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Rapid determination of free D-serine with chicken D-serine dehydratase☆

Chihiro Suzuki[a], Masahito Murakami[a], Hirokazu Yokobori[a], Hiroyuki Tanaka[b], Tetsuo Ishida[b], Kihachiro Horiike[b], Yoko Nagata[a,*]

[a] Department of Materials and Applied Chemistry, College of Science and Technology, Nihon University, Kanda-Surugadai, Chiyoda-Ku, Tokyo 101-8308, Japan
[b] Department of Biochemistry and Molecular Biology, Shiga University of Medical Science, Ohtsu, Shiga 520-2192, Japan

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ABSTRACT

We have developed a simple, rapid, and inexpensive method of measuring the concentration of intrinsic free D-serine in tissue samples. This method uses chicken D-serine dehydratase in an enzymatic reaction to produce pyruvate, which is detected spectrophotometrically. Pyridoxal 5′-phosphate (PLP), a cofactor of D-serine dehydratase, increased pyruvate formation by 28%. The presence of Zn^{2+} or ethylenediaminetetraacetic acid (EDTA) did not have any effect on pyruvate formation under the present assay conditions. In addition, this method was not affected by the presence of a large excess of L-serine, nor by the presence of tissue extracts, and accurately determined concentrations of 2–30 μM (200 pmol–3 nmol) of D-serine. The entire assay requires only 60 min. With this method, we determined the concentration of D-serine in various silkworm tissues. The results were in agreement with high performance liquid chromatography measurements. We found high concentrations of D-serine in silkworm larvae at day 3 of the fifth instar; specifically, 509 nmol g^{-1} wet tissue in the midgut, 434 nmol g^{-1} in the ovary, and 353 nmol g^{-1} in the testis.

1. Introduction

Silkworms (*Bombyx mori*) contain high concentrations of free D-serine, the optical isomer of L-serine. This isomer accounts for >50% of the total free serine content [1]. Free D-serine is also present in human plasma [2] and mammalian brain tissue [3,4]. In the rat cerebrum, approximately 25% of free serine is the D-isomer. D-Serine is synthesized from L-serine by the enzyme serine racemase [5,6], and it functions as a physiological co-agonist of *N*-methyl-D-aspartate (NMDA) receptors, modulating neurotransmission by binding to the glycine-binding site of NMDA [7,8]. D-Serine is associated with NMDA receptor dysfunctions [9], and has been employed in therapeutic treatment of schizophrenia [10], and post-traumatic stress disorder [11]. In the brain, D-serine levels are inversely related to D-amino acid oxidase (DAO, EC 1.4.3.3) activity [12–14]. Additionally, we have identified the importance of D-serine in the growth and development of the silkworm [15,16]. Taken together, a simple, rapid, and accurate method of determining D-serine in biological samples is suggested to be useful in studies on the physiological implications of D-serine, as well as for the diagnosis and treatment of neurological disorders.

High performance liquid chromatography (HPLC) has generally been used to accurately assay small amounts of D-serine [13,14]. Unfortunately, analytical methods that use HPLC are expensive and time-consuming. In addition, chiral columns and chiral solvents are costly, and both are required for assaying optical isomers such as D-serine. HPLC systems also tend to require significant maintenance. As such, we initially aimed to develop a simple colorimetric method for D-serine estimation by using porcine kidney DAO, a commercially available enzyme that catabolizes free, neutral D-amino acids. However, the activity of DAO toward D-serine is approximately 10% its activity toward D-alanine. Therefore, we report here a method for D-serine quantification using chicken D-serine dehydratase (DSD, EC 4.3.1.18 [17]), which catalyzes the dehydration of D-serine with high substrate specificity. This dehydration yields pyruvate and ammonia: the pyruvate can be easily determined spectrophotometrically. We applied the method to assay D-serine concentrations in silkworm tissues and compared the results to those obtained by HPLC [18].

2. Experimental

2.1. Chemicals and materials

Non-diapause eggs of the silkworm *B. mori* N4 were reared on an artificial diet (Silkmate 2S; Nihon Nosan, Yokohama, Japan)

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* Corresponding author.
E-mail addresses: nagata@chem.cst.nihon-u.ac.jp, ocean_nagata@yahoo.co.jp (Y. Nagata).

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at around 25 °C with a 12-h light/dark photoperiod, produced by 25 W fluorescent light tubes. D-Serine and D-alanine were purchased from Wako Pure Chemicals (Osaka, Japan) and 2,4-dinitrophenylhydrazine was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Preparation of chicken D-serine dehydratase

Chicken D-serine dehydratase (DSD, DDBJ accession number: AB284370) was purified from recombinant *Escherichia coli* BL21 Star (DE 3) (Invitrogen, Carlsbad, CA, USA) cells containing the pET DSD plasmid inserted into a pET100/D-TOPO vector (Invitrogen). The purification of DSD was performed at 4 °C throughout the procedure. The *E. coli* cells were grown on Luria-Bertani (LB) medium at 37 °C, and then suspended in Tris–HCl buffer (pH 8.0) with 100 μM pyridoxal phosphate (PLP) and sonicated (50 W for 60 min). The cell-free extract was centrifuged at 140,000 × *g* for 30 min. Subsequently, 0.5 M NaCl and 20 mM imidazole were added to the supernatant, which was loaded onto a Ni-NTA Superflow (Qiagen) column. After washing the column with buffer A (20 mM Tris–HCl buffer (pH 8.0), 100 μM PLP, 0.5 M NaCl, and 20 mM imidazole), the DSD fraction was eluted with buffer A supplemented with 0.5 M imidazole. The active fraction was dialyzed against buffer B (20 mM potassium phosphate buffer (pH 7.8) and 50 μM PLP), and then loaded onto a Q Sepharose column (GE Healthcare Life Sciences) equilibrated with buffer B. Finally, the DSD was purified to homogeneity by eluting the column with a linear gradient of 0–0.5 M KCl in buffer B.

2.3. Sample preparation

The head, midgut, testis, ovary, silk gland, and Malpighian tubule of silkworm larvae were excised. The midgut typically contained a mixture of partially to completely digested artificial food, which was carefully removed. Four volumes of 50 mM Tris–HCl buffer (pH 8.5) were added to each organ. The organs were minced with scissors, and then homogenized on ice with a glass homogenizer at 1200 rpm for 3 min. The homogenate was clarified by centrifugation at 12,000 × *g* for 10 min at 4 °C: the resulting cell-free extract was used for the assay.

2.4. Enzymatic assay of D-serine

The reaction mixture (100 μL), which contained 10 μL of the cell-free extract, 0.5×10^{-3} U of DSD, 10 μM PLP, and 50 mM Tris–HCl buffer (pH 8.5), was incubated for 10 min at 37 °C in a 96-well polystyrene microtiter plate with a flat bottom (Asahi Technoglass, Tokyo, Japan). The reaction was stopped by adding 20 μL of phenylhydrazine solution (1 mM 2,4-dinitrophenylhydrazine dissolved in 1 M HCl), followed by another 5 min incubation at room temperature. For the control, DSD was added after the addition of phenylhydrazine. Finally, 160 μL of 0.6 M NaOH was added to all wells. After standing at room temperature for 2 min, the absorbance was measured at 450 nm (A_{450}) with a microplate reader (MTP-450, Corona, Ibaragi, Japan). Calibration curves for various amounts of D-serine in 50 mM Tris–HCl buffer (pH 8.5) containing 40 mg/mL (protein concentration) cell-free extracts of the silkworm testis were generated. The amounts of D-serine in the reaction mixtures were calculated from the A_{450} and the calibration curves.

The protein concentrations of the samples were determined by using the Bradford method [19] with bovine serum albumin as the standard.

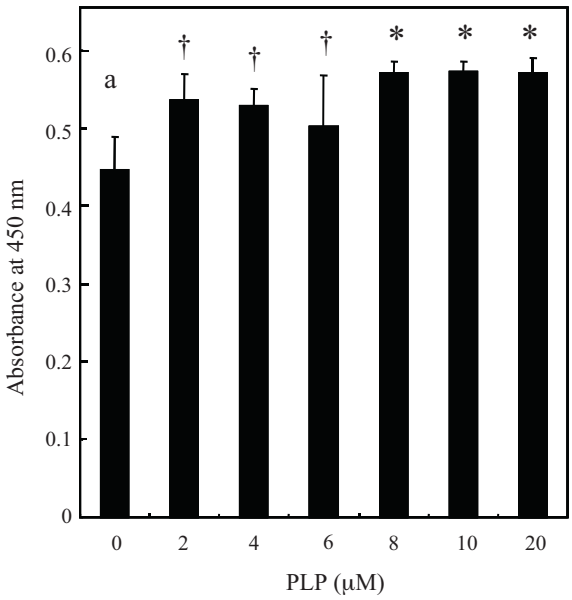

Fig. 1. Effects of PLP on pyruvate formation from D-serine dehydration. Various amounts of PLP were added to 50 mM Tris–HCl buffer (pH 8.5) containing 1.0 mM D-serine and 0.5×10^{-3} U chicken D-serine dehydratase (DSD). The reaction was performed at 37 °C for 10 min. Data are expressed as the mean ± SD of 4 assays. †Statistically significant difference between the value for no PLP and those for 2, 4, or 6 μM PLP ($p < 0.05$). *Statistically significant difference between the value for no PLP and those for 8, 10, or 20 μM PLP ($p < 0.002$).

3. Results and discussion

3.1. Determination of assay conditions

Because DSD is a PLP-dependent enzyme, we determined the optimal concentration of PLP for the assay (Fig. 1). The addition of 2–6 μM and 8–20 μM PLP increased the rate of pyruvate formation by approximately 17% and 28%, respectively. PLP concentrations above 10 μM did not increase the absorbance relative to that of 10 μM PLP. Hence, we added 10 μM PLP to the reaction mixtures in the assay. Although we have found that chicken DSD requires Zn^{2+} for the enzymatic activity (our unpublished results), and other groups have found the same for yeast DSD [20], 1 mM EDTA did not affect the reaction in this assay. Moreover, the addition of 100 μM Zn^{2+} ($ZnSO_4$) to a reaction mixture containing no EDTA did not affect the reaction rate (data not shown).

Then, we investigated the relationship between the amount of DSD in the reaction mixture and the maximum absorbance at 450 nm (Fig. 2). Higher enzyme concentrations typically led to greater absorbances when D-serine concentrations were above 10 μM. No differences in absorbance were observed among reactions containing 0.1×10^{-3}, 0.5×10^{-3}, and 1.0×10^{-3} U DSD, when <10 μM D-serine was present in the reaction mixtures. In this range of D-serine concentration, the reaction rate was linearly dependent upon DSD. Therefore, we determined that the appropriate amount of DSD for the reaction mixture was 0.5×10^{-3} U.

3.2. Quantification limits and linearity

Fig. 3 shows the calibration curves for D-serine that was catabolized with DSD in Tris–HCl buffer (pH 8.5). No cell-free extract was added to these reactions. Values have been normalized by subtracting the systemic absorbance shown by control experiments. In this way, a statistically significant ($p < 0.05$) absorbance was found

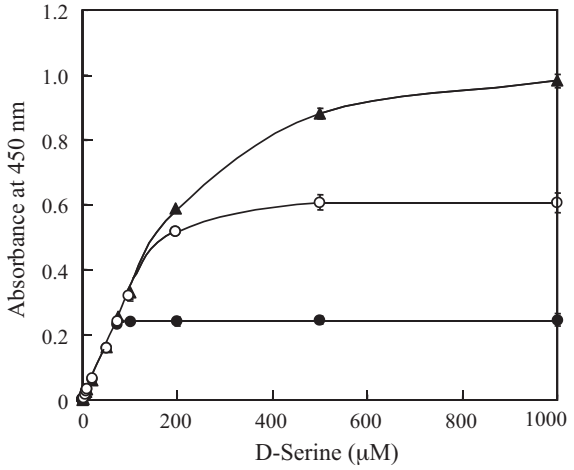

Fig. 2. Absorbance at 450 nm of varied amounts of DSD. The reaction was performed in 50 mM Tris–HCl buffer (pH 8.5) with 10 μM PLP and 0.1×10^{-3} U (●), 0.5×10^{-3} U (○), or 1.0×10^{-3} U (▲) DSD at 37 °C for 10 min. The absorbance of the reaction without DSD was subtracted from the absorbance of the reaction with DSD. Data are expressed as the mean ± SD of 4 assays. When not shown, the error bars were smaller than the symbol.

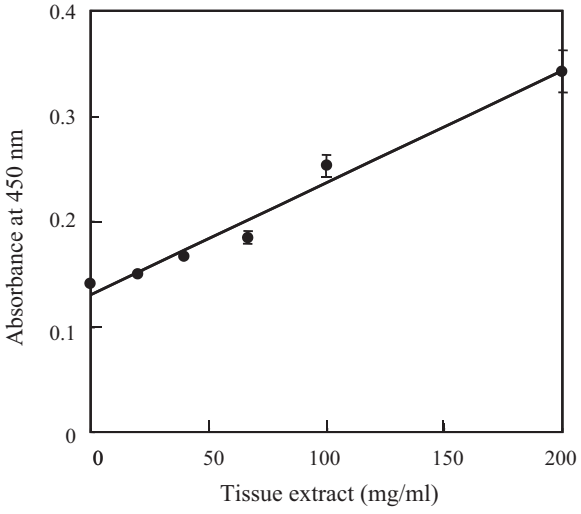

Fig. 4. Absorbance of cell-free extracts relative to their dilution factor. Testes from fifth instar larvae of silkworm were homogenized with an equal volume of Tris–HCl buffer (pH 8.5), and the cell-free extract was diluted with various volume of the buffer. To the diluted extracts, phenylhydrazine was added and incubated for 10 min. The absorbances were measured after the addition of NaOH, as described in Section 2. Data are expressed as the mean ± SD of 4 assays. When not shown, the error bar was smaller than the symbol.

for 200 pmol (2 μM) of D-serine, indicating the lower sensitivity threshold of our assay. The relationship between A_{450} (y) and the amount of D-serine (x) is expressed by the equation, $y = 0.0328x$, and the correlation coefficient (r^2) was 0.999. The K_m value for the enzymatic reaction was 0.149. We examined the effects of adding a large amount of L-serine (20 mM, approximately 10^4-fold higher concentration than D-serine) to the reaction mixture. As clearly shown in Fig. 3B, the additional L-serine had no significant effect on the reaction. The equation for the curve is $y = 0.0323x$ and $r^2 = 0.997$.

Fig. 4 shows the inherent absorbance at 450 nm of the tissue extract itself, across several dilutions. The silkworm tissue (testis) extract contains D-serine, as well as high concentrations of proteins, lipids, free amino acids, and organic acids that may react with phenylhydrazine. The absorbance increased with extract protein concentration. Without any extract, an A_{450} of 0.13 was found, likely due to the presence of DSD and phenylhydrazine that have absorbance at 450 nm.

Then, we investigated the contribution of the tissue extract (40 mg/mL) on the calibration curves for D-serine (Fig. 5). The curve is represented by the equation $y = 0.0322x + 0.167$, with $r^2 = 0.998$. The K_m value for the reaction was 0.145. These values are very close to those obtained in the absence of the extract (Fig. 3). Therefore, it appears that the addition of cell-free extracts does not affect the reaction, and that D-serine levels can be determined accurately, even in the presence of cell-free extracts. Moreover, the amounts of D-serine in various tissues can be determined without generating new calibration curves for each tissue. This is because, in the presence of cell-free extract from rat cerebra, the D-serine reaction curve fit to the very similar equation, $y = 0.0316x$, $r^2 = 0.996$. The K_m value for the reaction was 0.137, indicating the affinity of DSD toward D-serine does not vary among the silkworm extract, the rat extract, or the Tris–HCl calibration buffer.

3.3. Intrinsic amounts of free D-serine in silkworm

We assayed the amounts of D-serine in the midguts of fifth instar larvae, spinning larvae, and pupae of the silkworm by using the

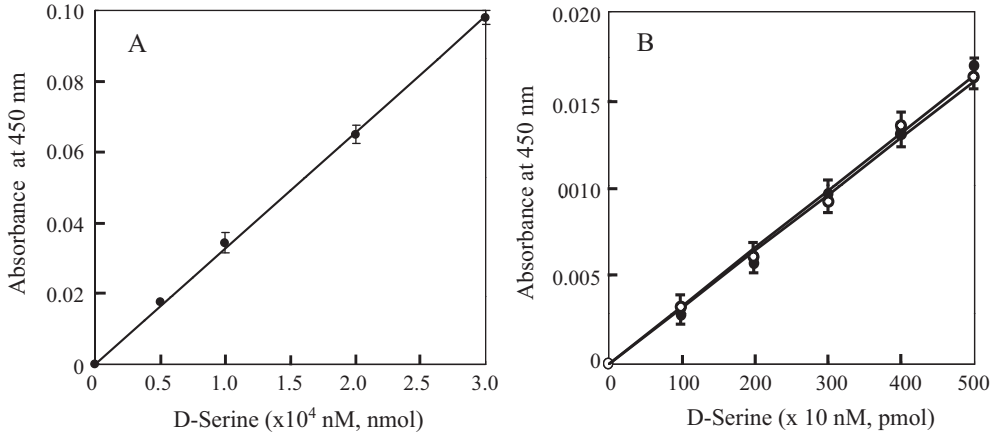

Fig. 3. Calibration curve of D-serine in buffer. (A, B, ●) Various amounts of D-serine dissolved in 50 mM Tris–HCl buffer (pH 8.5) containing 10 μM PLP were digested with 0.5×10^{-3} U DSD at 37 °C for 10 min. After adding phenylhydrazine, the formation of phenylhydrazone was measured by A_{450}. The control value, the absorbance of the reaction in which DSD was added after phenylhydrazine, was subtracted from the absorbance of the reactions with DSD. (B, ○) Calibration curve of D-serine in the buffer containing 20 mM L-serine. Data are expressed as the mean ± SD of 4 assays. When not shown, the error bar was smaller than the symbol.

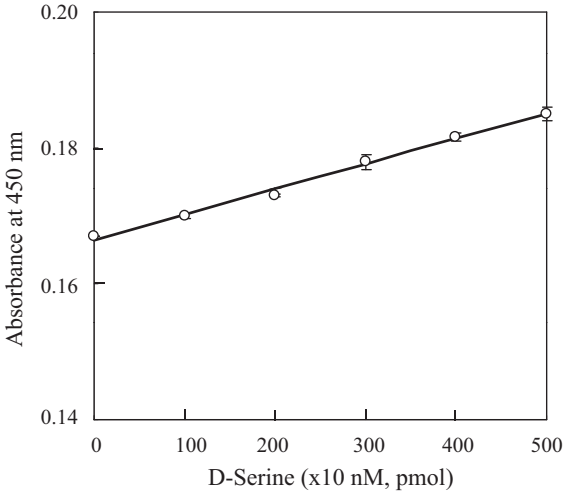

Fig. 5. Calibration curves of D-serine in cell-free extracts of silkworm testes. Various amounts of D-serine (dissolved in 50 mM Tris–HCl buffer (pH 8.5) containing the cell-free extract (40 mg/mL protein) and 10 μM PLP) were digested with 0.5×10^{-3} U DSD at 37 °C for 10 min. After adding phenylhydrazine, the formation of phenylhydrazone was measured by A_{450}. Data are expressed as the mean ± SD of 4 assays. When not shown, the error bar was smaller than the symbol.

presently described method as well as by HPLC, and compared the values obtained by the different methods (Fig. 6). Because the values coincided with each other, the reliability of our method was confirmed. We then investigated changes in D-serine concentration in the silkworm midgut with age (Fig. 7). We found that the D-serine concentration gradually increases until day 3 of pupation, markedly increases before day 5, and then decreases after day 5.

The amounts of D-serine in the various organs of silkworm larvae are shown in Fig. 8. The silkworm larvae at day 3 of the fifth instar were found to contain high concentrations of D-serine in the midgut (509 nmol g^{-1}), ovary (434 nmol g^{-1}), and testis (353 nmol g^{-1}).

3.4. Advantages of the method

DSD has been purified from *E. coli* [21], the yeast *Saccharomyces cerevisiae* [20], and chicken. The substrate specificity of these enzymes is summarized in Table 1. Chicken DSD is more useful for our assay than yeast or bacterial DSDs, as yeast DSD has a higher affinity toward D-threonine than D-serine, and bacterial DSD shows considerable activity toward D-threonine, as shown by V_{max} values. Although chicken DSD reacts with D-threonine, 1 mM D-threonine gave A_{450} = 0.002, as compared to A_{450} = 0.0025 for 1 μM D-serine (3 orders of magnitude less). Usually, the D-threonine content in organisms is low. Hence, it seems unlikely that D-threonine concentrations significantly influence our assay.

In a similar method that uses yeast DSD to determine D-serine concentrations [22], the absorbance of phenylhydrazone (formed from a reaction between pyruvate and 2,4-dinitrophenylhydrazine) was 2.5-fold lower than that observed in our assay: the lower limit of D-serine detection was 20 μM, as compared to 2 μM in our method. Recently, an improved method for detecting sub-micromolar concentrations of D-serine was reported [23]. In this method, hydrogen peroxide is detected fluorospectrophotometrically with a fluorescent dye: this technique required several reagents and equipment for the detection of fluorescent signals. The

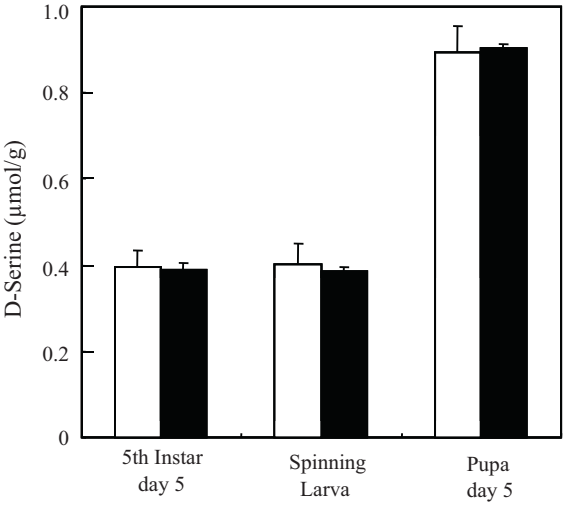

Fig. 6. Comparison of the values for D-serine concentration obtained with the presently described method (□) and HPLC (■). Midguts from fifth instar larvae, spinning larvae, and day 5 pupa were homogenized and the extracts were prepared as described under Experimental. Data are expressed as the mean ± SD of 4 assays.

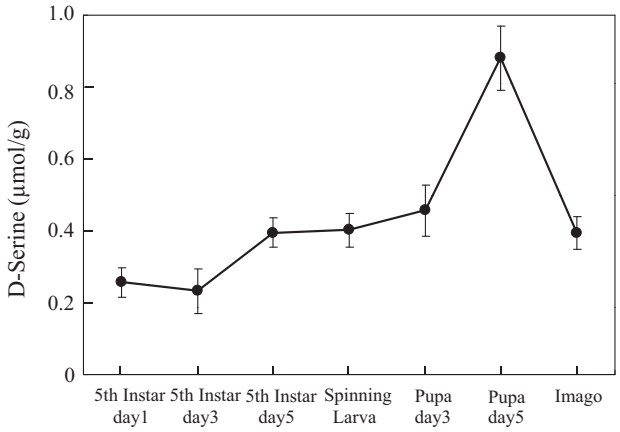

Fig. 7. D-Serine concentration in silkworm midgut with developmental age. The extracts of midguts were prepared as described under Experimental. Data are expressed as the mean ± SD of 4 assays.

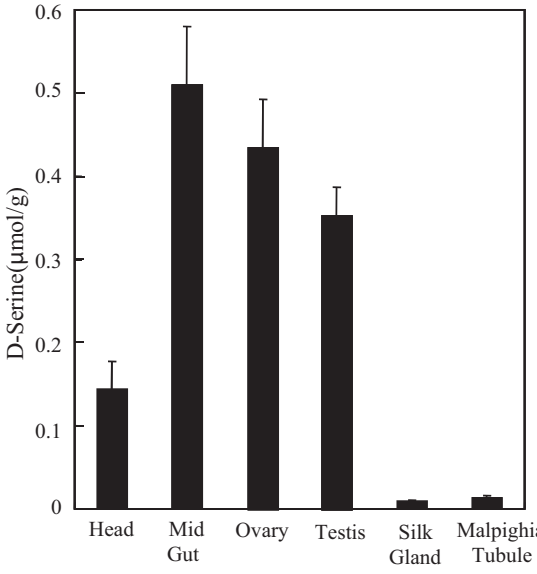

Fig. 8. Intrinsic D-serine concentration (nmol g^{-1} wet weight) in various organs from of fifth instar day 3 silkworms. The reaction mixture (100 μL) containing 10 μL of approximately 60 mg/mL cell-free extract of each organ, 0.5×10^{-3} U DSD, 10 μM PLP, and 50 mM Tris–HCl buffer (pH 8.5) was incubated at 37 °C for 10 min. Data are expressed as the mean ± SD of 4 assays.

Table 1
Substrate specificity of DSD.

	Chicken[a]		Yeast[b]		*E.coli*[c]	
	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	V_{max} (mmol/min/mg)
D-Serine	0.131	0.811	0.39	13.1	1.3	300
D-Threonine	0.312	0.051	0.13	0.43	3.2	50
L-Serine	12.3	0.197	ND	ND	ND	ND

ND: not detected.
[a] Tanaka et al. [17].
[b] Ito et al. [20].
[c] Dupourque et al. [21].

method presented here, however, requires only standard test tubes and a spectrophotometer, or a microtiter plate and a microplate reader. In addition, all procedures, including preparation of tissue extracts, can be completed in only 1 h, while the HPLC method [18] takes 4 days.

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