# Utilization of Kynurenic Acid Produced from D-kynurenine in an *in Vitro* Assay of D-Amino Acid Oxidase Activity

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(Received January 29, 2010; Accepted March 17, 2010)

D-2-Amino-4-(2-aminophenyl)-4-oxobutanoic acid (D-kynurenine) was used to assay D-amino acid oxidase (DAAO, EC 1.4.3.3) activity. D-Kynurenine is enzymatically converted by DAAO to 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid), which emits fluorescence at 398 nm (excitation wavelength 251 nm) in the presence of zinc ions. Using Dkynurenine as a substrate, fluorescence intensity originating from kynurenic acid can be used to assess DAAO activity. Under enzymatic reaction at pH 8.5 (37°C), the  $K_m$  and  $V_{max}$  of D-kynurenine were 148  $\mu$ M and 5.53  $\mu$ mol/min per mg, respectively. Inhibition of DAAO by a commercial DAAO inhibitor was clearly observed in this assay.

**Key words**—— D-amino acid oxidase, D-kynurenine, kynurenic acid, enzymatic assay, enzymatic inhibitor

## INTRODUCTION

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidative deamination of D-amino acids to produce their corresponding  $\alpha$ -keto acids.<sup>1,2)</sup> D-Serine, which is an endogenous coagonist at the glycine-binding site of *N*-methyl-D-aspartate (NMDA) receptors, is metabolized by DAAO.<sup>3,4)</sup>

The gene encoding DAAO is one of the susceptibility genes for schizophrenia<sup>4)</sup> and is asso-

## -Research Letter -

ciated with hypofunction of the NMDA receptors in the brain. Schizophrenia is a severe psychiatric disease with symptoms that include auditory hallucinations, paranoid or bizarre delusions, and disorganized speech and thinking with significant social or occupational dysfunction.<sup>5)</sup> We previously reported that serum D-serine concentrations were significantly decreased in schizophrenic patients.<sup>6)</sup> In addition, co-administration of D-serine with antipsychotic drugs is effective for the treatment of schizophrenia.<sup>7)</sup> Further, because brain Dserine is metabolized by DAAO, suppression of Dserine metabolism may improve the symptoms of schizophrenia. Thus, a screening assay for a DAAO inhibitor is crucial for the discovery of novel drugs for the treatment of schizophrenia.

Kynurenic acid (KYNA) antagonizes both NMDA receptor function by interacting with the glycine-binding site and  $\alpha_7$  nicotinic acetylcholine receptor activity.<sup>8,9</sup> In the course of our studies, we found that D-kynurenine (D-KYN), a D-amino acid, may be metabolized to KYNA by DAAO *in vivo*.<sup>10,11</sup> We reported that an increase of plasma KYNA concentration was observed in rats after the administration of D-KYN.<sup>10</sup> In addition, a remarkable amount of KYNA was produced in the extracellular fluid of rat prefrontal cortex during D-KYN infusion.<sup>11</sup> Considering these findings, oxidative deamination of the  $\alpha$ -amino group of D-KYN by DAAO may result in the production of KYNA through an intermediate  $\alpha$ -keto acid (Fig. 1).

In the present study, the enzymatic formation of KYNA from D-KYN was investigated *in vitro* by using pig kidney DAAO (pkDAAO). Additionally, we developed a fluorescence assay based on the intrinsic fluorescence property of KYNA, to test whether DAAO could convert D-KYN into KYNA for the screening of DAAO inhibitors.

There have been reports of DAAO inhibitors such as benzoic acid,<sup>12)</sup> 5-methylpyrazole-3-carboxylic acid (MPC; AS057278),<sup>13)</sup> 6-chlorobenzo-[*d*]-isoxazol-3-ol (CBIO),<sup>14, 15)</sup> 3-hydroxyquinoline-2(1 H)-one,<sup>16)</sup> and 5-carboxyfuro[3,2-*b*]pyrrole.<sup>17)</sup> Of these inhibitors, MPC was chosen as an authentic compound for our studies, and the IC<sub>50</sub> value of MPC for pkDAAO was determined. We also examined the inhibition of DAAO by Dalanine, D-serine, and chlorpromazine when D-KYN was used as a substrate.

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Fig. 1. Speculative Metabolic Pathway of D-KYN to KYNA

## MATERIALS AND METHODS

Materials — pkDAAO was obtained from Calzyme Laboratories Inc. (San Luis Obispo, CA. U.S.A.). D-KYN, L-KYN, KYNA, MPC, bovine serum albumin (BSA), and 3-hydroxyanthranilic acid were obtained from Sigma (St. Louis, MO, U.S.A.). FAD was obtained from Nacalai Tesque (Kyoto, Japan). Sodium hydroxide and boric acid were purchased from Wako Pure Chemicals (Osaka, Japan). Zinc acetate dihydrate and D-serine were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore Co. Ltd., Bedford, MA, U.S.A.). All other reagents were of reagent grade and were used without further purification.

DAAO Assay with D-KYN as a Substrate-The enzymatic reaction conditions were optimized by varying the pH of the buffer solution, the concentrations of D-KYN, and the reaction time. For the DAAO assay, 50 µl of 0.05 mg/ml DAAO in 0.2 M borate buffer solution (pH 8.5),  $^{18, 19)}$  50 µl of 200 µM FAD solution, 50 µl of 0.4 mg/ml BSA in 0.2 M borate buffer solution (pH 8.5), and 150 µl of 0.2 M boric acid buffer solution (pH 8.5) were mixed and incubated at 37°C<sup>20, 21)</sup> for 20 min. Then, varying concentrations of D-KYN (50 µl) were added and incubated at 37°C for 30 min. To stop the reaction, 50 µl of 0.6 M hydrochloric acid was added, and the resulting solution was vortexed vigorously. A 100-µl aliquot of the reaction mixture was added to 2000 µl of 0.2 M borate buffer solution (pH 8.5) and 50 µl of 300 mM zinc acetate in H<sub>2</sub>O. The final solution was vortexed and centrifuged at 4000 rpm for 1 min in a KUBOTA 2010 centrifuge (Kubota Corporation, Tokyo, Japan), and the fluorescence of the resulting supernatant was measured by a JASCO FP-6300 fluorescence spectrometer (Jasco Co. Ltd., Tokyo, Japan). The fluorescence intensity of each solution was measured at an excitation wavelength of 251 nm and emission wavelength of 398 nm.

According to the following equation, the  $\Delta F$  value was determined and used to calculate the kinetic parameters,  $K_{\rm m}$  and  $V_{\rm max}$ , by using a

Lineweaver-Burk plot.

$$\Delta F = F - F_0 \tag{1}$$

F and  $F_0$  are fluorescence intensities of the sample and blank sample (a sample treated without D-KYN), respectively.

Standard Curve of KYNA — KYNA was dissolved in 0.2 M borate buffer solution (pH 8.5) to prepare 0.01, 0.02, 0.05, 0.1, 0.15, and  $0.2 \mu g/ml$  KYNA solutions as the standards. Fluorescence intensity of these standards was measured as described above. The standard curve was prepared by plotting fluorescence against KYNA concentrations (n = 3).

Fluorescence Spectra and Mass Spectrometry (MS) — A fluorescence spectrum of the reaction sample was measured in the wavelength range of 200–500 nm. For measuring the spectra,  $20 \,\mu\text{M}$  D-KYN concentration was used; the other conditions were as described above.

To confirm the results by MS, D-KYN (100  $\mu$ M) was reacted with DAAO as described, and the resulting sample was diluted with methanol without adding zinc acetate. Subsequently, the diluted sample was infused at a rate of 10  $\mu$ l/min into the mass spectrometer. All MS data were acquired on an LCQ ion trap MS (ThermoFisher Scientific, Yokohama, Japan) equipped with an electrospray ionization source in the positive-ion mode. The experimental conditions were as follows: spray needle voltage, 5 kV; heated capillary temperature, 200°C; and sheath gas flow rate, 40 (arbitrary units). MS were obtained using full ion-scan mode over a mass-to-charge (m/z) range of 100–2000.

**High-performance Liquid Chromatography** (HPLC) — After the enzymatic reaction of D-KYN (100  $\mu$ M) with DAAO as described, the resulting solution was diluted 200-fold with H<sub>2</sub>O/CH<sub>3</sub>CN (95/5) containing 50 mM ammonium acetate (mobile phase), and a 5.0- $\mu$ l aliquot of the final solution was injected onto column-switching HPLC with fluorescence detection (ex. 251 nm, em. 398 nm) as reported.<sup>10, 11</sup> Five  $\mu$ l of the KYNA standard (5.0 nM in the mobile phase) was also injected for the comparison of retention times.

**DAAO Assay with Test Compounds** — For the inhibition assay of DAAO,  $50 \,\mu$ l of the test compounds (concentrations, 2.5–10000  $\mu$ M) was added to 1000  $\mu$ M D-KYN, and DAAO activity was assayed as described. For the inhibition curve, the final concentrations of the test compounds were plotted on the *x*-axis, and the  $\Delta F$  value in the absence

of the inhibitor was set as 100% on the *y*-axis. The degree of inhibition of each test compound was expressed as a percentage according to the following equation:

Remaining activity of pkDAAO (%)

$$= \Delta F' / \Delta F \times 100 \tag{2}$$

 $\Delta F'$  is the  $\Delta F$  value at each concentration of the inhibitor.

## **RESULTS AND DISCUSSION**

#### In Vitro Production of KYNA

Our recent findings on KYNA production from D-KYN *in vivo*<sup>10,11)</sup> suggested that D-KYN is metabolized to KYNA by DAAO. In the present study, we examined whether KYNA formation from D-KYN could occur *in vitro* by using a commercial pkDAAO. As shown in Fig. 2 (a), the excitation and emission spectra of the product generated by the enzymatic reaction of DAAO with D-KYN were consistent with those reported for KYNA, and the fluorescence intensity was dependent on the D-KYN concentration. The mass spectrum of the enzymatic product clearly showed a pseudomolecular ion ( $[M + H]^+$ ) peak at m/z 190.0, which is consistent with KYNA, in the positive-ionization mode

[Fig. 2 (b)]. In addition, a peak with a retention time that was identical to that of KYNA was observed [Fig. 2 (c)] when the enzymatic product was injected onto a column-switching HPLC system we used previously.<sup>10,11</sup> These results suggest that DAAO was responsible for the production of KYNA from D-KYN in this in vitro assay. To date, the  $K_{\rm m}$ and  $V_{\text{max}}$  of D-KYN for DAAO have not been published; however, based on the Lineweaver-Burk plot of our data (Fig. 3),  $K_{\rm m}$  and  $V_{\rm max}$  were 148  $\mu$ M and 5.53 µmol/min per mg, respectively. These values indicate that D-KYN has a high affinity for DAAO. From the increased fluorescence,  $\Delta F$  in Eq. (1), the amount of KYNA produced from D-KYN can be determined from the standard curve of KYNA ( $r^2$ = 0.9958). The  $\Delta F$  value can be used to evaluate DAAO activity as described below. When zinc acetate is added to the boric acid buffer solution, a slight sediment is produced. However, this sediment can be removed by centrifugation without affecting fluorescence measurements. The assay precision (i.e., the relative standard deviation) was within 3.25% (*n* = 3).

In previous research involving DAAO assays, mainly  $\alpha$ -keto acid or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), both of which are produced in the enzymatic reaction of D-amino acids with DAAO, have been used in the subsequent colorimetric assay. Since  $\alpha$ -keto



Fig. 2. Instrumental Analyses of the Reaction Product after Incubation of D-KYN with DAAO

(a) Excitation (left) and emission (right) spectra of the product in the presence of zinc ions. Wavelengths from 200 nm to 500 nm were scanned. (b) Mass spectrum of the product. (c) Chromatograms of a KYNA standard (upper) and the product (lower) by column-switching HPLC. Experimental details are described in the text. acid can react with 2,4-dinitrophenylhydrazine to produce its hydrazone, the visible absorption of hydrazone can be assessed to monitor DAAO activity.<sup>22)</sup> On the other hand, the produced  $H_2O_2$  is used for the oxidation of a colorimetric reagent such as *o*-dianisidine by peroxidase,<sup>12)</sup> and the visible absorption of the oxidized reagent can be assessed to monitor DAAO activity. Compared to these previous methods, the proposed assay is simple and facile because it uses the fluorescence intensity of KYNA produced from D-KYN through one enzymatic reaction step by DAAO.

## Inhibition by MPC, a DAAO Inhibitor

KYNA itself emits fluorescence, especially in the presence of zinc ions;<sup>23–26)</sup> therefore, the procedure described above can be used to evaluate *in vitro* DAAO activity. A test compound was added to the enzymatic reaction of DAAO with D-KYN. If the compound inhibits DAAO activity, a significant attenuation in the  $\Delta F$  value [*i.e.*, a small  $\Delta F'$  value, Eq. (2)] will be observed. Figure 4 shows dosedependent inhibition curves of pkDAAO by test



Fig. 3. Lineweaver-Burk Plot of the Enzymatic Reaction of D-KYN to produce KYNA by DAAO

compounds. The remaining activity of pkDAAO (%) can be used as a measure of the inhibitory activity of the test compound at each concentration.

We first tested MPC, which was originally developed as a specific inhibitor of DAAO.<sup>13</sup> As shown in Fig. 4 (a), a remarkable attenuation of fluorescence, which reflects an inhibition of pkDAAO activity, was clearly observed in the presence of MPC at concentrations above 0.5  $\mu$ M. In the present study, the IC<sub>50</sub> of MPC was approximately 3.4  $\mu$ M, which is slightly higher than the reported value (0.91  $\mu$ M).<sup>13</sup> This slight difference may be due to differences in the origin of the DAAO enzymes or in the assay procedures between the studies.

#### Inhibition by D-serine or D-alanine

As shown in Fig. 4 (b) and 4 (c), D-serine and Dalanine, which are endogenous D-amino acids, inhibited DAAO activity in our assay (n = 4). The IC<sub>50</sub> values of D-serine and D-alanine were approximately 0.85 mM and 0.89 mM, respectively. These D-amino acids are known to be easily metabolized by DAAO, and the  $K_m$  of D-serine and D-alanine for pkDAAO is reported to be 41 mM and 3.1 mM, respectively.<sup>18)</sup> Therefore, competitive inhibition at the reactive site of DAAO may occur between D-KYN and these D-amino acids.

#### Inhibition by Chlorpromazine

We tested chlorpromazine, a tricyclic antipsychotic drug, because it has been reported to inhibit DAAO activity *in vitro*.<sup>27, 28)</sup> In the present study, chlorpromazine at concentrations above 10 mM clearly inhibited DAAO activity, and the IC<sub>50</sub> was



Fig. 4. Dose-dependent Inhibition Curves of pkDAAO by AS057278 (MPC) (a), D-Serine (b), D-Alanine (c), and Chlorpromazine (d)

approximately 1.9 mM [n = 3, Fig. 4 (d)]. The inhibitory mechanism of chlorpromazine is believed to be different from that of D-alanine and D-serine. One report suggested that chlorpromazine displaces FAD, a cofactor necessary for DAAO activity.<sup>27)</sup>

In this study, we verified that our assay can be used to monitor DAAO activity by testing several compounds that inhibit DAAO. We have established a facile assay for measuring DAAO activity using D-KYN as a substrate. This method will be used to screen for additional inhibitors of DAAO.

Acknowledgements The authors thank Drs. H. Yamada and S. Iwasa, Toho University for their kind technical assistance. This research was advanced by a Scientific and Educational Cooperation between Shenyang Pharmaceutical University, China and Toho University, Japan.

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