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A highly sensitive enzymatic assay for D- and total serine detection using D-serine dehydratase from *Saccharomyces cerevisiae*

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ABSTRACT

D-Serine acts as a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor, an excitatory glutamate receptor in the mammalian brain that is thought to be involved in higher brain functions such as memory and study. A relationship between the concentration of D-serine in cerebrospinal fluid and neurological disorders such as schizophrenia and amyotrophic lateral sclerosis has been hypothesized. A simple and accurate D-serine assay system would be useful in the study and diagnosis of these diseases. Previously, we developed an enzymatic assay of D-serine using D-serine dehydratase from *Saccharomyces cerevisiae*, lactate dehydrogenase and NADH. Although this method can detect 10 μM D-serine, a 10-fold increase in sensitivity is required for the assay to be useful for the study and diagnosis of neurological disorders. In this study, we increased the sensitivity of this assay to detect submicromolar concentrations of D-serine. In the new assay, D-serine dehydratase converts D-serine to pyruvate, which is in turn oxidized by pyruvate oxidase. Then, in the presence of horseradish peroxidase, hydrogen peroxide formed during the oxidation converts 10-acetyl-3,7-dihydroxy-phenoxazine (Amplex® Red) to resorufin, which exhibits a strong fluorescence. We show that this improved assay can be used to determine the concentration of D-serine in calf serum.

1. Introduction

With the recent development of analytical methods, several free D-amino acids have been found to have important physiological functions in eukaryotes, including mammals. For example, D-serine modulates brain functions by acting as a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor in the mammalian forebrain [1,2]. Dysfunction of the NMDA receptor may be associated with neurodegenerative disorders, such as schizophrenia and Alzheimer's disease [3]. Interestingly, the ratio of D-serine to the total (D- + L-) serine concentration in the cerebrospinal fluid of schizophrenics [4] and in the serum of Alzheimer's disease patients [5] is significantly lower than in control subjects. This leads the hypothesis that a lack of D-serine lowers the function of the NMDA receptor and results in the neuronal disorders. Excess D-serine also may be related to neuronal disease. For example, in the mouse model of amyotrophic lateral sclerosis (ALS), the concentration of D-serine in the cerebrospinal fluid increase as the disease progresses [6]. This may be the result of glutamate toxicity to neurons due to the overactivation of the NMDA receptor by excess D-serine. D-Serine is also found in human urine [7], at a much higher concentration than in mouse or rat urine [8,9]. However, the physiological function of urinary D-serine is not known.

A rapid and simple method for assaying D-serine would be useful for studying its physiological role and clinical importance. D-Amino acids are typically detected by high performance liquid chromatography (HPLC), after they are derivatized to fluorescent diastereomers [10,11]. This method is highly sensitive and can determine the presence of D- and L-amino acids simultaneously, but it is time consuming and requires proficiency and expensive equipments.

As a result, we have developed an inexpensive, high-throughput enzymatic assay method of D-serine [12]. This assay of D-serine uses D-serine dehydratase from *Saccharomyces cereviciae* (DsdSC), which catalyzes the dehydration of D-serine to pyruvate and ammonia [13]. DsdSC is an eukaryotic D-serine dehydratase that is both structurally and evolutionally distinct from the bacterial enzyme discovered by Snell and co-workers [14,15]. Unlike the bacterial enzymes showing a little activity towards L-serine, DsdSC acts on D-serine dominantly [13]. Previously we took advantage of the strict substrate specificity of DsdSC to develop our enzymatic assay of D-serine. In the assay, DsdSC converts D-serine to pyruvate, which is detected by a reduction in the concentration of NADH in the coupling reaction with lactate dehydrogenase (LDH) [12]. This assay

Abbreviations: ALS, amyotrophic lateral sclerosis; APF, aminophenyl fluorescein; DsdSC, D-serine dehydratase of *Saccharomyces cerevisiae*; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; LDH, lactate dehydorogenase; NMDA, *N*-methyl-D-asparatate; PLP, pyridoxal 5′-phosphate; XO, xylenol orange.

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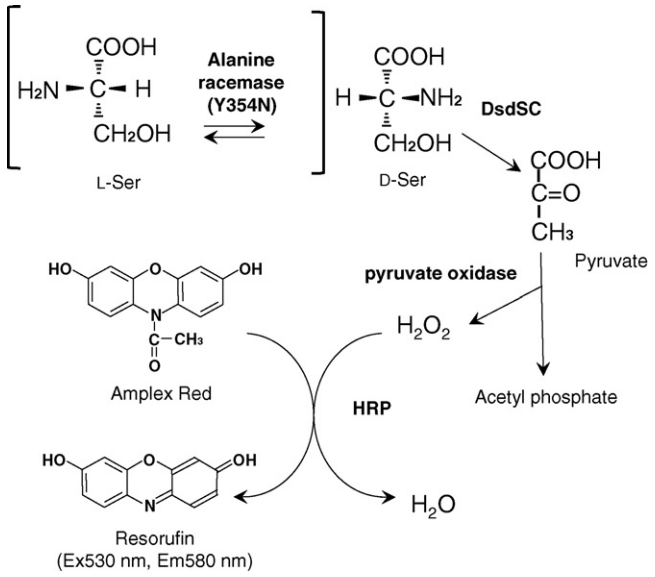

Fig. 1. D-Serine and total (D- + L-) serine assay with DsdSC. The enzymatic assay for D-serine utilizes three enzymes; DsdSC, pyruvate oxidase and HRP, and the fluorescent reagent Amplex® Red. DsdSC converts D-serine to pyruvate, which is oxidized by pyruvate oxidase. Hydrogen peroxide that is formed during the oxidation is detected with HRP and Amplex® Red. D-Serine concentrations can be determined from the fluorescence intensity of the resorufin product. To measure total (D- + L-) serine content, mutant alanine racemase (mAR/Y354N) catalyzing serine racemization can be coupled to the D-serine assay.

can also be used for the determination of total (D- + L-) serine by coupling it with the mutant alanine racemase (mAlaR/Y354N), which exhibits increased serine racemase activity [16]. L-Serine concentration could be obtained by the subtraction of the D-serine concentration from the total serine concentration [12]. With the LDH-coupling method, we can detect 10–250 μM D-serine or total serine, which is sufficient to detect D-serine in human urine [12].

However, since the concentration of D-serine in human cerebrospinal fluid and human serum is around 2–5 μM [4–6], a 10-fold higher sensitivity is required for the application of the assay to the study and diagnosis of neurological disorders by detecting D-serine in the cerebrospinal fluid or in serum. In this study, we increased the sensitivity of the assay by using pyruvate oxidase, horseradish peroxidase (HRP), and a fluorescence reagent, 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red) (Fig. 1). In the new assay, D-serine is converted to pyruvate with DsdSC, followed by the oxidization of pyruvate with pyruvate oxidase to produce hydrogen peroxide. Finally, in the presence of HRP, the hydrogen peroxide oxidizes Amplex® Red to resorufin, which exhibits a strong fluorescence. Using this assay, we can detect submicromolar concentration of D-serine, and determine the D- and total serine concentrations in calf serum.

2. Experimental

2.1. Preparation of enzymes

In the previous studies, we used the DsdSC with an *N*-terminal His_6 Tag [12,13]. However, we found that the DsdSC with a C-terminal His_6 Tag showed similar properties and more efficient productivity as compared to those of the *N*-terminal His-tagged enzyme [Ito et al. manuscript in preparation]. Therefore, we used the *C*-terminal His-tagged DsdSC in the present study. The expression vector of the *C*-terminal His-tagged enzyme was constructed as follows. Briefly, the *YGL196W* gene (*DSD1*, gene symbol for *Saccharomyces* Genome Database) was amplified from *S. cerevisiae* BY4742 using polymerase chain reaction (PCR) and ligated into the pET15b vector as described previously in Ref. [13]. The resultant plasmid encoding the DsdSC with a *C*-terminal His_6 tag was cloned into *Escherichia coli* KRX cells (Promega, Madison, WI, USA). DsdSC was overexpressed and then purified to homogeneity with the Ni-chelating, anion exchanger DEAE-Toyopearl, and gel permeation chromatography [13].

Geobacillus stearothermophilus alanine racemase (AR) with an asparagine mutation at Tyr354 (referred to as "mAR/Y354N" hereafter) catalyzes the racemization of L-serine at a rate of 80 times that of the wild-type enzyme [16]. To construct the mAR/Y354N gene, the AR gene with *N*-terminal His_6 tag and thrombin cleavage sequences in the pET15b vector was mutated with the QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara CA, USA) using the complementary primer pairs, 5′-GGAAACGATCAACAACGAAGTGCCTTGC-3′ (forward) and 5′-GCAAGGCACTTCGTTGTTGATCGTTTCC-3′ (reverse). The mutated plasmid was cloned into *E. coli* BL21(DE3) cells to overexpress mAR/Y354N. Subsequently, mAR/Y354N was purified to homogeneity with heat treatment, Butyl-Toyopearl, DEAE-Toyopearl and gel permeation chromatography [17].

Pyruvate oxidase and horseradish peroxidase (HRP) were purchased from TOYOBO (Tokyo, Japan) and Sigma–Aldrich (St. Louis, MO, USA), respectively.

2.2. Reagents

Amino acids were purchased from Wako (Osaka, Japan). The optical purity of the D- and L-serine used in this study was over 99%. His-Bind resin was bought from Novagen (EMD Bioscience, San Diego, CA, USA). DEAE-Toyopearl 650 M and Butyl-Toyopearl 650 M were obtained from Tosoh (Tokyo, Japan). Amplex® Red was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.3. Equipment and analytical conditions

Fluorescence measurements were made using a RF5399-PC fluorospectrophotometer (Shimadzu, Kyoto, Japan) with a 3 mm cell, or with a Fluoroskan Ascent microplate fluorometer (Thermo Electron Corporation, Vantaa, Finland). To prepare D- and L-serine for the HPLC analysis, the amino acids were derivatized to fluorescent diastereomers with *N*-tertbutyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde (Boc-L-cysteine/OPA) as described previously in Refs. [10,11]. The HPLC analysis was performed on a Shimadzu SLC-10A system (Shimadzu) equipped with a COSMOSIL C-18 column (Nacalai Tesque, Kyoto, Japan). The derivatized amino acids were separated by a linear gradient of 0–60% mobile phase B (47% acetonitrile in a 0.1 M acetate buffer, pH 6.0) in mobile phase A (7% acetonitrile in a 0.1 M acetate buffer, pH 6.0) for 120 min at a flow rate of 0.8 ml/min at room temperature. Elution was monitored with an RF-10A fluorescence detector (Shimadzu) with excitation and emission wavelengths of 344 and 443 nm, respectively.

2.4. Enzymatic assay of D- and total serine

Upon completion of the enzymatic assay of D-serine with DsdSC, the amount of hydrogen peroxide formed from the oxidation of pyruvate with pyruvate oxidase was measured with Amplex® Red and HRP. For the analysis of D-serine, the reaction mixture consisted of 100 mM sodium phosphate buffer (pH 7.5), 20 μM pyridoxal phosphate (PLP), D-serine (sample), 0.1 unit or no DsdSC, 0.4 unit pyruvate oxidase, 1 unit of HRP and 40 μM Amplex® Red, in a final volume of 250 μl. For the analysis of total serine, 0.6 unit of mAlaR/Y354N was added to this reaction mixture.

One unit of DsdSC is defined as the amount of the enzyme converting 1 μmol of D-serine to pyruvate per minute at 30 °C with 10 mM D-serine. One unit of mAlaR/Y354N was defined as the amount of the enzyme forming 1 μmol of D-serine per minute at 37 °C with 10 mM L-serine. The reaction was started by the addition of DsdSC and completed by the incubation at 37 °C for 30 min. Finally, the fluorescence intensity was measured with excitation and emission wavelengths of 530 and 580 nm, respectively.

2.5. Enzymatic assay of D- and total serine in calf serum

To demonstrate the usefulness of the enzymatic assay, we measured the D- and total serine concentrations in calf serum. Calf serum was obtained from Thermo (Melbourne, Australia). It was filtered with an Amicon Ultra-15 (Millipore, Billerica, MA) to remove proteins, and then treated with borohydride to removal endogenous pyruvate using the following procedure. $NaBH_4$ (1 M) was diluted 100-fold into filtrated serum, and the solution was incubated for 30 min at 30 °C. Then, to remove the unreacted $NaBH_4$, 0.2 M HCl was added (1% of the volume), and the solution was incubated at 30 °C for another 30 min. The resulting serum solution was diluted prior to using it in the enzymatic serine assay. The D- and total serine concentrations in the serum were obtained from the calibration curve for D- and L-serine in the serum solution (see Fig. 4). The mean value (y) of the difference in fluorescence intensity between samples with or without DsdSC was plotted against the amount of D- or L-serine added (x). Then a line, $y = ax + b$, was fit to the data. The slope (a) is the fluorescence intensity per unit concentration of D- or L-serine, and the y-intercept (b) is the fluorescence intensity per unit concentration of D- or total serine naturally occurring in the serum. Therefore, the concentration of D- or total serine in the assay mixture is calculated from the ratio b/a.

3. Results and discussion

3.1. Comparison of three pyruvate detection methods using Amplex® Red, aminophenyl fluorescein and xylenol orange

In this study, we attempted to increase the sensitivity of the enzymatic assay of D-serine with DsdSC [12] more than 10-fold to make it useful for clinical studies of neuronal diseases. One way to increase the sensitivity is to improve the detection of pyruvate formed from D-serine. We compared the three different reagents for detecting pyruvate: Amplex® Red, aminophenyl fluorescein (APF), and xylenol orange (XO). All of these reagents detect the hydrogen peroxide that is formed during the reaction between pyruvate and pyruvate oxidase. APF is converted by a hydroxyl radical to fluorescein, which fluoresces at 538 nm. XO forms a complex with Fe(III) released during the Fenton reaction with Fe(II) and hydrogen peroxide. The resulting XO–Fe(III) complex absorbs at 580 nm. All three reagents can be used to detect 1 μM D-serine in a pure solution (data not shown). However, the presence of calf serum in the assay mixture reduced the sensitivities of APF and XO, even in the absence of other proteinaceous components (data not shown). Among the three reagents, Amplex® Red method was the least affected by calf serum. Therefore, we used the Amplex® Red reagent in the enzymatic assay of D-serine with DsdSC.

3.2. Enzymatic assay of D- and L-serine with Amplex® Red

D- And L-serine were detected by using the hydrogen peroxide formed from the pyruvate oxidase reaction to convert Amplex® Red to fluorescent resorufin. There is a linear relationship between the fluorescence intensities and the concentrations of D- or L-serine from 0.1 to 5.0 μM (Fig. 2). The correlation coefficients for D- and L-serine were very similar, even when pyruvate or hydrogen peroxide was substituted for serine in the assay (data not shown). These results suggest that both D-serine dehydratase and pyruvate oxidase catalyzed substrates nearly completely under the assay conditions.

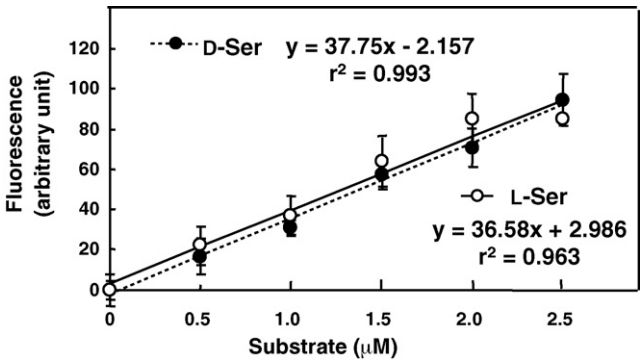

Fig. 2. Calibration curves for D- and L-serine standards using the enzymatic assay. Fluorescence intensity at 580 nm is plotted versus the concentration of L-serine (open circle) and D-serine (closed circle). The y-axis shows the difference in the fluorescence intensity between that obtained with DsdSC and that obtained without DsdSC. Average values of three measurements were plotted. Other conditions are described in the text.

3.3. Removal of endogenous pyruvate from calf serum

We attempted to apply the enzymatic D-serine assay system to the measurement of the D-serine concentrations of the calf serum. Addition of the serum in the assay mixture increased the background and decreased the sensitivity of the assay system. Such unfavorable effects of the serum were partially eased by the elimination of the serum protein by the filtration with an Amicon Ultra-15 (data not shown). However, a high background signal was still observed when the filtered calf serum was added to the assay system. Specifically, the fluorescence intensity (Fig. 3, bar B) of the reaction mixture containing the calf serum, pyruvate oxidase, HRP, and Amplex® Red was about two times greater than that for the reaction mixture without pyruvate oxidase (bar A). The closed part of the bar B, which shows the difference in fluorescence intensity between A and B, is most likely due to endogenous pyruvate in the calf serum. To eliminate this endogenous pyruvate, the calf serum was pretreated with $NaBH_4$, which reduces pyruvate to lactate. The closed part of bar D, which indicates the difference in fluorescence intensity between C and D, is most likely due to residual pyruvate in the $NaBH_4$-treated calf serum. These results indicate that pretreatment of calf serum with $NaBH_4$ decreased the concentration of endogenous pyruvate by 83%. In Fig. 3, bars E and F show the fluorescence intensity of the reaction mixtures containing DsdSC, pyruvate oxidase and HRP, with either the untreated or $NaBH_4$-treated serum, respectively. The closed parts of bars E and F show the difference in fluorescence intensity between E and B, and that between F and D, respectively. These differences correspond to the amount of endogenous D-serine in the untreated and $NaBH_4$-treated serum. Since the areas of the closed parts of bars E and F are nearly equal, it is unlikely that the pretreatment with $NaBH_4$ has significant effect on the D-serine assay.

3.4. Enzymatic assay of D- and total serine in the calf serum

Despite the pretreatments, i.e., the membrane filtration and the $NaBH_4$ reduction, the treated calf serum still affected the sensitivity of the D-serine assay system. Slope of each D- and L-serine

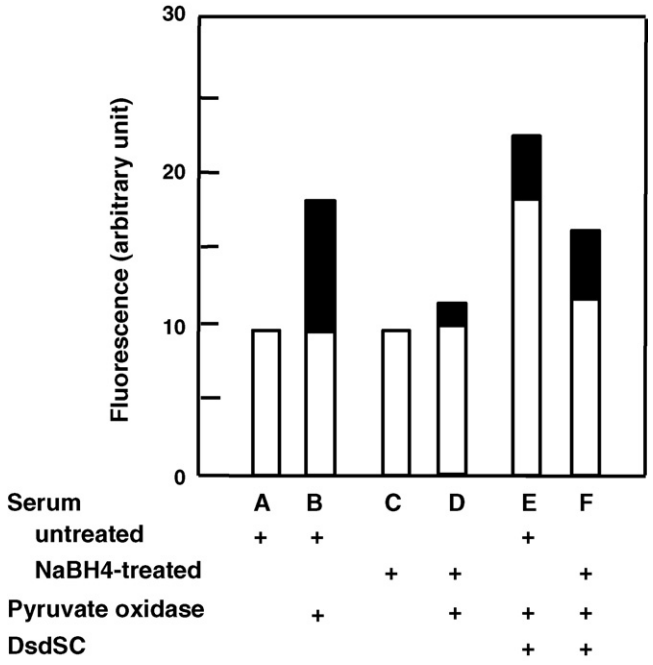

Fig. 3. Elimination of endogenous pyruvate in calf serum with $NaBH_4$. The reaction mixture (250 μl) contained 0.1 M potassium phosphate buffer (pH 7.5), 20 μM PLP, 40 μM Amplex® Red, 1 unit of HRP and 25 μl of the untreated (A, B, E) or $NaBH_4$-treated (C, D, F) calf serum. In addition to these ingredients, the reaction mixtures for B and D included 0.4 unit of pyruvate oxidase, and E and F included 0.4 unit of pyruvate oxidase and 0.1 unit of DsdSC. After the mixtures were incubated at 37 °C for 30 min, fluorescence intensities were measured with excitation and emission wavelengths of 530 and 580 nm, respectively.

calibration curve changed depending on the amount of the added serum (see Fig. 4). The reason is not clear, but the serum probably affects the steps after the DsdSC reaction of the present assay system (Fig. 1), because the serum did not inhibit our former assay system consisting of the mAlaR/Y354N, DsdSC and lactate dehydrogenase [12]. To perform the accurate serine assay, we obtained the calibration curve of D- or L-serine for each measurement (Fig. 4). For the assay of the total serine in the serum, the calibration curve for L-serine was obtained (Fig. 4B), because about 95% of serine in the calf serum is L-enantiomer (see Table 1). As shown in Fig. 4, there is a linear relationship between the fluorescence intensities and the concentration of D-serine (Fig. 4A) or L-serine (Fig. 4B) in the presence of the pretreated calf serum. The linear equations for the D-serine and total serine assays are $y = 17.456x + 10.19$ ($r^2 = 0.999$) and $y = 30.508x + 7.5954$ ($r^2 = 0.992$), respectively. Difference in the slopes reflected the difference in the amount of the added calf serum. The serine concentrations in the assay mixture were calculated by dividing the intercept by the slope. Taking account of the dilution ratio, the D- and total serine concentrations were determined to be 5.84 and 124 μM, respectively. The L-serine concentration determined by subtracting the D-serine concentration from total serine concentration was 119 μM. These values are in close agreement with those obtained by the conventional HPLC method, specifically 5.15 and 108 μM for D- and L-serine, respectively (Table 1).

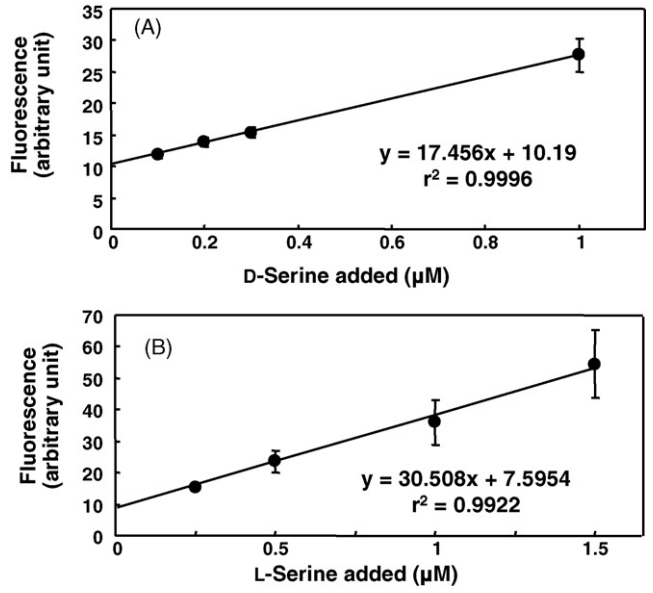

Fig. 4. Determination of D- and total serine concentrations of calf serum with the enzymatic assay. The reaction mixtures for the D-serine assay (A) contained 10% $NaBH_4$-treated calf serum and 0.1–1.0 μM D-serine in a final volume of 250 μl. The reaction mixture for the L-serine assay (B) contained 0.2% $NaBH_4$-treated serum and 0.25–1.5 μM L-serine. Average values of three measurements were plotted. Other conditions are described in the legend of Fig. 2 and the text.

Table 1
Comparison of the D- and L-serine concentrations of the calf serum obtained by the enzymatic assay and the conventional HPLC methods. Values obtained with HPLC method were the average of four measurements. Other conditions are described in the text. Details of the enzymatic assay were described in the legend of Fig. 4.

	Enzymatic assay	HPLC method
L-Serine	119 μM	108 ± 4.73 μM
D-Serine	5.84 μM	5.15 ± 0.200 μM
D/(D + L) Ratio	4.68%	4.55%

As mentioned above, D-amino acids are usually detected by HPLC, after they are derivatized to their fluorescent diastereomers [10,11]. This method is very sensitive and has the advantage of being able to determine the concentrations of multiple D- and L-amino acids simultaneously. However, the HPLC method has a significant disadvantage in the analysis of biological samples. Due to the relative abundance of L-amino acids compared to D-amino acids in organisms, the signals from L-amino acids can mask those from D-amino acids. In order to accurately measure the concentrations of D-serine with HPLC, the sample should be co-chromatographed with a D-serine standard or pretreated with a D-serine deaminase [18]. Recently, it has been shown that two-dimensional HPLC (2D-HPLC) can circumvent this inconvenience by first separating the mixture of D- and L-serine and then separating each enantiomer [19]. However, 2D-HPLC is expensive and impractical for the analyses of many samples. In contrast, our enzymatic D-serine assay using DsdSC is relatively inexpensive and can be used for high-throughput analyses. We anticipate that it will be useful for studying the relationship between D-serine and various neurological disorders.

4. Conclusions

We developed a highly sensitive enzymatic D-serine assay that can detect submicromolar concentrations of D-serine. The key components of the assay are D-serine dehydratase, pyruvate oxidase, horseradish peroxidase, and the fluorescence reagent Amplex® Red. In the assay, D-serine dehydratase converts D-serine to pyruvate, which is then oxidized by pyruvate oxidase. Hydrogen peroxide that is formed from the pyruvate oxidase reaction converts Amplex® Red to resorufin, which exhibits a strong fluorescence. The assay also can be extended to the measurement of total (D- + L-) serine assay by including of the mutant alanine racemase mAR/Y354N. Finally, we showed that this assay could accurately measure D-serine and total serine in calf serum.

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