BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 31–34 (1998)
ARTICLE NO. RC988561

Occurrence of Pyridoxal 5′-Phosphate-Dependent Serine Racemase in Silkworm, *Bombyx mori*

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Received March 28, 1998

D-Serine is known to occur in the silkworm *Bombyx mori* as well as in the mammalian central nervous systems. We found that serine racemase occurs in the insect, catalyzing the conversion of L-serine to its antipode. The enzyme was partially purified from pupae of the insect, and was inactivated by treatment with hydroxylamine and reactivated with pyridoxal 5′-phosphate (PLP). L-Alanine was racemized slowly by the enzyme at a rate of only about 6% of that of L-serine, and L-arginine and L-glutamine were inert as substrates. Therefore, the enzyme is a member of PLP-dependent amino acid racemases, and is distinct from alanine racemase (EC 5.1.1.1) and amino acid racemase with low substrate specificity (EC 5.1.1.10). This is the first report of the occurrence of serine racemase in eukaryotes producing D-serine.

Several D-amino acids such as D-alanine and D-glutamate are indispensable constituents in bacterial peptidoglycans. D-Serine has been demonstrated in fluids and tissues of multicellular organisms. It is produced by earthworms and incorporated into lombricine (1). In the blood of the silkworm, D-serine concentration is increased at particular stages of metamorphosis (2), although little is known about the role of D-serine in metamorphosis. D-Serine is also present at late larval and pupal stages in most other lepidopteran species so far studied (2).

Recent advances in analytical techniques have led to the demonstration of D-amino acids in vertebrates including human beings (3, 4). D-Serine occurs in forebrain structures, whereas D-aspartate is accumulated transiently in the brain at particular stages of development (4). D-Serine acts as an agonist at the glycine site of *N*-methyl-D-aspartate (NMDA) receptor, while D-aspartate serves as an agonist at its glutamate site (5, 6, 7).

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D-Serine is assumed to be produced from its antipode by racemization in both silkworm and mammals, but no one has succeeded in demonstrating serine racemase in these animals. We have attempted to clarify whether the enzyme does indeed occur in the silkworm. We know that it contains higher concentrations of D-serine than mammals and may therefore be expected to produce serine racemase abundantly. We here demonstrate the occurrence of serine racemase which depends on pyridoxal 5′-phosphate and acts with high specificity towards serine.

MATERIALS AND METHODS

Materials. D-Amino acid oxidase (EC 1.4.3.3) from pig kidney was purchased from Boehringer Mannheim (Germany); *o*-phthalaldehyde (OPA), D- and L-serine from Nacalai Tesque (Kyoto, Japan); L-homocysteic acid and L-*O*-phosphoserine from Sigma (St. Louis); *N-tert*-butyloxycarbonyl-L-cysteine (BOC-L-Cys) from Novabiochem (Läufelfingen, Switzerland); Butyl-TOYOPEARL and DEAE-TOYOPEARL from Tosoh (Tokyo, Japan). Silkworms *Bombyx mori* (type, Kinshu x Showa) were reared on fresh mulberry leaves. Three-days old pupae were collected and stored at −80°C until use. Commercially available L-serine contains about 1% (w/w) D-serine, which we removed with D-serine dehydratase, whose gene (*dsdA*) of *Escherichia coli* JM109 was cloned by polymerase chain reaction according to the reported sequence of the gene (accession number of GenBank, JO 1603). The enzyme was purified from the recombinant cells as reported previously (8). A mixture (40 ml) containing 400 mM potassium phosphate buffer (pH 7.8) and 466 units of the enzyme was incubated at 37°C for 12 h, and the product was purified with Dowex 50 × 8 (H^+). The optical purity of the L-serine obtained was more than 99.7% enantiomeric excess (ee) and comparable to that of commercial D-serine (99.5% ee) upon HPLC analysis as described below.

Purification of serine racemase from Bombyx mori pupae. All procedures were carried out at 4°C. The standard buffer used throughout the purification was: 10 mM potassium phosphate buffer (pH 7.2) containing 50 mM KCl, 10 μM PLP, 1 mM dithiothreitol (DTT) and 1 mM ethylenediamine-tetraacetic acid (EDTA).

Pupae (about 100 g) were homogenized with 5 volumes of the buffer, which was supplemented with 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM *p*-toluene sulfonyl-L-phenylalanine chloromethyl ketone, 50 μg/ml phenylthiourea, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A and 2 μg/ml chymostatin, in a Potter-Elvenhjem glass equipped with a motor-driven Teflon pestle (3,000 rpm). The homogenates were centrifuged at 20,000 × g for 20 min. The supernatant

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TABLE I

Partial Purification of Serine Racemase from Silkworm Pupae

Step	Total protein (mg)	Specific activity (units/mg)		Total activity (units)	
		L-serine[a]	D-serine[a]	L-serine[a]	D-serine[a]
Pupae homogenate	4420			N.D.[b]	N.D.[b]
Ammonium sulfate fractionation	1275	0.16	0.31	200	400
Butyl-TOYOPEARL	725	0.26	0.51	190	370
DEAE-TOYOPEARL	125	1.1	2.4	140	300

[a] Racemase activity was determined as described in Materials and Methods with either D- or L-serine as a substrate.
[b] N.D., not detectable.

solution was brought to 45% saturation with ammonium sulfate. The solution was stirred for 40 min, and centrifuged at 20,000 × g for 20 min. The resulting pellet was dissolved in 75 ml of the buffer, and dialyzed against the buffer overnight. The supernatant solution obtained by centrifugation at 20,000 × g for 10 min was brought to 20% saturation with ammonium sulfate. After standing for 1 h, the solution was centrifuged to remove insoluble materials. The supernatant solution was loaded onto a Butyl-TOYOPEARL column (150 ml) equilibrated with the buffer supplemented with ammonium sulfate (20% saturation). A stepwise elution was performed with 300 ml of the buffer supplemented with 20, 10, 5.0, 2.5, 1.0, and 0% saturation of ammonium sulfate in this order. Each fraction was brought to 45% saturation with ammonium sulfate. The pellet was dissolved in a small volume of the buffer and dialyzed overnight against the buffer. The active fraction, which was eluted with 10% saturation of ammonium sulfate, was loaded onto a DEAE-TOYOPEARL column (50 ml) equilibrated with the buffer. The column was washed with 100 ml of the buffer, and the enzyme was eluted with 100 ml of the buffer supplemented with 50 mM KCl. The enzyme was precipitated with 45% saturation of ammonium sulfate, and dialyzed against the buffer overnight.

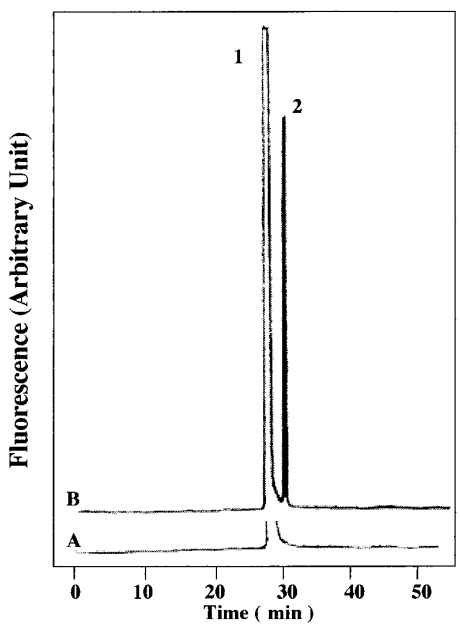

FIG. 1. Production of D-serine from L-serine with partially purified preparation of silkworm serine racemase. The reaction mixture containing 100 mM Bis-tris Propane (pH 8.5), 40 mM L-serine, 7 μM PLP and partially purified enzyme in a final volume of 200 μl was incubated at 37°C. A 50-μl aliquot was withdrawn from the reaction mixture and mixed with 450 μl of 0.05 M HCl to stop the reaction. After centrifugation, 50 μl of the supernatant solution was subjected to analysis of D-serine formed as described in MATERIALS AND METHODS. Peaks 1 and 2 correspond to L- and D-serine, respectively. A: the enzyme reaction was stopped immediately after initiation; B, reaction time, 4 h.

Assay of serine racemase. Serine racemase was assayed by measuring the rate of conversion of L- or D-serine to the corresponding antipode. The amino acids were derivatized to the fluorescent diastereomer and determined by HPLC according to the method of Hashimoto et al (9). The reaction mixture (100 μl) contained 100 mM 1,3-Bis[tris(hydroxymethyl)methylamino]-propane (Bis-tris Propane) buffer (pH 8.5), 40 mM L- or D-serine, 7 μM PLP, 0.7 mM DTT, 0.7 mM EDTA, and enzyme. After incubation at 37°C for 8 h, a 20 μl-aliquot was withdrawn from the reaction mixture and diluted 10-fold with 0.05 M HCl. After centrifugation, 50 μl of the supernatant solution was incubated with BOC-L-Cys and OPA for 3 min at room temperature. A 10 μl-aliquot of the reaction mixture was subjected to HPLC, whose system comprised two CCPD dual pumps, a CCP solvent programmer, a FS-8000 spectrofluorometer (Tosoh, Tokyo, Japan), an ERC-3310 degasser (Erma Optical Works, Tokyo, Japan), and a 4-μm Nova-Pack C18 column (3.9 × 300 mm) (Waters). Other conditions were the same as described by Hashimoto et al (9). One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of 1 nmol of the product enantiomer per minute. Protein was assayed according to the method of Bradford with bovine serum albumin as a standard (10).

RESULTS AND DISCUSSION

Srinivasan et al. (11) have demonstrated conversion of L-serine to its antipode with homogenates of silkworm pupae. However, the activity was found to be extremely labile. We succeeded in stabilizing the activity by addition of various protease inhibitors to the homogenate, and obtained a preparation of partially purified enzyme by ammonium sulfate fractionation, and Butyl-TOYOPEARL and DEAE-TOYOPEARL column chromatographies. A summary of the purification is shown in Table I. As the purification proceeded, a conversion of D- to L-serine was detectable in addition to that of L- to D-serine. Specific activities of the final preparation in the L- to D-serine and D- to L-serine directions were 1.1 and 2.4 units/mg, respectively. Amounts of D- and L-serine formed from their antipo-

TABLE II

Effect of Various Reagents on the Activity of Serine Racemase from Silkworm

Reagent	D-Serine formed (nmol)	D-Serine formed (ratio, %)
None	58.2	100
Sodium cyanoborohydride	44.1	76
Sodium borohydride	15.5	27
Hydroxylamine	0	0
Phenylhydrazine	0	0

Note. The reaction mixture (100 μl) containing 100 mM Bis-tris Propane (pH 8.5), 40 mM L-serine, 350 μg of partially purified enzyme, and 10 mM reagent indicated was incubated at 37°C for 8 h. D-Serine produced was determined as described in text.

TABLE III

Effect of PLP on the Activity of Serine Racemase from Silkworm

First dialysis	Second dialysis	Specific activity (units)	Ratio (%)
Not done	Not done	510	100
Hydroxylamine not added	PLP not added	110	22
Hydroxylamine not added	PLP added	480	94
Hydroxylamine added	PLP not added	0	0
Hydroxylamine added	PLP added	620	122

Note. The enzyme (200 μl, 1 mg of protein) was dialyzed against 1,250 volumes of 100 mM potassium phosphate (pH 7.2) containing 1 mM DTT, 1 mM EDTA, 50 mM KCl, and either 50 mM or no hydroxylamine for 18 h at 4°C (first dialysis), followed by dialysis against 2,500 volumes of 10 mM potassium phosphate buffer (pH 7.2) containing 50 mM KCl, 1 mM DTT, and 1 mM EDTA with or without 10 μM PLP for 12 h (second dialysis). Serine racemase activity was determined with L-serine as a substrate as described in Materials and Methods.

des were increased proportionally with increases in the amount of enzyme and incubation time (data not shown). These results clearly show occurrence of serine racemase.

However, it is possible that in addition to serine racemase some other enzymes also participate in the biosynthesis of D-serine in silkworm. D-Serine may be formed: from β-hydroxypyruvate by D-amino acid aminotransferase (EC 2.6.1.21) (12); from *O*-phosphoserine by an unknown enzyme analogous to *O*-phosphoserine phosphohydrolase (EC 3.1.3.3) (13, 14) catalyzing the inversion of C_2-configuration upon hydrolysis of L-*O*-phosphoserine; or from glycine by a hypothetical analog of glycine hydroxymethyltransferase (EC 2.1.2.1) (6, 15) with opposite stereospecificity. We examined the possible occurrence of such alternative pathways for the production of D-serine. L-Serine was incubated with the partially purified enzyme at 37°C for various periods of time (0, 0.5, 1, 2, 4, 6 and 8 h), and amino acids produced were analyzed after derivatization as described above. However, no amino acids except D-serine was produced (Fig. 1). Moreover, L-*O*-phosphoserine was not converted to D-serine by incubation with the enzyme (data not shown). These results suggest that serine racemase provides silkworm with the major route for the biosynthesis of D-serine.

Plots of specific activities of the partially purified enzyme against various concentrations of D-serine (and L-serine) gave hyperbolic curves (data not shown). Apparent K_m and V_{max} values were estimated: L-serine, 4.6 mM and 0.80 nmol/min/mg; D-serine, 16.5 mM and 2.1 nmol/min/mg. Thus, V_{max}/K_m values for the conversion from L- to D-serine and from D- to L-serine were similar: 0.17 and 0.13 nmol/min/mg/mM, respectively. The values are consistent with the theory of racemization reactions shown by Briggs and Haldane (16).

There are two types of amino acid racemases; one depends on pyridoxal 5′-phosphate (PLP), and the other is independent of any cofactors (17). Serine racemase activity of the partially purified preparation was inhibited strongly by sodium cyanoborohydride, sodium borohydride, phenylhydrazine and hydroxylamine (TABLE II). The enzyme was also inactivated by dialysis against the buffer containing hydroxylamine, and full activity was recovered by subsequent dialysis against the PLP-supplemented buffer (TABLE III). These results indicate that the enzyme depends on PLP. When the enzyme was dialyzed against the PLP-free buffer, about 78% of its

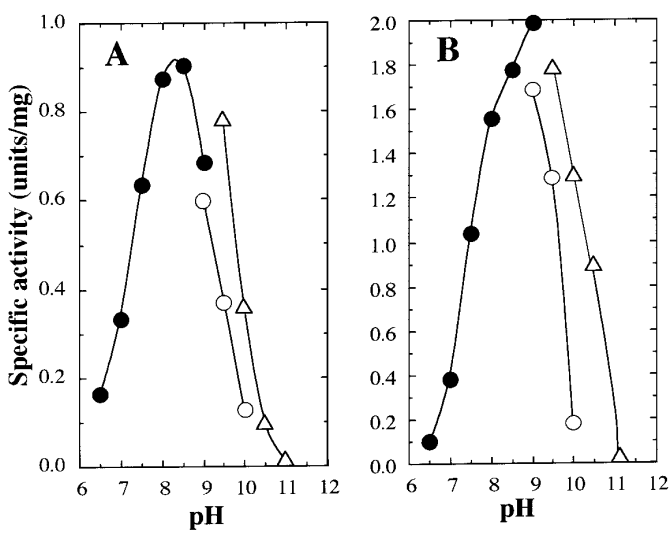

FIG. 2. Effect of pH on serine racemase activity with L-serine (A) and D-serine (B) as substrates. The reaction mixture containing 100 mM buffer and 350 μg of partially purified serine racemase was incubated at 37 °C for 8 h. Buffers used were: Bis-tris Propane (pH 6.5-9.0, closed circles), CHES (pH 9.0-10.0, open circles), and CAPS (pH 9.5-11.0, open triangles). Product enantiomers of serine were determined as described in MATERIALS AND METHODS.

original activity was lost. This suggests that PLP is loosely bound to the enzyme.

Several microbial amino acid racemases are known to act on serine. Serine undergoes racemization at a significant rate by alanine racemases from *Salmonella typhimurium* and *Tolypocladium niveum* (18, 19), although it is only a poor substrate of the enzymes. Amino acid racemase with low substrate specificity from *Pseudomonas putida* also racemizes serine, but basic and aliphatic straight-chain amino acids including arginine and glutamine are much better as substrates than serine (20). Two enzymes apparently functioning as serine racemases were demonstrated in *Streptomyces garyphalus*, but their substrate specificities have not yet been clarified (21). The enzyme we have demonstrated in silkworm is highly specific toward serine; L-alanine was racemized at a rate only about 6% of that of L-serine under the same conditions. Moreover, L-arginine and L-glutamine were inert as substrates. Thus, serine racemase of silkworm differs markedly from alanine racemase and amino acid racemase with low substrate specificity in this respect. The enzyme showed maximum activity toward L- and D-serine at pH 8.5 and 9.0, respectively (Fig. 2).

D-Serine serves as an agonist of the NMDA receptor in the mammalian brain. Recent reports have presented evidence that L-serine is probably the precursor to D-serine in mammalian brains (24, 25). The results presented here clearly show the occurrence of serine racemase in silkworm pupae. Thus, one can speculate that D-serine is also synthesized by serine racemase in mammalian central nervous systems. Two putative NMDA receptor genes of fruit fly *Drsophila melanogaster,* which are homologous to mammalian NMDAR1, are expressed synchronously with the development of the central nervous system (22, 23). Silkworm serine racemase and its gene may provide us with a useful means to clarify the role of D-serine in mammalian brains. Large-scale purification is in progress to obtain amino acid sequences of the silkworm serine racemase and antibodies against it.

ACKNOWLEDGMENT

We are grateful to Dr. Atsushi Hashimoto of Tokai University for his helpful discussion. This work was supported in part by Grants-in-Aid for Scientific Research 08680683 (to T.Y.) and 07308048 (to N.E.) from the Ministry of Education, Science, Sports and Culture of Japan, and a Research Grant from Japan Society for the Promotion of Science (Research for the Future) to N.E.

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