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Simultaneous measurement of D-serine dehydratase and D-amino acid oxidase activities by the detection of 2-oxo-acid formation with reverse-phase high-performance liquid chromatography ☆

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Abstract

N-methyl-D-aspartate receptors (NMDARs) play critical roles in excitatory synaptic transmission in the vertebrate central nervous system. NMDARs need D-serine for their channel activities in various brain regions. In mammalian brains, D-serine is produced from L-serine by serine racemase and degraded by D-amino acid oxidase (DAO) to 3-hydroxypyruvate. In avian organs, such as the kidney, in addition to DAO, D-serine is also degraded to pyruvate by D-serine dehydratase (DSD). To examine the roles of these two enzymes in avian brains, we developed a method to simultaneously measure DAO and DSD activities. First, the keto acids produced from D-serine were derivatized with 3-methyl-2-benzothiazolinone hydrazone to stable azines. Second, the azine derivatives were quantified by means of reverse-phase high-performance liquid chromatography using 2-oxoglutarate as an internal standard. This method allowed the simultaneous detection of DAO and DSD activities as low as 100 pmol/min/mg protein. Chicken brain showed only DSD activities (0.4 ± 0.2 nmol/min/mg protein) whereas rat brain exhibited only DAO activities (0.7 ± 0.1 nmol/min/mg protein). This result strongly suggests that DSD plays the same role in avian brains, as DAO plays in mammalian brains. The present method is applicable to other keto acids producing enzymes with minor modifications.

Keywords: D-Amino acid oxidase; 3-Hydroxypyruvate; 3-Methyl-2-benzothiazolinone hydrazone; D-Serine; D-Serine dehydratase; HPLC

N-Methyl-D-aspartate receptors (NMDARs)[1] are ubiquitously found in vertebrate brains. NMDARs play critical roles in excitatory synaptic transmission, involving aspects such as learning and memory [1–3 and references cited therein]. Glutamate, the neurotransmitter for the receptor, cannot activate NMDAR in the absence of glycine and/or D-serine [4]. In mammalian brains, glial cells produce D-serine from L-serine by serine racemase [5–8] and degrade it by D-amino acid oxidase (DAO; EC 1.4.3.3) [9]. The regional distribution of D-serine in the brain is the same as that of serine racemase [8] and shows an inverse correlation with that of DAO [9–11]. However, it is not known how the regional expression of these enzymes is controlled to modulate NMDAR activities.

Unlike mammals, birds have another D-serine-degrading enzyme, D-serine dehydratase (DSD; EC. 4.3.1.18), together with DAO [12]. As shown in Fig. 1, DSD catalyzes the dehydration of D-serine to pyruvate, whereas DAO catalyzes the oxidative deamination of D-serine to 3-hydroxypyruvate. D-Serine exists in significant levels in both avian and

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[1] *Abbreviations used:* NMDARs, *N*-methyl-D-aspartate receptors; DAO, D-amino acid oxidase; DSD, D-serine dehydratase; MBTH, 3-methyl-2-benzothiazolinone hydrazone; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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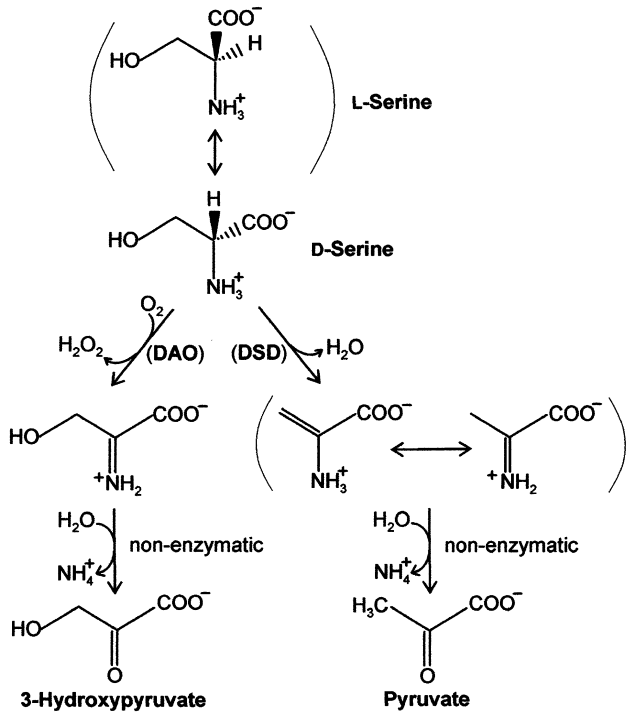

Fig. 1. Reactions catalyzed by D-serine dehydratase and D-amino acid oxidase. The primary enzymatic products undergo rapid nonenzymatic reaction with water to give 2-keto acids and ammonium ions.

mammalian brains [10]. The distribution of DAO and DSD activities in avian brains remains to be revealed. Because mammalian brains contain only DAO for the degradation of D-serine, a comparative study of avian and mammalian brains is important to better elucidate the region-specific modulation of NMDAR activities by D-serine in vertebrate brains.

In the present study, we developed a method to simultaneously determine both DSD and DAO activities by measuring the enzymatic formation of pyruvate and 3-hydroxypyruvate from D-serine. Spectrophotometric determination of aldehydes and keto acids with 3-methyl-2-benzothiazolinone hydrazone (MBTH) has been well established [13,14]. The chromatographic behavior of MBTH–azines of various aldehydes has also been examined in detail by Chiavari and Facchini [15]. On the basis of these previous studies, we first developed a high-performance liquid chromatographic quantification method for keto acids with precolumn MBTH derivatization. Using this method, we found that chicken brain contains only DSD activities. Because mammalian brains contain only DAO activities, this result has important evolutionary implications for the degradation of D-serine in vertebrate brains.

Materials and methods

Materials

Sodium pyruvate and 2-oxoglutaric acid were purchased from Nacalai Tesque (Kyoto, Japan). 3-Hydroxypyruvic acid was from Sigma. D-Serine, D-alanine, and hydroxylamine were from Wako Pure Chemical Industries (Osaka, Japan). 3-Methyl-2-benzothiazolinone hydrazone hydrochloride was from Aldrich. Catalase was obtained from Boehringer Mannheim. All other chemicals were of analytical grade.

Male rats (Sprague–Dawley, 6 weeks old, 200–250 g) were obtained from Japan Clea (Tokyo, Japan). Chicken organs (male White Leghorn, *Gallus domesticus*) were from a local slaughter house and kept at about −35 °C until use.

Preparation of organ homogenates

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and perfused with 10 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl [9,10]. Then, the organs were removed and washed with buffer. Each organ was homogenized on ice with 9 volumes of 20 mM Tris–HCl (pH 7.6) using a glass homogenizer. All these procedures were approved by the animal care committee of our university. The chicken organs were homogenized using the same method as described above.

Assay for DSD and DAO

Reactions were performed in 1.5-mL polypropylene tubes with a final assay volume of 200 μL. Reactions were carried out at 37 °C in 50 mM Tris–HCl, pH 7.6. A typical reaction mixture contained 50 mM D-serine and 20 μg catalase. The reaction was initiated by the addition of 2–10 μL of the organ homogenates and stopped by the addition of 40 μL of 12.5% trichloroacetic acid (TCA) after 0–60 min incubation. We added 10 μL of 1 mM 2-oxoglutaric acid (10 nmol) to each TCA-stopped reaction mixture as an internal standard. Then, the solutions were centrifuged at 5700*g* for 5 min. Aliquots of the supernatants (100 μL each) were transferred into 1.5-mL polypropylene tubes and treated with MBTH as follows according to the method described by Soda [14] with slight modifications. The supernatants were mixed with 200 μL of 1 M sodium acetate buffer (pH 5.0) and 80 μL of 0.1% MBTH (aqueous solution). The mixtures were incubated at 50 °C for 30 min and then allowed to cool to room temperature. Then 5 μL of each aliquot of the MBTH-derivatized samples was applied to a Cosmosil 3C18 column (4.6 × 100 mm; Nacalai Tesque). The column was preequilibrated with 20% acetonitrile in water, which contained 0.1% trifluoroacetic acid (TFA). The elution of the azines was carried out at 25 °C using a linear gradient of acetonitrile (from 20 to 90% over 8 min) in the presence of 0.1% TFA. The flow rate was 1.2 mL/min, and the absorbance at 350 nm was continuously monitored. A Tosoh HPLC system (Tokyo, Japan), incorporating DP-8020 pumps, a PX-8020 controller, and a Shimadzu SPD-10Avp detector, was used in this study. The peaks were integrated using a Smart Chrom data processor (Kya Tech Corp., Tokyo, Japan).