

Fluorimetric assay for D-amino acid oxidase activity in rat brain homogenate by using D-kynurenine as a substrate

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Summary

An easy fluorimetric assay for measuring D-amino acid oxidase (DAAO) activity by using one of the D-amino acids – D-kynurenine (D-KYN) – as a substrate was applied to assess DAAO activity in the cerebrum, cerebellum, and brainstem of Sprague-Dawley (SD) male rats. In this assay, DAAO produces kynurenic acid (KYNA) from D-KYN, and the fluorescence originating from KYNA can then be used to evaluate DAAO activity. Here, pellet fractions obtained by centrifugation of brain homogenates were allowed to react enzymatically with D-KYN. The addition of specific DAAO inhibitors, such as 3-methylpyrazole-5-carboxylic acid and 4*H*-thieno [3, 2-*b*] pyrrole-5-carboxylic acid (Compound 8), significantly attenuated the fluorescence intensity of KYNA, suggesting that DAAO present in the rat brain homogenates was responsible for the production of KYNA. In contrast, an inhibitor of aminotransferase (AT), aminooxyacetic acid, did not decrease KYNA production from D-KYN, meaning that AT could not metabolize D-KYN to KYNA under the present conditions. Moreover, the DAAO activity measured by the proposed assay correlated well with DAAO mRNA expression ($r = 0.9982$) determined by real-time polymerase chain reaction. Taken together, these findings show that the proposed fluorimetric assay can be used to evaluate DAAO activity in rat brain.

Keywords: D-amino acid oxidase, D-kynurenine, kynurenic acid, rat brain, fluorescence

1. Introduction

D-Amino acid oxidase (DAAO) (E.C. 1.4.3.3) can decompose neutral and basic D-amino acid oxidatively to generate hydrogen peroxide (H₂O₂), ammonia, and the corresponding α -keto acid (1,2). In mammalian brain tissue, D-serine, an endogenous co-agonist for the ionotropic glutamate receptor *N*-methyl-D-aspartate (NMDA) receptor (3), is decomposed by DAAO located in astroglial cells (3,4). Thus, the brain D-serine concentration controlled by DAAO is crucial for regular neurotransmission *via* the NMDA receptor,

and it has been suggested that a decrease in the D-serine concentration could induce NMDA receptor dysfunction. An example of this is the "glutamate hypothesis", which postulates that the cause of schizophrenia might arise from hypofunction of the NMDA receptor (5-7). In fact, the D-serine concentration in serum (8) and cerebrospinal fluids (9) is significantly decreased in schizophrenia patients. In addition, it has recently been reported that DAAO activity is significantly increased in the post-mortem brains of schizophrenia patients compared to controls (10).

DAAO activity in a tissue homogenate is usually assessed by measuring the H₂O₂ (11-14) or α -keto acid that is generated after addition of a substrate, D-alanine, or D-proline (10,15-17). Using the increased H₂O₂, an oxidative reaction that generates a colorimetric or fluorescent compound in the presence of a peroxidase is performed. Alternatively, the produced α -keto acid is reacted with a hydrazine compound to produce fluorescent or colorimetric substances. The generated

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substances are then quantified spectrophotometrically. However, both generated H_2O_2 and α -keto acid require the addition of next-step reagents, such as 2,4-dinitrophenyl hydrazine (DNPH) or Amplex Red[®], for the colorimetric or fluorimetric reaction to proceed.

A previous study by our group has shown that D-kynurenine (D-KYN) is oxidatively deaminated by a standard of pig kidney (pk) DAAO to produce a fluorescent compound, 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid, KYNA) by one-step enzymatic reaction. The corresponding α -keto acid, 2-oxo-4-(2-aminophenyl)-4-oxobutanoic acid may be instantaneously produced as an intermediate in this reaction, but the α -keto acid appears to be immediately thereafter transformed to KYNA by intramolecular cyclization (18). Thus, the fluorescence intensity of KYNA can be utilized in conjunction with a standard of pig kidney (pk) DAAO in a fluorimetric assay to evaluate several compounds including drugs inhibiting DAAO activity (18,19).

The purpose of this study was to investigate the applicability of the proposed assay to rat brain homogenate. Here, we measured DAAO activity in the brain tissues of Sprague-Dawley (SD) rats according to our previously described *in vitro* assay using D-KYN as the substrate (18). The inhibition of the DAAO activity by 2 kinds of commercial DAAO inhibitors, as well as the correlation between DAAO mRNA expression and the enzyme activity was examined to determine the reliability of the proposed DAAO assay.

2. Materials and Methods

2.1. Chemicals

D-KYN, KYNA, 3-methylpyrazole-5-carboxylic acid (MPC), 3-methylpyrazole-4-carboxylic acid, Trizma[®] base (Tris) (min. 99.9%), D-alanine, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Flavin adenine dinucleotide (FAD) and dimethylsulfoxide (DMSO) were obtained from Nacal Tesque (Kyoto, Japan). Zinc sulfate heptahydrate was obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). 2-Aminoxyacetic acid (AOAA) and catalase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Compound 8 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and 2,4-dinitrophenyl hydrazine (DNPH) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore Co. Ltd., Bedford, MA, USA). All other reagents were of reagent grade and were used without further purification.

2.2. Animal experiments

All animal experiments were approved by the Committee

of Animal Care, Toho University (No. 12-51-165). Male Sprague-Dawley (SD) rats were purchased from Charles River Japan (Kanagawa, Japan) and were housed in an environmentally controlled room for at least 1 week before use.

2.3. Tissue homogenate preparation

The rats were sacrificed by drawing blood from the abdominal aorta under diethyl-ether anesthesia. The rat brain tissue was immediately dissected and rinsed in a chilled physiological saline. The brain was then divided into cerebrum, cerebellum, and brainstem on an ice-cooled plate. After weighing each piece, the tissue was homogenized in 10 volumes of chilled Tris buffer (pH 8.3) using a potter-type homogenizer under ice cooling. The prepared homogenates were stored at -80°C until analysis.

2.4. DAAO assay with D-KYN as a substrate

After thawing at 4°C , an aliquot of 2.0 mL of the homogenate was centrifuged at $600 \times g$ for 30 min, and the obtained supernatant was further centrifuged at $20,000 \times g$ for 20 min. The pellet was suspended in 150 μL of 0.4 M Tris-buffer (pH 8.3) and subjected to the DAAO assay as an enzyme source. The DAAO assay was carried out according to the previously published method (18,19) with minor modifications.

In brief, 20 μL of the pellet fraction was mixed with 50 μL of 200 μM FAD solution, 20 μL of 2.0 mg/mL BSA in H_2O , and 390 μL of 0.4 M Tris buffer solution (pH 8.3). Subsequently, the mixed solution was incubated at 37°C for 15 min. In some cases, 370 μL of 0.4 M Tris buffer solution (pH 8.3) was used instead of 390 μL , and 20 μL of inhibitor solution (in DMSO) was added before the incubation. The inhibitors used in the present study were AOAA (0, 1.0, 2.0, 4.0, 6.0, 8.0, and 10 mM), MPC (100 μM), 3,4-MPC (100 μM), and compound 8 (100 μM). Next, 20 μL of 7.0 mM D-KYN dissolved in Tris buffer (20 μL) were added, and the mixtures were then incubated at 37°C for 120 min. To determine the optimum D-KYN concentration, different concentrations (0, 1.4, 4.2, and 7.0 mM) of D-KYN were added. After the enzymatic reaction, 1.0 mL of $CH_3CN/MeOH$ (1:1, v:v) was added and vortex-mixed. The mixture was then added to 500 μL of 0.4 M Tris buffer solution (pH 8.3) and 50 μL of 300 mM zinc sulfate dissolved in H_2O . The final solution was vortex-mixed and filtered (Millex[®] GV, 0.22 μm , Nihon Millipore, Tokyo, Japan) to remove proteins denatured by $CH_3CN/MeOH$ (1:1, v:v).

The fluorescence of the resultant solution was measured by a HITACHI F-7000 fluorescence spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan). The fluorescence intensity of each solution was measured at an excitation wavelength of 346 nm and

an emission wavelength of 396 nm. According to the following equation (1), the ΔF value was determined:

$$\Delta F = F - F_0 \quad (1)$$

where F and F_0 are fluorescence intensities of the sample and blank sample (a sample treated without rat brain homogenate), respectively. Based on the ΔF value, the amount of KYNA produced (pmol) was calculated by the calibration curve for KYNA (Figure 1). Finally, DAAO activity was expressed as the production rate of KYNA per μg protein (fmol KYNA/min/ μg protein), where the protein concentration of the homogenate was determined by the Bradford method according to our previous paper (20). In the inhibition experiment, the mean fluorescence intensity (ΔF) value of the control sample, *i.e.*, the sample without inhibitor, was designated as 100%, and the inhibition degree was expressed as a percentage.

2.5. DAAO assay with D-alanine as a substrate

In the conventional DAAO assay using D-alanine as a substrate (10,16), 20 μL of the pellet fraction was mixed with 20 μL of 0.1 mg/mL catalase, 50 μL of 200 μM FAD solution, 20 μL of 2.0 mg/mL BSA in H_2O , and 390 μL of 0.4 M Tris buffer solution (pH 8.3). After incubation of the mixed solution at 37°C for 15 min, 20 μL of 50 mM D-alanine was added instead of D-KYN, and incubated for another 120 min. After the enzymatic reaction, 1.0 mL of $\text{CH}_3\text{CN}/\text{MeOH}$ (1/1) and 700 μL of 0.6 M NaOH solution were added and vortex-mixed. The final solution was filtered (Millex[®] GV, 0.22 μm , Nihon Millipore, Tokyo, Japan), and the absorbance of the resultant solution was measured at 445 nm (21,22) using a JASCO V-650 spectrophotometer (JASCO Corporation, Tokyo, Japan) using a quartz cell (1 cm \times 1 cm).

2.6. Stability of KYNA

In the incubated mixture described in 2.4. Section, 20 μL of KYNA (0.8 mg/mL, 4.2 μM) was added instead of 7.0 mM D-KYN after incubation at 37°C for 15 min. Samples were then incubated at 37°C for another 120 min and assayed as previously described. The mean fluorescence intensity (ΔF) value of the control sample, *i.e.*, the sample without a pellet fraction, was designated as 100%, and the decreased degree of ΔF was expressed as a percentage.

2.7. Real-time quantitative PCR

Total RNAs were extracted from each brain tissue using RNeasy Plus Universal kits (Qiagen, Courtaboeuf, France). Complementary DNAs (cDNAs) were made with a QuantiTect Reverse Transcription kit (Qiagen). DAAO gene expression was determined by RT-PCR using the glyceraldehyde 3-phosphate dehydrogenase

(GAPDH; accession number NM017008) gene as an internal control and primers specific for DAAO (accession number NM053626) mRNAs. Upstream primers were CCCTTTCTGGAAAAGCACAG (DAAO) and GTGGACCTCATGGCCTACAT (GAPDH), and downstream primers were CTCCTC TCACCACCTCTTCG (DAAO) and TGTGAGGGA GATGCTCAGTG (GAPDH). Expression of genes was assessed by real-time RT-PCR using the iQ SYBR Green Supermix and Chrome 4 (Bio-Rad Laboratories Inc., California, CA, USA) running 45 cycles of the following protocol: 3 min predenaturation at 95°C, 10 sec denaturation at 95°C, 10 sec at 62°C, followed by a 1 min extension at 65°C. The PCR products, DAAO and GAPDH, were purified by 2% agarose gel electrophoresis and subcloned into the pTZ19R vector. After transformation of these recombinants into XL1-Blue by the calcium chloride method, plasmid DNAs were recovered, and their nucleotide sequences were determined by a DNA sequencer, Model 4000 (LI-COR Inc., Lincoln, NE, USA).

3. Results and Discussion

3.1. DAAO activity

In our previous paper, we reported an easy fluorimetric assay for measuring compounds that can inhibit pig kidney DAAO using D-KYN as the substrate (18,19). The kynurenic acid (KYNA) produced enzymatically from D-KYN by DAAO emits an intense fluorescence at 396-398 nm in the presence of zinc ions. Thus, the inhibition potency of various compounds against DAAO can be evaluated by the degree of fluorescence produced.

In the present study, this fluorimetric assay was applied to assess the DAAO activity in rat brain tissues. As reported, KYNA emits fluorescence at 396-398 nm with an excitation wavelength at 251 nm (19,23) or 344-346 nm (24,25). An excitation wavelength of 346 nm was used in the present study to increase the signal to noise ratio and to avoid interference fluorescence stemming from endogenous substances included in the brain homogenate. Under the present conditions, a linear calibration curve for standard KYNA in the range of 26.1-81.2 pmol per assay tube was obtained (Figure 1), and this calibration curve was used to calculate KYNA production.

To assess DAAO activity in rat brain tissues, the brain tissue homogenates were centrifuged as previously described (15,17), and the pellet fraction was then used as an enzyme source for the proposed assay. Increasing concentrations of D-KYN were added to the reactions to determine its optimum concentration as a substrate, which was set at the plateau level of 7.0 mM (Figure 2). The pellet fraction was incubated with the D-KYN substrate in Tris buffer (pH 8.3), and

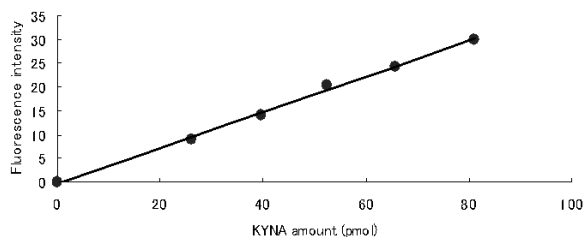


Figure 1. Calibration curve for standard KYNA.

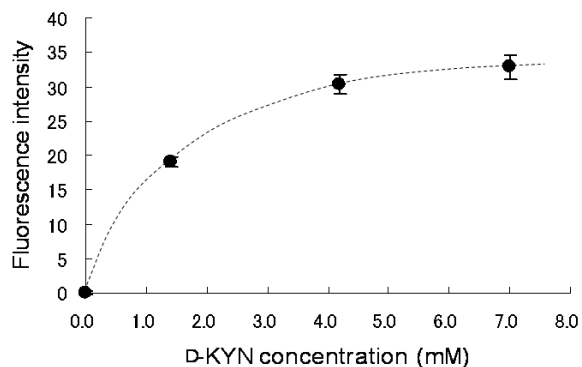


Figure 2. Effect of D-KYN concentration on the fluorescence intensity of the produced KYNA.

the fluorescence intensity of KYNA produced from D-KYN was measured. As shown in Figure 3, the fluorescence of KYNA at 396 nm increases linearly with time, suggesting that the added D-KYN was converted to KYNA by DAAO present in the pellet fraction. Conversely, incubation with D-KYN alone for 120 min in the absence of the pellet fraction gradually showed fluorescence at 396 nm. Based on this finding, the fluorescence derived from KYNA produced enzymatically from D-KYN by DAAO was calculated by subtracting the fluorescence value without the pellet fraction (blank) from the sample fluorescence intensity at 396 nm. As might be expected, DAAO activity expressed as fmol KYNA/min/ μ g protein was the highest in rat cerebellum, followed by brain stem and cerebrum (Figure 4), in agreement with previous reports (26,27).

Although endogenous KYNA is present in rat brain tissues, the concentration is reported to be relatively low, approximately 200-600 fmol/mg protein among the tissues assayed here (28). In this previous study, a centrifugation fractionation procedure was not carried out, while the assay here uses the pellet fraction by centrifugation. As a result, endogenous KYNA present in the brain tissues hardly affected the fluorescence intensity under the present experimental conditions. Indeed, a blank sample without adding D-KYN consistently showed a background level of fluorescence (Figure 3).

To determine whether the KYNA produced from D-KYN was further metabolized into other metabolites under the present experimental conditions, a standard

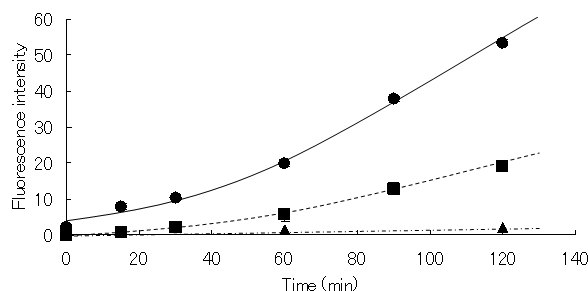


Figure 3. Changes in the fluorescence intensity at 396 nm with an excitation wavelength of 346 nm as a function of time. A closed circle with a solid line and a closed square with a dotted line represent the fluorescence intensities of a sample with D-KYN in the presence or absence of brainstem homogenate, respectively. A closed triangle represents the fluorescence intensity of the sample without D-KYN in the presence of brainstem homogenate.

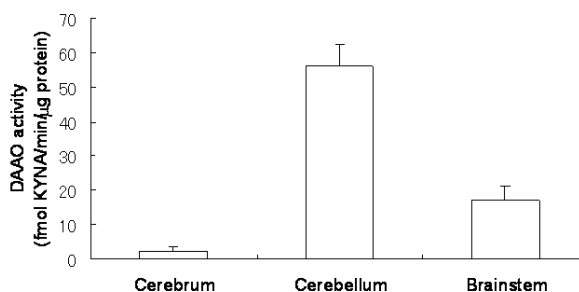


Figure 4. DAAO activity (fmol KYNA/min/ μ g protein) determined by the proposed method in rat cerebrum, cerebellum, and brainstem ($n = 5$).

of 4.2 μ M KYNA instead of D-KYN was added to the enzymatic reaction, and stability of KYNA was investigated. No remarkable decrease in fluorescence intensity of KYNA after the enzymatic reaction at 37°C for 120 min was observed (Figure 5). In addition, to our knowledge, no information on the metabolites of KYNA in the brain has been reported. Considering these observations, KYNA itself does not appear to be drastically metabolized or decomposed under the present conditions. In the fluorimetric assay, the stability of KYNA that is produced provides an advantage over H_2O_2 or α -keto acids. Thus, the fluorescence originating from the produced KYNA can be used as a reliable indicator of DAAO activity.

To compare the present data with data obtained by the conventional method, the homogenate sample was also reacted with D-alanine, followed by the addition of 2,4-DNPH, and the absorbance was measured at 445 nm as previously described (21,22). Unfortunately, the conventional method failed to assess DAAO activity since the obtained absorbance value hardly differed from the blank value (data not shown). Thus, a more concentrated homogenate sample might be needed to determine DAAO activity by the conventional method. In contrast, the proposed method employs fluorescence detection, allowing brain DAAO activity to be determined in spite of a small amount of homogenate

sample.

3.2. Inhibition of brain DAAO activity by specific inhibitors

To examine whether the enzymatic formation of KYNA from D-KYN was indeed caused by DAAO or other unknown enzymes, specific inhibitors for DAAO (12,16) were added to the reaction mixture. 3-Methylpyrazole-5-carboxylic acid (MPC) (12) and 4*H*-thieno [3,2-*b*] pyrrole-5-carboxylic acid (Compound 8) (17) were chosen as specific inhibitors in the present study because both compounds are commercially available. Both MPC and Compound 8 (each 100 μ M) could completely attenuate the DAAO activity (Figure 6). It has been reported that the IC_{50} value of Compound 8 is 114 nM (17), while that of MPC is 0.91 μ M (12), indicating that Compound 8 is a more potent inhibitor of DAAO activity. However, the results from this study (Figure 6) indicated that both specific inhibitors (100 μ M) appropriately inhibited the DAAO activity. In contrast, 3-methylpyrazole-4-carboxylic acid (3,4-MPC), a structural isomer of MPC, failed to inhibit the production of KYNA (Figure 6). In agreement with

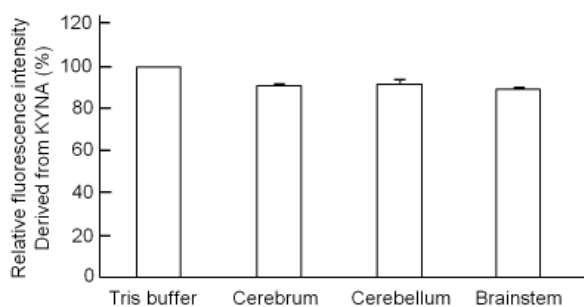


Figure 5. Relative fluorescence intensity of added KYNA in rat cerebrum, cerebellum, and brain stem after the enzymatic reaction of the pellet fraction ($n = 3$). The fluorescence intensity of the added KYNA in Tris buffer (pH 8.3) was designated as 100%.

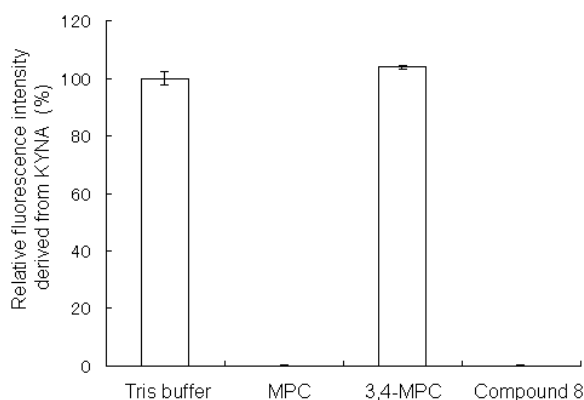


Figure 6. Relative fluorescence intensity of the produced KYNA after the enzymatic reaction of D-KYN with rat brainstem homogenates in the presence or absence of inhibitors, 3,4-MPC, MPC, and Compound 8 (each 100 μ M; $n = 3$).

this finding, 3,4-MPC has previously been shown to hardly inhibit DAAO in an *in vitro* study (19). Taken together, these findings suggest that DAAO in the pellet fraction is responsible for the enzymatic production of KYNA from D-KYN.

Recently, an *in vivo* microdialysis study demonstrated that D-KYN was metabolized to KYNA in rat striatum by kynurenine aminotransferase (KAT) based on evidence showing that addition of an inhibitor of KAT, AOAA, remarkably inhibited KYNA production from D-KYN (29). Considering this recent report, the effect of AOAA on the production of KYNA from D-KYN in our brain homogenates was investigated. As shown in Figure 7, however, no considerable decrease in the production of KYNA by D-KYN was observed in the present study with the addition of AOAA. Therefore, it seems unlikely that KAT contributed to the production of KYNA under the present experimental conditions. The reason for this discrepancy is unknown, but it may stem from differences between the *in vivo* state examined in the first study and the homogenates examined here, which are not at physiological pH, but at a weakly basic pH in Tris buffer (pH 8.3).

3.3. mRNA expression

In addition to DAAO activity, DAAO mRNA expression was also investigated by real-time quantitative PCR. Table 1 shows DAAO activity and mRNA expression in the cerebrum, brainstem, and cerebellum of SD rats. The present data revealed a similar tendency to those reported previously (30,31), namely, the highest DAAO mRNA expression was found in the cerebellum, while slight expression was observed in the cerebrum. DAAO mRNA expression plotted against DAAO activity, as shown in Figure 8, reveals a linear relationship between DAAO mRNA and DAAO activity ($r = 0.9982$), indicating that the DAAO activity shown by the present assay might be reflected in DAAO expression in rat brain.

In summary, DAAO activity in the cerebrum,

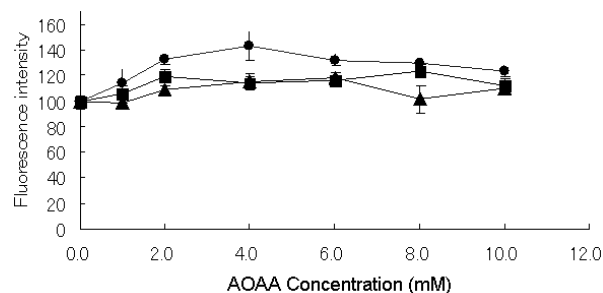
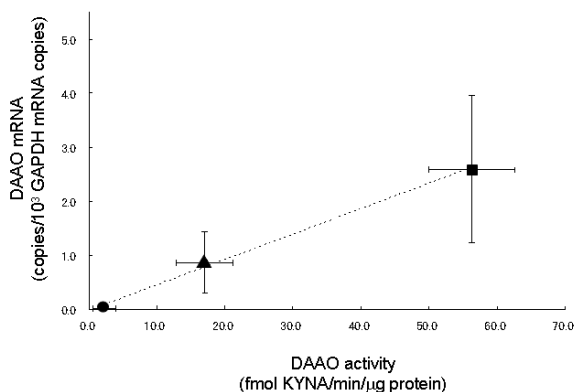


Figure 7. Fluorescence intensity at 396 nm with an excitation wavelength of 346 nm after the enzymatic reaction of D-KYN with rat cerebrum (circle), cerebellum (square), and brainstem (triangle) in the presence of several concentrations of an aminotransferase inhibitor, AOAA ($n = 3$).

Table 1. DAAO activity and mRNA expression in the cerebrum, brainstem, and cerebellum of control rats (n = 5)

| Items | Activity (fmol KYNA/min / μ g protein) | mRNA(copies/ 10^3 GAPDH mRNA copies) |
|------------|--|--|
| Cerebrum | 2.24 \pm 1.67 | 0.0143 \pm 0.00623 |
| Brainstem | 17.0 \pm 4.15 | 0.858 \pm 0.567 |
| Cerebellum | 56.3 \pm 6.31 | 2.59 \pm 1.36 |

**Figure 8. Expression of DAAO mRNA plotted against the DAAO activity in the cerebrum (circle), brainstem (triangle), and cerebellum (square) determined by the proposed assay (n = 5).**

cerebellum, and brainstem of rats can be measured by an easy fluorimetric assay using D-KYN as a substrate. The produced KYNA was stable in the homogenate under the current experimental conditions. Recently, Wong *et al.* reported a microplate fluorescence assay for KAT I using fluorescence originating from KYNA (32). Since the fluorimetric assay proposed here also uses the fluorescence of KYNA, a rapid microplate version of this assay will be performed with a plate reader in the future.

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