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

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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.

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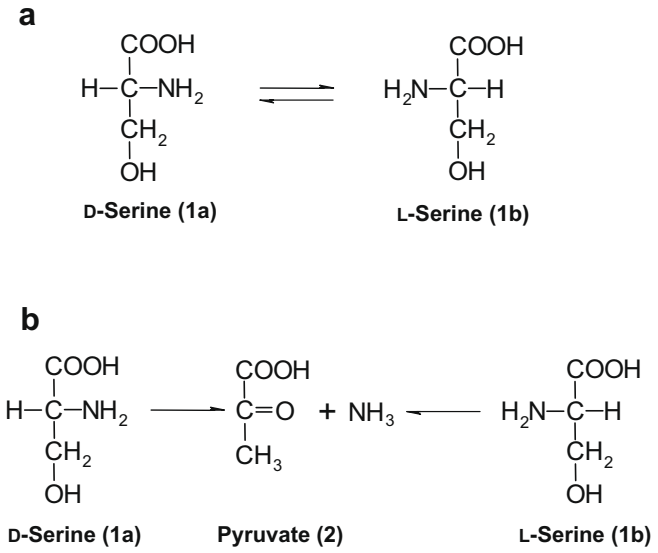

Fig. 1. Reaction pathways of D-serine (**1a**) and L-serine (**1b**) by serine racemase from *Oryza sativa* L. (a) Racemase reaction; (b) Dehydratase reaction.

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.

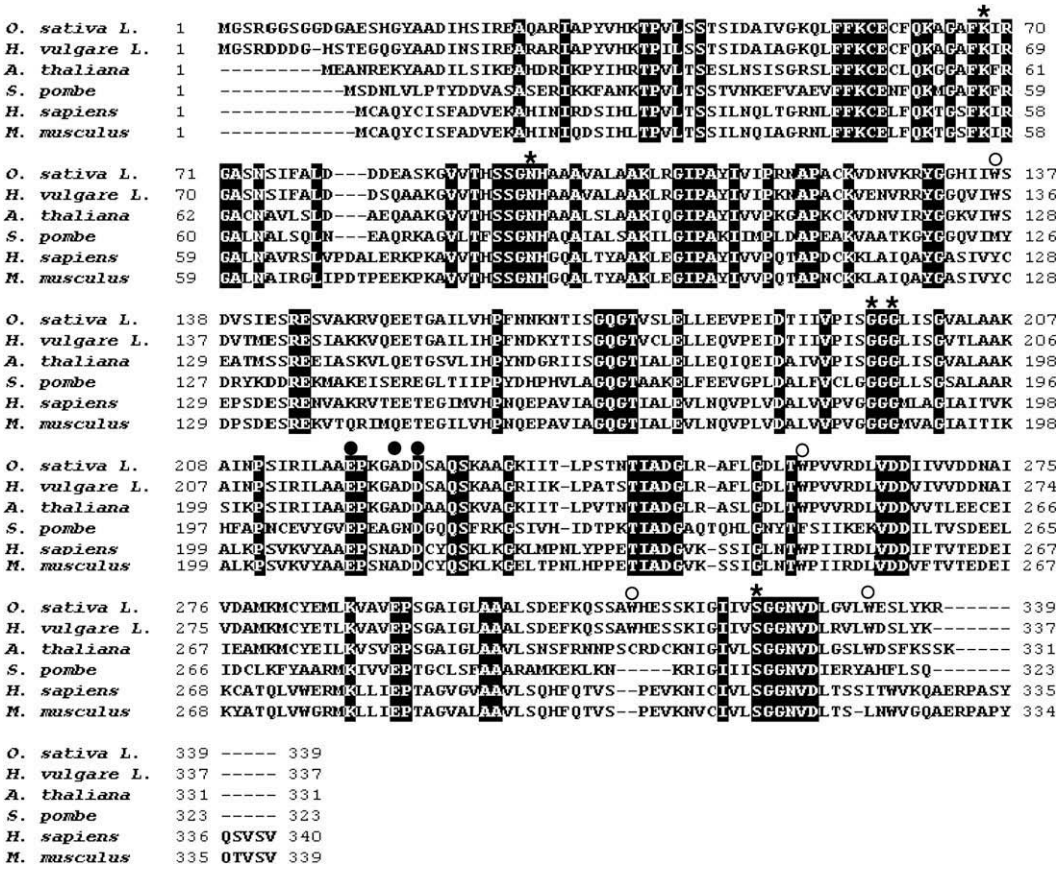

Fig. 2. Alignment of primary structures of various serine racemases *O. sativa* L., *Hordeum vulgare* L., *A. thaliana*, *S. pombe*, *H. sapiens*, and *M. musculus* represent the serine racemase amino acid sequences from *Oryza sativa* L. (accession No. AB425957), *H. vulgare* L. (accession No. BAF63026), *Arabidopsis thaliana* (accession No. NP192901), *Schizosaccharomyces pombe* (accession No. NP587715), *Homo sapiens* (accession No. NP068766), and *Mus musculus* (accession No. NP038789). The shaded area represents the amino acid residues conserved between all serine racemases. ●, probable amino acid residues composing a magnesium binding site; ○, tryptophan residues in SerR; ∗, amino acid residues composing a PLP binding site.

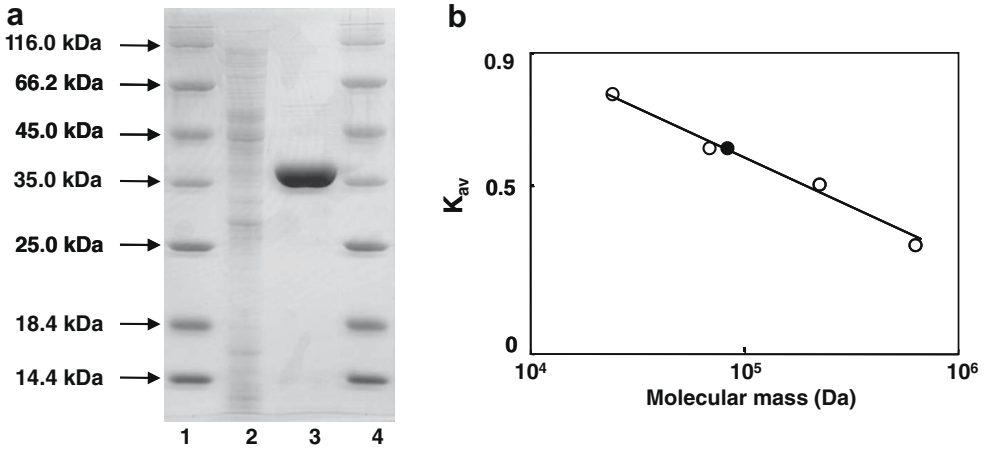

Fig. 3. Subunit and quaternary structures of the serine racemase from *Oryza sativa* L. (a) SDS–PAGE Electrophoresis was conducted with a Ready gel cell 108 BR (Bio-Rad Japan, Tokyo) equipped with a Power pac 300 (Bio-Rad). The concentration of acrylamide was 12%T. Lane 1: protein marker: β-galactosidase from *Escherichia coli*, 116.0 kDa; bovine serum albumin from bovine plasma, 66.2 kDa; ovalbumin from chicken egg white, 45.0 kDa; lactate dehydrogenase from porcine muscle, 35.0 kDa; restriction endonuclease *Bsp* 981 from *E. coli*, 25.0 kDa; β-galactoglobulin from bovine milk, 18.4 kDa; lysozyme from chicken egg white, 14.4 kDa. Lane 2: a crude extract of *E. coli* BL21 (DE3) cells harboring pET-21b/*serr* (protein: 8 μg). Lane 3: purified SerR (protein: 8 μg). Lane 4: protein marker. b. Gel filtration. K_{av} values were calculated according to the equation of $K_{av} = V_e - V_o/V_t - V_o$, where V_e is the elution volume for the protein, V_o is the column void volume, i.e., an elution volume for Blue Dextran 2000, and V_t is the total bead volume. The mobile phase used was 10 mM potassium phosphate buffer containing 300 mM NaCl. The flow rate was 0.3 ml/min. The column and protein marker used are described in Section 3.11.

Table 2
Purification of a putative serine racemase from *Oryza sativa* L.

Step	Total activity* (nkatal)	Total protein (mg)	Specific activity (nkatal/mg)	Yield (%)	Purification (fold)
Crude extract	52.0	396	1.31×10^{-1}	100	1.00
Ni–NTA agarose	13.0	6.00	2.17	25.0	16.9

* Activity is expressed as serine dehydratase activity.

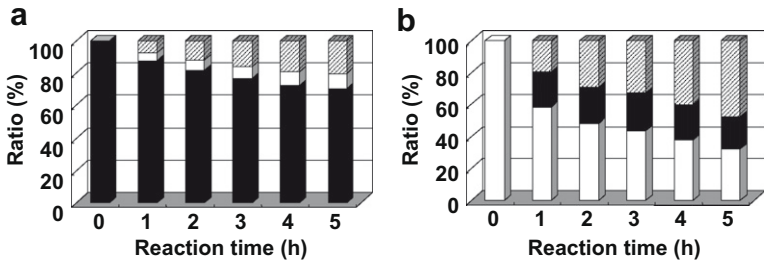

Fig. 4. Time course of the reaction products of serine racemase from *Oryza sativa* L. (a) D-Serine (**1a**) was used as substrate. (b) L-Serine (**1b**) was used as substrate. The preparation of a reaction mixture and the measurement of D- and L-serine (**1a** and **1b**) and pyruvate were conducted according to the method described in Section 3.6. An aliquot (500 μl) was taken from the reaction mixture after 0, 1, 2, 3, 4, and 5 h. Black bar: D-serine, white bar: L-serine, striped bar: pyruvate.

2.5. Effects of temperature and pH

The optimum temperature for the serine racemase and dehydratase activities of SerR was determined to be 35 °C, which agrees well with the optimum germination temperature of *O. sativa* L. (30–35 °C). A similar optimum temperature (37 °C) was found for the serine racemase from *Rattus norvegicus* (Wolosker et al., 1999a). SerR was stable up to about 40 °C and became inactivated at 50 °C. The activation energies for the serine racemase and serine dehydratase reactions catalyzed by SerR were calculated to be 61.8 kJ and 36.2 kJ, respectively. The optimum pH for the serine racemase reaction was 9.5, while that for the serine dehydratase was pH 9.0. These values are slightly higher than those of the enzymes from mammals such as *R. norvegicus* (pH 8–9) (Wolosker et al., 1999a) and *Mus musculus* (pH 8–9.5) (Strísovský et al., 2003). SerR was most stable at pH 8.5 and was almost inactivated at pH 6 and 11.

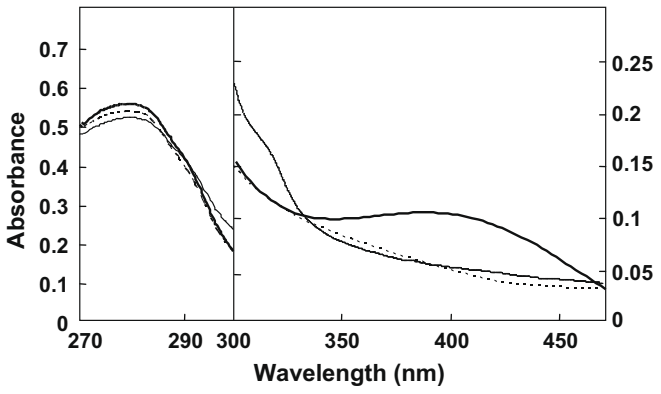

Fig. 5. Absorption spectra of serine racemase from *Oryza sativa* L. Solid line: the holo SerR (1 mg/ml in a 10 mM sodium phosphate buffer (pH 8.0) containing 10 mM PLP and 0.02% 2-mercaptoethanol); Narrow line: the reduced form of SerR (1 mg/ml in a 10 mM sodium phosphate buffer (pH 8.0) containing 0.02% 2-mercaptoethanol after reduction with $NaBH_4$); Dashed line: the apo SerR (1 mg/ml in a 10 mM sodium phosphate buffer (pH 8.0) containing 0.02% 2-mercaptoethanol). The spectra were taken with a Jasco V550 spectrophotometer (Jasco, Tokyo) at room temperature (about 25 °C).

2.6. Effects of inhibitors

Since the salicylaldehyde method that is used for the determination of the serine dehydratase activity interfered with the inhibitors used, we examined only the effects of various inhibitors on serine racemase activity. Like the serine racemase from *R. norvegicus* (Wolosker et al., 1999a,b), SerR was completely inhibited by 1 mM of semicarbazide, hydroxylamine, aminooxyacetate, sodium borate, or phenylhydrazine, respectively. SerR showed maximum absorption at 417 nm, and this disappeared after the enzyme solution was treated with hydroxylamine to form an apoenzyme (Fig. 5). These results suggest that SerR probably contains PLP as a coenzyme.

2.7. Effect of metal ions and ATP

ATP, Mg^{2+}, and Ca^{2+} are known to affect both serine racemase and dehydratase activities of serine racemases from mammalian brain (Foltyn et al., 2005), *M. musculus* (Cook et al., 2002; Neidle and Dunlop, 2002), and *A. thaliana*. In contrast to the mammalian brain and *M. musculus* enzymes, the serine racemase activity of SerR characteristically decreased upon addition of ATP, but the serine dehydratase activity of SerR increased (for [ATP] > 0.1 mM), as it does for those enzymes (Fig. 6a). ADP had no effect on either activity. Various metal ions affected the serine racemase activity of SerR in many of the same ways that they affect the activities of other serine racemases: Mg^{2+} and Ca^{2+} increased activity, and Zn^{2+} and Cu^{2+} decreased it (Fig. 6b). However, the serine dehydratase activity of SerR decreased with increasing the Mg^{2+} concentration (Fig. 6c). The serine racemase activity decreased in the presence of both ATP and Mg^{2+} (Fig. 6d). These results suggest that ATP, Mg^{2+}, and Ca^{2+} affect serine racemase and dehydratase activities of SerR differently from how they affect those of other serine racemases.

2.8. Kinetic parameters

To clarify the effects of Mg^{2+} on serine racemase and dehydratase activities of SerR, we determined the k_{cat}, K_m, and k_{cat}/K_m values for both activities in the presence or absence of Mg^{2+} (1 mM) using L-serine (**1b**) as a substrate (Table 3). Mg^{2+} decreased the

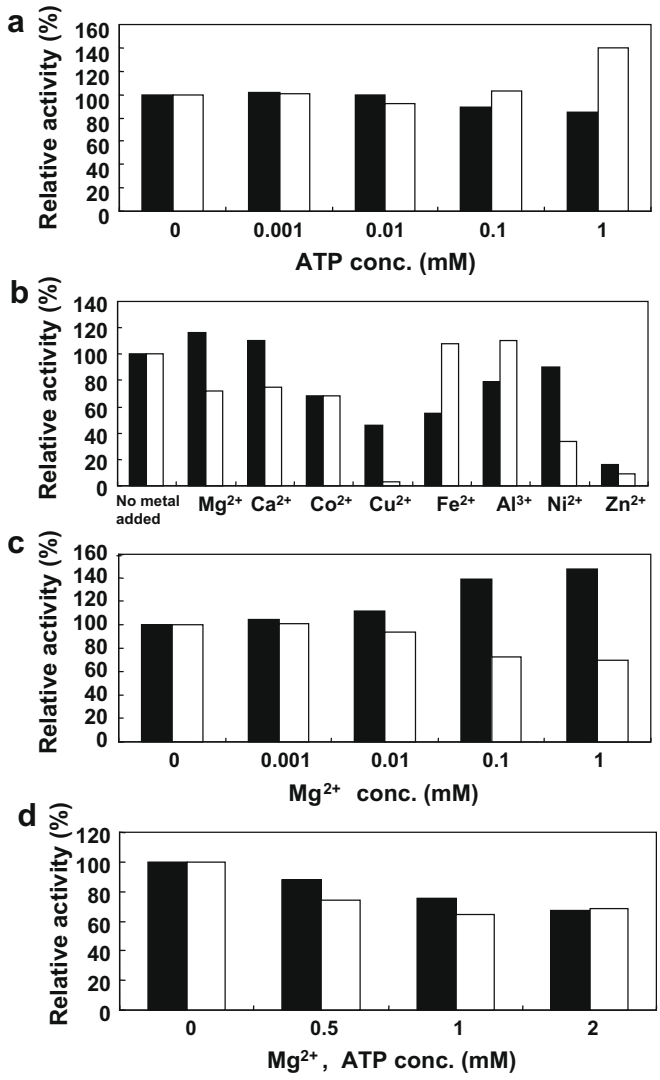

Fig. 6. Effects of metal ions and ATP on serine racemase activity. (a) Effect of ATP; (b) effects of various metal ions; (c) effect of Mg^{2+}; (d) effects of Mg^{2+} and ATP. Reactions were carried out according to the methods described in Section 3.6. Black bar: serine racemase activity of SerR; white bar: L-serine dehydratase activity of SerR.

Table 3
Kinetic parameters.

	k_{cat} (s^{-1})	K_m for L-ser (mM)	k_{cat}/K_m ($S^{-1} \cdot mM^{-1}$)
For racemase activity			
None	3.6×10^{-1}	18	2.0×10^{-2}
+Mg^{2+} (1 mM)	3.1×10^{-1}	10	3.1×10^{-2}
For dehydratase activity			
None	6.1×10^{-1}	28	2.2×10^{-2}
+Mg^{2+}(1 mM)	3.6×10^{-1}	18	2.0×10^{-2}

K_m value but did not affect the overall k_{cat} value of the serine racemase activity, and, subsequently, the k_{cat}/K_m value increased about 1.6 times. Regarding the serine dehydratase activity, both the K_m and k_{cat} values decreased; therefore, the k_{cat}/K_m value decreased slightly. These results suggest that Mg^{2+} characteristically increases the catalytic efficiency of the serine racemase activity and decreases that of the serine dehydratase activity (Table 3). As compared with the k_{cat} (4.0 s^{-1} for the dehydratase activity; 3.8 s^{-1} for the racemase activity) and K_m (3.8 mM for the dehydratase activity; 4.0 mM for the racemase activity) values of the serine racemase from *M. musculus* (Strísovský et al., 2003), those of SerR were one order of magnitude lower and higher, respectively. The mammalian brain enzyme has a k_{cat}/K_m value for the L-serine dehydratase activity (0.22 s^{-1}) about 10 times higher than that of SerR (Foltyn et al., 2005), and a higher K_m value (1.9 mM), suggesting that SerR has lower affinity to L-serine (Baumgart et al., 2007).

2.9. Quenching of SerR tryptophan fluorescence by acrylamide

We estimated the positions of the four tryptophan residues (Trp136, Trp257, Trp312, and Trp333) of SerR using the Swiss-PDB viewer and the three-dimensional structure of the serine racemase from *S. pombe* (PDB 1V71) (Fig. 7a and b). Trp257 probably exists next to a Mg^{2+}-binding site in the active site, and the other tryptophan residues may be distributed on the surface of the enzyme. There are no tryptophan residues in the serine racemase from *S. pombe*, and those in SerR may serve as probes for a fluorescence-quenching analysis (Fig. 2). To examine the regulation mechanism of Mg^{2+}, we tried to analyze the fluorescence quenching of tryptophan residues in SerR by acrylamide in the presence or absence of Mg^{2+} (1 mM) (Fig. 8a and b). Fig. 8c shows the Stern-Volmer plots for SerR, and the Stern-Volmer constants (K_{sv}) determined are summarized in Table 4. The K_{sv} constants in the presence of Mg^{2+} were lower than those in the absence of Mg^{2+} regardless of whether or not D- or L-serine (**1a** and **1b**) were added as substrates. These results suggest that the accessibility of the SerR tryptophan residues to the solvent is decreased by the addition of Mg^{2+}. Accordingly, the structure of SerR is distorted by the addition of Mg^{2+}, and this structural change probably regulates the two activities (serine racemase and dehydratase). Lys57 and Ser82 in the serine racemase of *S. pombe* are thought to be involved in abstracting the α-proton and forming a hydrogen bond to the β-OH in L- and D-serine (**1a** and **2b**), respectively (Yoshimura and Goto, 2008), but the regulation mechanism for both reactions is still unknown. Lys68 and Ser93 in the primary structure of SerR probably correspond to Lys57 and Ser82 of *S. pombe*, respectively (Fig. 2).

3. Experimental

3.1. Materials

Unpolished rice (*Oryza sativa*. L. cv. Koshihikari) was purchased from JA, Japan. *o*-Phtalaldehyde (OPA), *N*-acetyl-L-cysteine (NAC), D- and L-serine (**1a** and **1b**), and pyridoxal 5′-phosphate (PLP) were purchased from Wako (Osaka, Japan), whereas Ni–NTA Agarose was purchased from Qiagen (Hilden Germany). The rice full-length cDNA clone (clone name: 001-110-B03) was obtained from the Rice Genome Resource Center, National Institute of Agrobiological Science, Tsukuba, Ibaraki, Japan. *E. coli* BL21 (DE3), *E. coli* NovaBlue, pT7 Blue-2 T-vector, and pET-21b were purchased from EMD Biosciences, Inc. (San Diego, CA, USA). Blend Taq polymerase was purchased from Toyobo, Osaka. NdeI and XhoI were purchased from Roche Diagnostics (Basel, Switzerland). All other reagents were the best grade commercially available unless otherwise stated.

3.2. Germination of unpolished rice

Unpolished rice (20 g) was sterilized with EtOH–H_2O (7:3, v/v) and 5% NaOCl containing 0.01% Triton X-100 and washed five times with sterilized H_2O. The washed, unpolished rice, was put into a sterilized Erlenmeyer flask (500 ml) and statically incubated at 30 °C for 22 h.

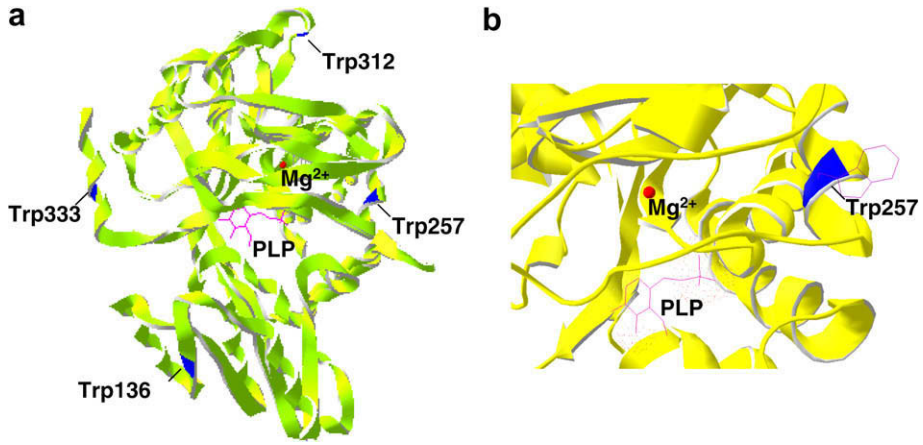

Fig. 7. Proposed three-dimensional subunit structure of serine racemase from *Oryza sativa* L. (a) Subunit structure: The three-dimensional subunit structure of SerR (yellow ribbon) was superimposed on that of serine racemase from *Schizosaccharomyces pombe* (yellowish-green ribbon). Mg^{2+} is shown as a red sphere. (b) Active site of SerR: Try257, PLP, and Mg^{2+} are shown as a blue ribbon with purple sticks, pink sticks, and a red sphere, respectively. The structures were estimated by the method described in Section 3.11.

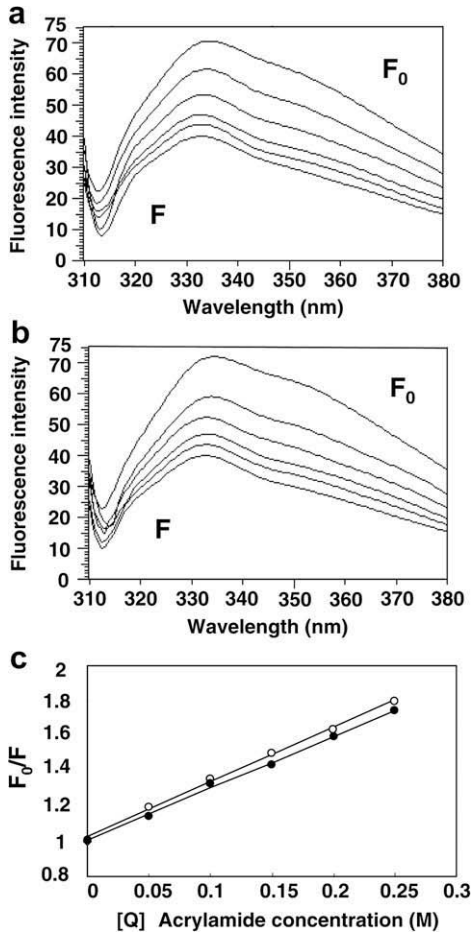

Fig. 8. Quenching of SerR tryptophan fluorescence by acrylamide. (a) Fluorescence spectra in the absence of Mg^{2+}. (b) Fluorescence spectra in the presence of Mg^{2+}. (c) Stern-Volmer plot. The experiment was carried out according to the method described in Section 3.10.

3.3. Extraction of D-amino acids in germinated, unpolished rice

The germinated, unpolished rice (3 g) was frozen in liquid N_2 and ground in a mortar with EtOH–H_2O (7:3, v/v, 27 ml). The homogenate was put into a centrifuge tube (50 ml, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and stored in a cold room (about 4 °C). After 20 h, the homogenate was centrifuged at 26,100×*g* for 30 min at 4 °C. The amino acids in the supernatant solution were derivatized with OPA and NAC according to the method of Aswad (1984). The HPLC analyses of D- and L-amino acids were carried out under the same conditions as described previously (Ono et al., 2006).

Table 4
Stem-Volmer constants.

Substrate	Mg^{2+} (mM)	K_{sv}
None	0	3.12
	+1	2.96
L-Ser (**1b**)	0	3.26
	+1	3.07
D-Ser (**1a**)	0	3.10
	+1	2.89

3.4. Cloning of the putative serine/threonine racemase gene in the rice genome

The oligonucleotides *serac1* (5′-TCATATGGGGAGCAGAGGTG-3′) and *serac2* (5′-CTCGAGACGTTTATAGAGAGACTCCC-3′), based on the 5′- and 3′- sequences of the insert in the *O. sativa* cDNA clone (*O. sativa* L. ssp. Japonica cv. Nipponbare, 001-110-B03), were used as PCR primers (0.2 pmol) to amplify the *serr* gene from the cDNA clone (1 ng). The PCR involved 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. PCR amplification was carried out with Blend Taq polymerase in a Gene Amp PCR system 9700 (PE Applied Biosystems, Piscataway, NJ, USA). The resulting 1,020-bp *serr* fragment with NdeI and XhoI restriction sites was sequenced with a DNA sequencing system, SQ5500 (Hitachi, Tokyo, Japan), ligated into the pT7 Blue-2 T-vector (50 ng/μl) and transformed into *E. coli* NovaBlue. After cultivation in an LB medium containing ampicillin, the plasmid was extracted with the alkaline mini-prep method and precipitated with polyethylene glycol 6,000. The purified DNA obtained was digested with NdeI and XhoI and ligated into an NdeI- and XhoI-digested pET-21b vector to form pET-21b/*serr*, which was transformed into *E. coli* BL21 (DE3).

3.5. Purification of the serine racemase homologue of O. sativa L.

After the *E. coli* BL21 (DE3) cells harboring pET-21b/*serr* were selected on an LB agar medium containing 100 μg/ml ampicillin, the clone was grown in 1 l of an LB medium containing 100 μg/ml ampicillin at 37 °C until the absorption at 600 nm reached 0.6. The cells were collected by centrifugation (14,000×*g*, 20 min), suspended in 24 ml of a 10 mM sodium phosphate buffer, pH 8.0, and stored at −20 °C. The frozen cells were restored with a 10 mM sodium phosphate buffer (pH 8.0) containing 10 μM PLP, 10 mM imidazole, and 300 mM NaCl on ice and disrupted by ultrasonication (model UD-201, Tomy Seiko Co., Tokyo; output 6, duty cycle, 30; 2 min×5 times). The suspension was centrifuged (26,100×*g*, 20 min) and ultracentrifuged (100,000×*g*, 1 h), and the supernatant was dialyzed against a 10 mM sodium phosphate buffer (pH 8.0) containing 10 mM PLP, 10 mM imidazole, and 300 mM NaCl. The crude extract obtained was applied to a column of Ni–NTA ($\varphi 1 \times 2$ cm), which was equilibrated with a 10 mM sodium phosphate buffer containing 10 mM imidazole and 300 mM NaCl and washed (first with 100 ml of the same buffer, then with 200 ml of a 10 mM sodium phosphate buffer containing 20 mM imidazole and 300 mM NaCl, and finally with 60 ml of a 10 mM sodium phosphate buffer containing 50 mM imidazole and 300 mM NaCl). The flow rate was 0.5 ml/min. The adsorbed protein was eluted with 50 ml of a 10 mM sodium phosphate buffer containing 250 mM imidazole and 300 mM NaCl. The elution was collected with Bio-collector (ATTO, Tokyo, 2 ml/tube), and the protein concentration was monitored spectrophotometrically at 280 nm.

3.6. Assay methods

The serine racemase and serine dehydratase activities were measured with the following assay mixture: a 500 mM CHES–NaOH buffer, pH 9.0 (100 μl), 100 mM L- or D-serines (**1a** and **1b**) (50 μl), 1 mM PLP (5 μl), a 1 mg/ml crude extract (100 μl), and deionized water (245 μl), for a total volume of 500 μl. The assay mixture, except for the crude extract, was put into a microtube (1.5 ml, Greiner, Heidelberg, Germany) and incubated at 30 °C. The enzymatic reaction was started by the addition of the crude extract. After the reactions were stopped by heat treatment (100 °C, 5 min), any D- or L-serines (**1a** and **1b**) produced were measured by HPLC (for serine racemase activity), and any pyruvate produced was measured using the salicylaldehyde method (for serine dehydratase activity) (Saier and Jenkins, 1967). One unit of the enzyme was defined as the amount of enzyme that produces 1 μmol of D- or L-serine (**1a** and **1b**) from racemization, or pyruvate from dehydration, per min at 30 °C.

3.7. Basic enzymatic properties

Serine racemase and serine dehydratase activities of SerR were examined at various reaction temperatures: 10, 20, 30, 35, 40, 50, 60, 70, 80, and 90 °C. The activation energies for the serine racemase and serine dehydratase reactions were calculated from Arrhenius plots. The thermal stability of SerR was estimated by both the serine racemase and the serine dehydratase activities after the enzyme was treated at various temperatures (10, 20, 30, 35, 40, 50, 60, 70, 80, and 90 °C) for 1 h. The effects of pH on the serine racemase and serine dehydratase activities of SerR were measured under the standard assay conditions at various pHs (a PIPES–NaOH buffer for pH 6.0–7.0; a HEPES buffer for pH 7.0–8.5; a CHES–NaOH buffer for pH 8.5–10.0; and a CAPS–NaOH buffer for pH 10.0–11.0), and the pH stability of the enzyme was estimated by both the serine racemase and the serine dehydratase activities after the enzyme was treated in buffers with various pHs at 4 °C for 5 h. The substrate specificities of the serine racemase and serine dehydratase activities of SerR were determined under the standard assay conditions in the presence of various 10 mM substrates. The effects of various enzyme inhibitors on SerR were determined as follows: the enzyme solution was incubated with 1 mM of reagents such as semicarbazide, hydroxylamine, aminooxyacetate, sodium borate, or phenylhydrazine for 30 min at pH 9.0 on ice, and the residual activities were measured under the standard assay conditions.

3.8. Effect of metal ions, ADP, and ATP

The effects of metal ions, ADP, or ATP on the serine racemase and serine dehydratase activities of SerR were examined in the presence of 1 mM $AlCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $MgCl_2$, NaCl, $NiCl_2$, $ZnCl_2$, ADP, or ATP under the standard assay conditions (pH 9.0, 30 °C). The effects of the concentrations of $MgCl_2$ (0, 0.001, 0.01, 0.1, and 1.0 mM), ATP (0, 0.001, 0.01, 0.1, and 1.0 mM), or ATP (0, 0.5, 1, and 2 mM) in the presence of $MgCl_2$ (1 mM) were also examined.

3.9. Kinetic analysis

Kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) were determined for both serine racemase and serine dehydration reactions of SerR. L-serine was used as a substrate, and k_{cat}, K_m, and k_{cat}/K_m values were calculated in the presence or absence of Mg^{2+} according to Lineweaver-Burk double-reciprocal plots.

3.10. Quenching of SerR tryptophan fluorescence by acrylamide

A fluorescence-quenching experiment was conducted by addition of various concentrations (0, 0.05, 0.10, 0.15, 0.20, and 0.25 M) of acrylamide dissolved in a 10 mM CHES–NaOH buffer, pH 9.0 (0.25 ml), to the enzyme solution (protein conc.: 0.5 mg/ml, 0.1 ml) in the presence or absence of the substrate (L- or D-serines (**1a** or **1b**)) dissolved in the same buffer (0.65 ml). The fluorescence intensity was measured with the Hitachi 650-60 fluorescence spectrophotometer (Hitachi, Ibaragi) equipped with a data processor under a 5 nm slit width. The excitation wavelength used for the tryptophan residue was 295 nm, and fluorescence was measured at 330 nm at room temperature (about 20 °C). The Stern-Volmer quenching constant (K_{sv}) was determined according to the Stern-Volmer equation

$$Fo/F = 1 + K_{sv}[Q],$$

where *Fo* is the fluorescence intensity in the absence of a quencher (i.e., acrylamide), *F* is the fluorescence intensity at the molar quencher concentration [Q]. K_{sv} is obtained experimentally from the slope of the plot of *Fo*/*F* versus [Q].

3.11. Other methods

Metal analysis was conducted using a polarized Zeeman atomic absorption spectrophotometer Z-8200 (Hitachi High-Technologies Co., Tokyo). Ni was monitored at 232.0 nm, with measurements repeated at least twice. These protein concentrations were measured using the Bradford method, with bovine serum albumin (Wako Chemical Co., Osaka; product No. 011-07493) as a standard. The molecular mass of SerR was estimated by gel filtration with a column (1.6 by 70 cm) of Superdex 200 Hiload (16/60) (GE Healthcare UK Ltd., Amersham Place, England), and with thyroglobulin (669 k), catalase (232 k), albumin (69 k), and chymotrypsinogen A (25 k) as standards. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out by the method of Laemmli (Laemmli, 1970). Gels were stained with Coomassie Blue R-250.

The three-dimensional structure of SerR was estimated by the molecular modeling program, MDL Chime (http://www.mdl.com/).

3.12. Nucleotide sequence accession number

The DNA sequence of the gene encoding serine racemase from *O. sativa* L. ssp. Japonica cv. Nipponbare is available from GenBank under the accession number AB425957.

4. Conclusions

In this study, we clarified the enzymological properties of SerR in rice. The function of SerR is probably either catabolic (the degradation of D- or L-serine (**1a** and **1b**) to pyruvate and ammonia) or anabolic (the production of D-serine (**1a**) from L-serine (**1b**)) but this still remains unknown. We are currently studying the three-dimensional structure of SerR by X-ray crystallography to clarify the reaction and regulation mechanisms of the bifunctional enzyme, as well as the physiological functions of SerR in germinated, unpolished rice.

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