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Occurrence of D-serine in rice and characterization of rice serine racemase

Yoshitaka Gogami, Katsuyoshi Ito, Yuji Kamitani, Yuki Matsushima, Tadao Oikawa *

Department of Life Science and Biotechnology, Faculty of Chemistry, Materials, and Bioengineering, Kansai University, 3-3-35 Yamate-Cho, Suita, Osaka-Fu 564-8680, Japan

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ABSTRACT

Germinated, unpolished rice was found to contain a substantial amount of D-serine, with the ratio of the D-enantiomer to the L-enantiomer being higher for serine than for other amino acids. The relative amount of D-serine (D/(D + L)%) reached approximately 10% six days after germination. A putative serine racemase gene (*serr*, clone No. 001-110-B03) was found in chromosome 4 of the genomic DNA of *Oryza sativa* L. ssp. Japonica cv. Nipponbare. This was expressed as *serr* in *Escherichia coli* and its gene product (SerR) was purified to apparent homogeneity. SerR is a homodimer with a subunit molecular mass of 34.5 kDa, and is highly specific for serine. In addition to a serine racemase reaction, SerR catalyzes D- and L-serine dehydratase reactions, for which the specific activities were determined to be 2.73 and 1.42 nkatal/mg, respectively. The optimum temperature and pH were respectively determined for the racemase reaction (35 °C and pH 9.0) and for the dehydratase reaction (35 °C and pH 9.5). SerR was inhibited by PLP-enzyme inhibitors. ATP decreased the serine racemase activity of SerR but increased the serine dehydratase activity. Kinetic analysis showed that Mg^{2+} increases the catalytic efficiency of the serine racemase activity of SerR and decreases that of the serine dehydratase activity. Fluorescence-quenching analysis of the tryptophan residues in SerR indicated that the structure of SerR is distorted by the addition of Mg^{2+}, and this structural change probably regulates the two enzymatic activities.

1. Introduction

D-amino acids were once considered as unnatural and unusual compounds that were not essential amino acids for humans. Accordingly, studies of D-amino acids and their metabolism have focused intensively on microbial amino acid racemases and D-amino acid transaminases (Inagaki et al., 1986; Uo et al., 2001; Yorifuji et al., 1971), since D-alanine and D-glutamate are known to be two essential components of the peptide glycan in the microbial cell wall (Osborn, 1969). But, with development of improved analytical and detection techniques, D-amino acids have recently been found in a much broader range of living organisms (Hashimoto and Oka, 1997), and our understanding of them has gradually changed. In particular, D-serine (**1a**) (Fig. 1) has recently been demonstrated to act as a neuromodulator in humans (De Miranda et al., 2002), and it is currently undergoing clinical testing in patients as a treatment for schizophrenia (Tsai et al., 1998). Furthermore, several kinds of D-amino acids have been discovered in plant seedlings, with the first occurrence of an amino acid racemase in plants being alanine racemase (E.C.5.1.1.1) in alfalfa (*Medicago sativa* L.) (Ono et al., 2006). During the course of this study, the D-amino acid content of various vegetables and fruits (Gougami et al., 2006) was analyzed. We estimated that germinated, unpolished rice (*Oryza sativa* L.) contains a substantial amount of D-serine (**1a**) and the ratio of the D-enantiomer (**1a**) to the L-enantiomer (**1b**) was higher for serine than for other amino acids. To attempt to discern the biosynthetic pathway to D-serine **1a** in *O. sativa* L., we searched for a putative metabolic gene encoding a protein affording D-serine (**1a**) formation in the rice genome from the International Rice Genome Sequencing Project (IRGSP) in 2004. Only one candidate, a putative serine/threonine racemase (*serr*) was found. Moreover, the cloning of the *serr* into *Escherichia coli* was reported at the Annual Meeting of the Vitamin Society of Japan in 2006 (Ito et al., 2006), and later by Fujitani et al. (2007). In the current study, we first describe our initial finding, as well as demonstrate both the occurrence of D-serine (**1a**) in germinated seeds of *O. sativa* L. and the enzymological characterization of the rice serine racemase.

2. Results and discussion

2.1. Detection of D-serine (*1a*) in germinated unpolished rice

Germinated unpolished rice was found to contain a high amount of D-serine (**1a**) relative to the total amount of L-serine (**1b**) (Table 1). The fraction of D-serine (D/D + L%) reached approximately

Abbreviations: CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NAC, *N*-acetyl-L-cysteine; OPA, *o*-phtalaldehyde; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); PLP, pyridoxal 5′-phosphate; SerR, serine racemase form *Oryza sativa* L.

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

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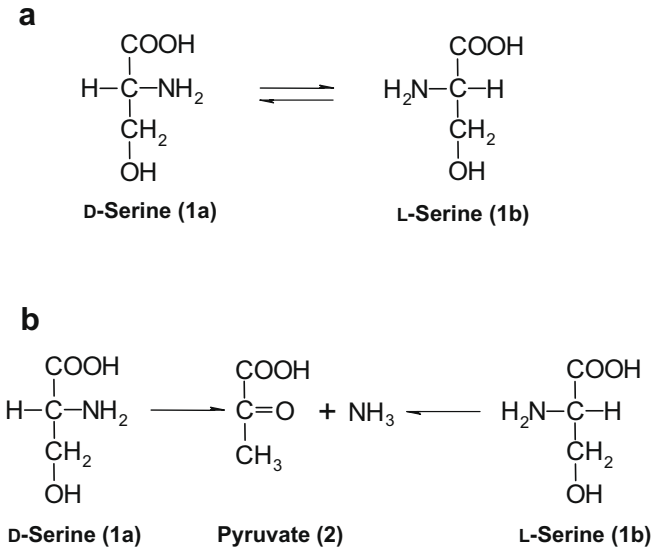

Fig. 1. Reaction pathways of D-serine (**1a**) and L-serine (**1b**) by serine racemase from *Oryza sativa* L. (a) Racemase reaction; (b) Dehydratase reaction.

10% six days after germination. The D-serine (**1a**) content did not increase with the germination period from 0 to 6 days, and it was detected even before germination (0 day). In previous studies, D-alanine, D-aspartate, and D-glutamate were detected in both free and conjugated forms from pea seedlings (*Pisum sativum*) (Ogawa et al., 1977), barley grains (*Hordeum vulgare* L.) (Erbe and Brückner, 2000), and hops blossoms (*Humulus lupulus* L.). D-alanine and D-alanyl-D-alanine were also found in wild rice (*Oryza australiensis Domin*) (Manabe, 1985), but there has been no report of the occurrence of D-serine (**1a**) in any plant including germinated, unpolished rice.

2.2. Cloning of a putative serine/threonine racemase gene, serr, in the genomic DNA of Oryza sativa L.

The project to sequence the full-length genome of rice (*O. sativa* L. ssp. Japonica cv. Nipponbare) was completed in the early 2000s (Sasaki et al., 2002). Three research institutes – the National Institute of Agrobiological Sciences (NIAS), the Foundation for Advancement of International Science (FAIS), and the Institute of Physical and Chemical Research (RIKEN) – collaborated under the coordination of the Bio-oriented Technology Research Advancement Institute (BRAIN) on this project. Using the Knowledge-based Oryza Molecular Biological Encyclopedia (KOME, http://cdna01.dna.affrc.go.jp/cDNA/), we tried to identify putative candidate genes related to D-serine (**1a**) metabolism in *O. sativa* L. One putative serine/threonine racemase gene (*serr*, clone No. 001-110-B03) was detected on chromosome 4 of the genomic DNA of *O. sativa* L. ssp. Japonica cv. Nipponbare, which encodes an open reading frame of 1,020 bp, having 50.1% GC content. The primary structure of the serine racemase from *O. sativa* L. (SerR, E.C.5.1.1.18) was similar to that of *H. vulgare* L. (identity, 88.5%; accession No. BAF63026), *Arabidopsis thaliana* (identity, 64.4%; accession No. NP192901), *Schizosaccharomyces pombe* (identity, 40.8%; accession No. NP587715), *Homo sapiens* (identity, 46.7%; accession No. NP068766), and *Mus musculus* (identity, 46.3%; accession No. NP038789) (See Fig. 2). The amino acid residues in the binding motifs for the cofactor, PLP (Lys68, Ser323, Asn95, Gly195, Gly196, and Gly197), and for Mg^{2+} (Glu219, Ala223, and Asp225) were highly conserved among these organisms. SerR was expressed in insoluble fractions of the cell-free extract of *E. coli* harboring pET-21b/*serr*, but, by decreasing the cultivation temperature from 37 to 15 °C, the enzyme was gradually localized in the soluble fractions. There were twenty-three rare codons (AGA (4), AGG (1), CGG (1), ATA (9), CTA (1), and CCC (1), and GGA (6)) contained in *serr*, which is probably one of the reasons for the low yield of SerR.

Table 1
D- and L-serine (**1a** and **1b**) concentration in germinated unpolished rice.

Germination (day)	D-ser (μmol/g)	L-ser (μmol/g)
1	0.37×10^{-2}	2.7×10^{-2}
2	0.76×10^{-2}	1.4×10^{-2}
3	0.67×10^{-2}	0.87×10^{-2}
4	0.59×10^{-2}	0.74×10^{-2}
5	0.64×10^{-2}	2.2×10^{-2}
6	0.67×10^{-2}	0.63×10^{-2}

2.3. Purification of serine racemase from O. sativa L.

SerR was purified to homogeneity using a Ni–NTA column according to the purification protocol of Qiagen (Fig. 3). About 3.5 mg of purified enzyme was obtained from one liter of culture (Table 2). The atomic absorption analysis indicated that Ni did not contaminate the purified enzyme (data not shown). During purification of the enzyme, 20 μM PLP and 0.01% 2-mercaptoethanol were present in the buffer. The enzyme can be stored in a 20 mM sodium phosphate buffer (pH 8) at 4 °C for 5 days without loss of activity, but in a 20 mM sodium or potassium phosphate buffer (pH 7), the enzyme was unstable and became inactivated, forming aggregates. About 30% of the initial activity was lost after the enzyme was stored in a 20 mM sodium phosphate buffer (pH 7) for 3 days. The purified enzyme migrated as a single band in SDS–PAGE (Fig. 3a) with an apparent molecular mass of 34.5 kDa. The molecular mass of the native enzyme determined by gel filtration with Superdex 200 Hiload was 87.0 kDa, suggesting that the enzyme was a homodimer (Fig. 3b), like the serine racemases from *A. thaliana* (Fujitani et al., 2006) and *Mus musculus* (Strísovský et al., 2003). The FPLC elution profile of mouse brain serine racemase showed that the enzyme exists in solution in equilibrium between dimeric and tetrameric forms (Cook et al., 2002). The elution profile of SerR by gel filtration indicated that the monomeric and tetrameric forms were completely absent, and that the subunit association properties of SerR were therefore quite different from those of mouse brain racemase.

2.4. Enzyme reactions and substrate specificity of SerR

We found that SerR catalyzes D- and L-serine (**1a** and **1b**) dehydratase reactions in addition to a serine racemase reaction. This catalytic feature is shared with the serine racemases from *M. musculus* (Strísovský et al., 2003), *S. pombe* (Yoshimura and Goto, 2008), and *Bombyx mori* (Uo et al., 1998). Fig. 4 shows the time course for the reaction products of SerR in the presence of D-serine (**1a**) (Fig. 4a) or L-serine (**1b**) (Fig. 4b) as a substrate. D-Serine (**1a**) and L-serine (**1b**) were converted to, respectively, L-serine (**1b**) and pyruvate or D-serine (**1a**) and pyruvate. The specific activities for the L-serine and D-serine dehydratase activities were determined to be 2.73 and 1.42 nkatal/mg, respectively. SerR is highly specific for serine (**1**), whereas other amino acids, such as L-alanine, L-arginine, L-threonine, L-glutamate, and L-aspartate, did not serve as substrates. By contrast, the serine racemases from *A. thaliana* and *H. vulgare* L. act on L-alanine, L-arginine, and L-glutamine in addition to serine. Although these three enzymes are all derived from plants, the substrate specificities are characteristically quite different from each other.