Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 67 (2006) 856–860
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

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

Kazutoshi Ono [a], Kazuki Yanagida [a], Tadao Oikawa [a,b,*], Tadashi Ogawa [c], Kenji Soda [a]

[a] *Department of Biotechnology, Faculty of Engineering, Kansai University, 3-3-35 Yamate-Cho, Suita-Shi, Osaka-Fu 564-8680, Japan*
[b] *Kansai University High Technology Research Center, Suita-Shi, Osaka-Fu 564-8680, Japan*
[c] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji-Shi, Kyoto-Fu 611-0011, Japan*

Received 28 November 2005; received in revised form 7 February 2006
Available online 17 April 2006

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

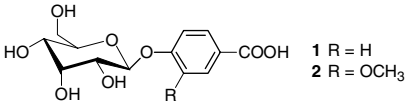
Abbreviations: OPA, *o*-phthalaldehyde; NAC, *N*-acetyl-L-cysteine; PLP, pyridoxal 5′-phosphate; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid.

* Corresponding author. Tel.: +81 6 6368 0812; fax: +81 6 6388 8609.
E-mail address: oikawa@ipcku.kansai-u.ac.jp (T. Oikawa).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.02.017

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).

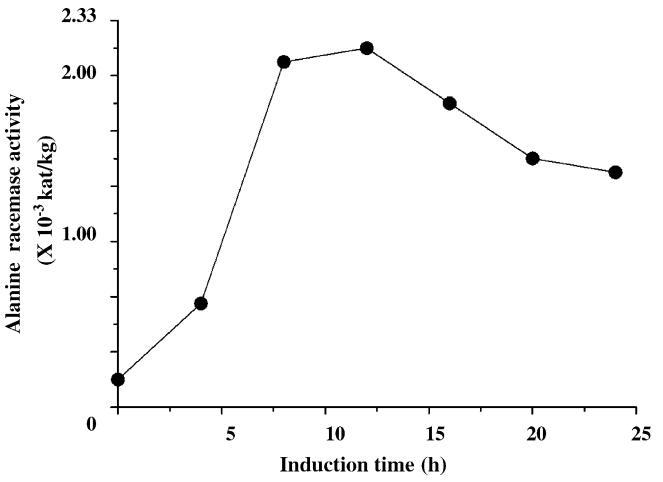


Fig. 1. Time course of induction pattern of alanine racemase in alfalfa seedlings. About 800 g of alfalfa seedlings were subjected to enzyme induction for 24 h, other conditions were the same as described in Section 4. Seedlings were collected every 4 h and homogenized with buffer B. After centrifugation,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