, Japan). Elution was performed under the same conditions as described previously (Hashimoto et al., 1992). Protein was assayed according to the method of Bradford (1976) with bovine serum albumin as a standard.

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