Substrate Selectivity of Monoamine Oxidase A, Monoamine Oxidase B, Diamine Oxidase, and Semicarbazide-Sensitive Amine Oxidase in COS-1 Expression Systems

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The substrate selectivity of monoamine oxidase A (MAO-A), monoamine oxidase B (MAO-B), diamine oxidase (DAO), and semicarbazide-sensitive amine oxidase (SSAO) was investigated in the absence of chemical inhibitors using the COS-1 cells expressed with respective amine oxidase. Serotonin (5-hydroxytryptamine), 1-methylhistamine, and histamine were preferentially oxidized by MAO-A, SSAO, and DAO, respectively, at a low substrate concentration. In contrast, benzylamine, tyramine, and β-phenylethylamine served as substrates for all of MAO-A, MAO-B, and SSAO. Each amine oxidase showed broad substrate selectivity at a high substrate concentration. The cross-inhibition was remarkable in MAO-A and MAO-B, especially in MAO-A, but not in SSAO and DAO. A study of the substrate selectivity of amine oxidases should include consideration of the effects of substrate concentration and specific chemical inhibitors.

Key words　substrate selectivity; COS-1; monoamine oxidase (MAO)-A; MAO-B; diamine oxidase (DAO); semicarbazide-sensitive amine oxidase (SSAO)

Amine oxidases (AOs) are widely distributed among all living organisms.[1] This class of enzymes is divided into two subclasses: FAD-containing amine oxidases (FAD-AOs) and Cu-containing amine oxidases (Cu-AOs). Two types of mitochondrial monoamine oxidases, monoamine oxidases (MAO)-A and MAO-B, and cytosolic polyamine oxidase are included in FAD-AOs.[2] The Cu-AOs consist of retina amine oxidase, diamine oxidase (DAO), and semicarbazide-sensitive amine oxidase (SSAO), which is known as the tissue-bound or plasma form of amine oxidase. The classification of MAO into an A and B form was primarily based on Johnston's presentation that both enzymes could be differentiated with the aid of the specific inhibition of MAO-A by clorgyline and of MAO-B by deprenyl.[3] Classification by means of specific inhibitors has been more convenient for the identification of different types of amine oxidase. However, this concept has been considered somewhat of an oversimplification and the premises on which it was based have come under increasing scrutiny.[4—9] One major problem was that serotonin and β-phenylethylamine, which are generally used to distinguish MAO-A and MAO-B, are not entirely specific for the respective MAO form. Another problem was that the substrate selectivity of MAO seems to be affected by minor cross-inhibition caused by specific inhibitors.

SSAO essentially metabolizes amine compounds as well as MAO-A and MAO-B. However, the enzyme activity is not inhibited by clorgyline and deprenyl, whereas it is almost completely inhibited by semicarbazide. In contrast, semicarbazide has a weak inhibitory effect on MAO at a concentration of 0.1—1 mM.[10] Clorgyline and deprenyl have been regarded as virtually inactive against the tissue-bound SSAO, nevertheless they showed moderate reversible competitive inhibition against bovine[11] and human[12,13] plasma SSAO at a concentration of 0.1—1 mM. DAO, belonging to the Cu-AOs, is responsible for histamine metabolism.[14] Comparative substrate selectivity studies of DAO have not always been performed in detail. Aminoguanidine has been used for many

years as an inhibitor of Cu-AO, but it is a relatively weak and nonselective inhibitor of SSAO.[15]

Thus, the results of the substrate selectivity of these amine oxidases using specific inhibitors are very confused and complex, and might not reflect natural biochemical properties. In this study, we utilized a single amine oxidase expressed in COS-1 cells to investigate the substrate selectivity of amine oxidases in the absence of chemical inhibitors.

MATERIALS AND METHODS

Animals　Nine-week-old male Wistar rats purchased from Japan SLC (200—220 g, Hamamatsu, Japan) were housed at a constant temperature (23±1 °C) and humidity (55±5%) with automatically controlled lighting (07:00—19:00).

Plasmid Construction　Standard protocols were used for all recombinant DNA technology.[16] The full-length ORF AOs plasmid was constructed as follows. Total RNA was isolated using the Wizard SV Total RNA Extraction Kit (Promega, Madison, WI, U.S.A.) from rat liver for MAO-A and MAO-B, small intestine for DAO, and aorta for SSAO. The cDNAs of each amine oxidase were synthesized by reverse transcriptase-PCR using the SuperScript First Strand System (Invitrogen, Carlsbad, CA, U.S.A.). The cDNAs for the respective amine oxidases were amplified by means of the PCR method with *Ex Taq* Polymerase (TaKaRa Bio Inc., Shiga, Japan) using the reverse transcriptase-PCR products as templates and the primers listed in Table 1. The amplified DNA fragment was cloned into a pCR4 TOPO vector (Invitrogen). Sequences of products were determined using a CEQ 8000 automated DNA sequencer (Beckman-Coulter Inc., Fullerton, CA, U.S.A.) and a DTCS DNA Sequence Kit (Beckman-Coulter) according to the manufacturer's instructions. The resulting plasmids were cleaved with *Bam*HI and *Eco*RI (MAO-A and MAO-B) and *Acc65*I and *Not*I (DAO and SSAO), and each DNA fragment obtained was cloned

Table 1. Sequence of Primers

Primer name	Sequence
*Bam*HI-MAOA-F	5′-cggg<u>atcc</u>catgacggatctggag-3′
*Bam*HI-MAOA-R	5′-cgg<u>aattc</u>cgtggcccaaaccatag-3′
*Bam*HI-MAOB-F	5′-cggg<u>atcc</u>cgagcaccatgagcaac-3′
*Eco*RI-MAOB-R	5′-cgg<u>aattc</u>cggctgtggatatgc-3′
*Acc*65I-DAO-F	5′-<u>ggtacc</u>aatgtgtctggccttcggct-3′
*Not*I-DAO-R	5′-<u>gcggccgc</u>gctgacccatcacacaggcttgt-3′
*Acc*65I-SSAO-F	5′-<u>ggtacc</u>aatgacccagaagaccaccctag-3′
*Not*I-SSAO-R	5′-<u>gcggccgc</u>gaattcgcccttccattactccag-3′

Underline indicates a recognized restriction site.

into the *Bam*HI/*Eco*RI (MAO-A and MAO-B) and *Acc*65I/*Not*I (DAO and SSAO) site of the pcDNA/His MAX A plasmid (Invitrogen).

Cell Cultures and Transfections The African green monkey kidney COS-1 cell line (gift from Prof. Yamazoe and Dr. Nagata, Tohoku University, Japan) was maintained in DMEM (Sigma, St. Louis, MO, U.S.A.) containing 10% FBS (Sigma), and 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Grand Island, NY, U.S.A.) were added to both media. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Semiconfluent COS-1 cells were transfected with each of pcDNA4/His MAX A-MAO-A, pcDNA4/His MAX A-MAO-B, pcDNA4/His MAX A-DAO, and pcDNA4/His MAX A-SSAO plasmid. As a negative control, COS-1 cells were also mock transfected with the unrelated pcDNA/His MAX A plasmid. All plasmids were extracted using the Wizard® PureFection Plasmid DNA Purification System (Promega) according to the manufacturer's instructions. Cells were transfected using the Lipofectamine 2000™ reagent (Invitrogen) following the manufacturer's instructions. The cells were detached from dishes by trypsin treatment after 48 h incubation and collected by centrifugation at 1000 ***g*** for 3 min. They were suspended in PBS (pH 7.6) and then solubilized by ultrasonication. Protein concentrations in cell lysates were measured by Bradford's method using bovine serum albumin as the standard and a protein assay kit (Dojindo Molecular Technologies Inc., Gaitherburg, MD, U.S.A.).

Amine Oxidase Activity Benzylamine hydrochloride, β-phenylethylamine hydrochloride, tyramine hydrochloride, and 1-methylhistamine dihydrochloride were purchased from Sigma Co. Ltd. Histamine dihydrochloride was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Clorgyline and deprenyl were obtained from Research Biochemical International (Natick, MA, U.S.A.). Semicarbazide, hydrogen peroxide, vanillic acid, 4-aminoantipyrine, and peroxidase (type II from horseradish) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Amine oxidase activities were measured by a reported spectrometric method[17] in a volume of 200 μl each in 96-well plates using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). As the substrate selectivity of amine oxidases has been investigated at the substrate concentration of 10 μM— 1 mM, two concentrations of low (100 μM) and high (1 mM) were chosen in this study. When necessary, the samples were preincubated with amine oxidase inhibitors for 30 min before assay. The deamination reaction was initiated by adding the substrate and linear over 90 min. The absorbance at 490 nm was read after 60-min incubation.

LC/MS/MS Assay of Serotonin Deamination Activity 5-Hydroxytryptamine hydrochloride (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), and 5-hydroxyindole-2-carboxylic acid (5-HICA) were purchased from Sigma Co., Ltd. Serotonin oxidative deamination activities were measured essentially according to the method reported by Manini *et al.*[18] A Thermo Electron (San Jose, CA, U.S.A.) Surveyor LC-MS/MS system consisting of a chromatograph pump, an auto-sampler, a gradient unit, an on-line degasser, a column oven, and a TSQ Quantum mass spectrometer was used. A reversed-phase Discovery RP-Amide C16 (50 mm×2.0 mm i.d., particle size 5 μm; Supelco, Bellefonte, PA, U.S.A.) was used. The mobile phase was 25% methanol/0.05% formic acid. The flow rate of the mobile phase and the column oven temperature were set at 0.2 ml/min and 30 °C, respectively. The effluent from the LC column flowed without splitting into the ion source of the mass spectrometer. The LC-MS/MS, TSQ Quantum mass spectrometer, was operated in the negative ESI mode. The TSQ Quantum was tuned using the built-in auto-tuning system. The spray needle voltage was 4.0 kV, the capillary temperature was 265 °C, sheath gas pressure 47, and auxiliary gas setting 25. Both the sheath gas and auxiliary gas used were nitrogen obtained from an N_2 supplier 12ES (Sic, Tokyo, Japan). The collision gas was argon at a pressure of 1.5 mTorr for all studies. For the MS/MS analysis, the optimized relative collision energy for collision-induced dissociation (CID) was -14 V. The collision energy was 27 eV. MS acquisition was performed in the selected-reaction monitoring (SRM) mode by monitoring the reaction m/z 190.1→146.3 (5-HIAA as serotonin metabolite) and 176.0→132.1 (5-HICA as internal standard).

Statistical Analysis All values were expressed as the mean±S.E. of triplicate assays. Statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test. A probability value of less than 0.05 was regarded as being statistically significant.

RESULTS

Amine Oxidase Activity at Low Substrate Concentration The oxidative deamination activity of six typical amine compounds was investigated in the absence of inhibitors using a COS-1 transient transfection system. The substrate concentration was 100 μM. The results are summarized in Table 2. Benzylamine, tyramine, and β-phenylethylamine were all oxidized by three monoamine oxidases of MAO-A, MAO-B, and SSAO in the approximately same order. It seems that there is no specific amine oxidase for these substrates. Histamine and 1-methylhistamine were predominantly deaminated by DAO and SSAO, respectively, showing the involvement of different enzymes in the metabolism of the two structurally related amines. Serotonin oxidative deamination activity was only observed in the MAO-A that was well in agreement with the reported results.[19]

Amine Oxidase Activity at High Substrate Concentration The effects of substrate concentration on substrate selectivity of amine oxidases were studied (Table 3). The concentration utilized was 1 mM. Benzylamine, tyramine, and β-phenylethylamine were oxidized by DAO in addition to MAO-A, MAO-B, and SSAO. The oxidation of histamine

Table 2. Substrate Selectivity of Rat Amine Oxidases in COS-1 Expressing Systems at Low Substrate Concentration

Substrate	Activity (nmol/mg protein/min)			
	MAO-A	MAO-B	DAO	SSAO
Benzylamine	0.30±0.08	0.19±0.04	N.D.	0.15±0.04
Histamine	N.D.	N.D.	0.17±0.02	N.D.
Tyramine	0.11±0.03	0.18±0.06	N.D.	0.25±0.03
β-Phenylethylamine	0.21±0.05	0.09±0.04	N.D.	0.23±0.07
1-Methylhistamine	N.D.	N.D.	N.D.	0.78±0.04
Serotonin	3.16±0.03	N.D.	N.D.	N.D.

A respective cDNA of rat amine oxidases were transfected into COS-1 cells. The cell lysates were assayed for amine oxidase activity at a concentration of 100 μM. The activity of COS-1 cells transfected with mock was not detected. N.D.: not detected. The detection limit of oxidative deamination activity for serotonin and the other amines was 8.84 and 33.3 pmol/min/mg protein, respectively.

Table 3. Substrate Selectivity of Rat Amine Oxidases in COS-1 Expressing Systems at High Substrate Concentration

Substrate	Activity (nmol/mg protein/min)			
	MAO-A	MAO-B	DAO	SSAO
Benzylamine	0.54±0.10	1.25±0.60	0.75±0.17	1.93±0.57
Histamine	0.04±0.01	0.54±0.10	2.26±1.15	N.D.
Tyramine	1.75±0.11	1.55±0.50	0.74±0.01	1.08±0.66
β-Phenylethylamine	1.63±0.62	0.92±0.08	0.37±0.38	1.36±0.19
1-Methylhistamine	0.36±0.01	0.95±0.05	N.D.	1.65±0.05
Serotonin	4.60±0.19	N.D.	N.D.	3.88±0.04

A respective cDNA of rat amine oxidases were transfected into COS-1 cells. The cell lysates were assayed for amine oxidase activity at a concentration of 1 mM. The activity of COS-1 cells transfected with mock was not detected. N.D.: not detected. The detection limit of deamination activity for serotonin and the other amines was 8.84 and 33.3 pmol/min/mg protein, respectively.

Fig. 1. Influence of Amine Oxidase Inhibitors on MAO-A Activity

Rat MAO-A cDNA was transfected to COS-1 cells and cell lysates were used directly for enzyme assays. The concentration of the substrates, benzylamine (BEZ), histamine (HA), tyramine (TYR), β-phenylethylamine (PEA), 1-methylhistamine (MHA), and serotonin (5-HT), was 1 mM. The concentration of the specific inhibitors, clorgyline, deprenyl, and semicarbazide, was 0.1 mM. The data are expressed as percentage of activity *versus* control activities±S.E. in triplicate determinations. * Statistically different value ($p < 0.05$) from no addition control.
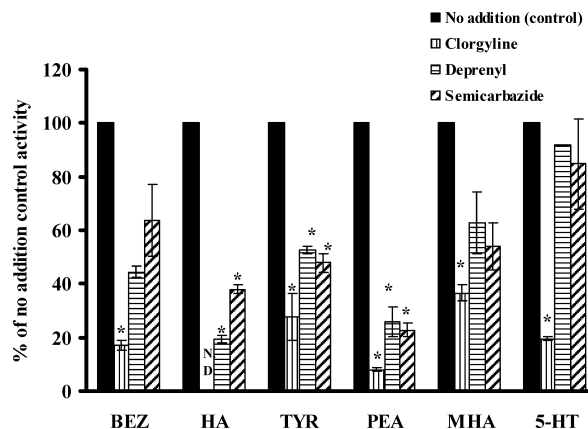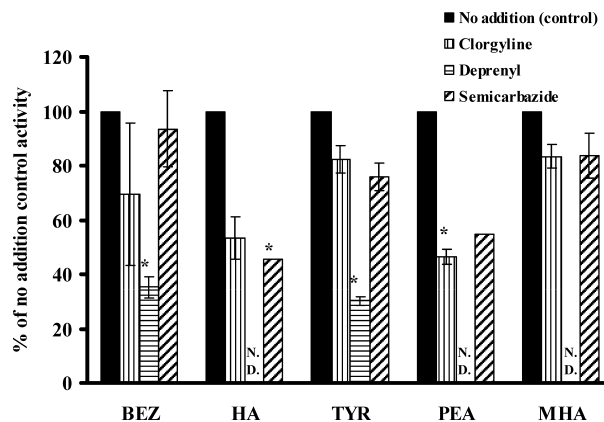

Fig. 2. Influence of Amine Oxidase Inhibitors on MAO-B Activity

Rat MAO-B cDNA was transfected to COS-1 cells and cell lysates were used directly for enzyme assays. The concentration of substrates and inhibitors, the expression manner of data and statistics are all the same as those in Fig. 1.

was catalyzed by MAO-B in addition to DAO. 1-Methylhistamine was deaminated by MAO-A and MAO-B in addition to SSAO. Serotonin was oxidized by SSAO in addition to MAO-A. In general, other enzymes were involved in the oxidation of every substrate in addition to the enzymes that functioned at a low substrate concentration.

Influence of Amine Oxidase Inhibitors The specific inhibitor for the particular amine oxidase form has been shown to have an inhibitory effect on other amine oxidases that cannot be disregarded and is called "cross-inhibition".[11—13] Here, the single amine oxidase system expressed in COS-1 was used to evaluate the inhibition selectivity of three typical amine oxidase inhibitors, clorgyline, deprenyl, and semicarbazide. The oxidative deamination activity was determined using six substrates, benzylamine, histamine, tyramine, β-phenylethylamine, 1-methylhistamine, and serotonine (5-HT).

MAO-A The MAO-A specific inhibitor, clorgyline, had undoubtedly strong inhibition activity for every substrate. However, deprenyl and semicarbazide significantly inhibited the oxidation activity of histamine, tyramine, and β-phenylethylamine catalyzed by MAO-A (Fig. 1).

MAO-B In a similar way, the MAO-B specific inhibitor, deprenyl, decreased the oxidative deamination activity of the five substrates, particularly suppressing almost completely that of histamine, β-phenylethylamine, and 1-methylhistamine. Pretreatment with clorgyline and semicarbazide significantly inhibited the metabolism of β-phenylethylamine and

histamine catalyzed by MAO-B, respectively (Fig. 2).

DAO Semicarbazide binds covalently to the prosthetic group of the DAO cofactor site to cause very strong inhibition. The oxidative deamination activities of the four substrates by DAO were almost completely inhibited by the addition of semicarbazide. In this case, clorgyline and deprenyl did not decrease DAO activity in any way (Fig. 3).

SSAO Semicarbazide caused significant decreases in the oxidative deamination activity of four among the five substrates catalyzed by SSAO. Clorgyline and deprenyl did not significantly inhibit the activities. The results were in good agreement with the reports that SSAO was resistant to clorgyline, deprenyl, and pargyline (Fig. 4).[20—22]

DISCUSSION

Several types of amine oxidases catalyze the oxidative deamination of various endogenous and xenobiotic amines in mammalian tissues. By now, determination of the substrate selectivity of amine oxidases has been based on the sensitiv-
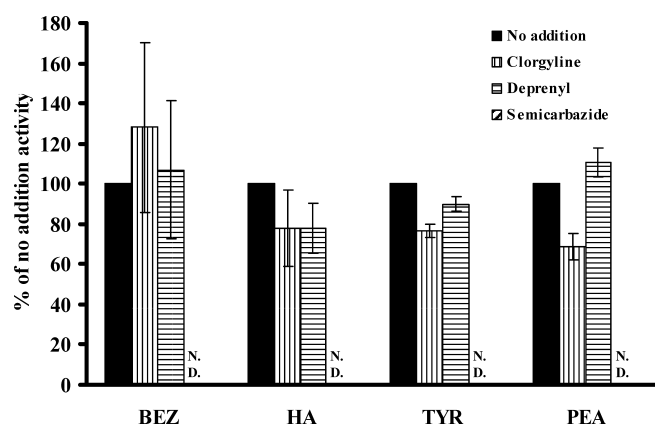
Fig. 3. Influence of Amine Oxidase Inhibitors on DAO Activity

Rat DAO cDNA was transfected to COS-1 cells and cell lysates were used directly for enzyme assays. The concentration of substrates and inhibitors, the expression manner of data and statistics are all the same as those in Fig. 1.
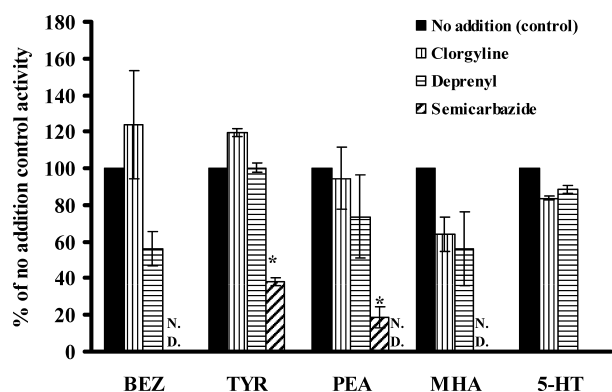

Fig. 4. Influence of Amine Oxidase Inhibitors on SSAO Activity

Rat SSAO cDNA was transfected to COS-1 cells and cell lysates were used directly for enzyme assays. The concentration of substrates and inhibitors, the expression manner of data and statistics are all the same as those in Fig. 1.

ity to specific inhibitors. However, this criterion has caused some confusion regarding the substrate selectivity of amine oxidases present in different animal species or tissues. Because the specific inhibitors against the particular amine oxidase have minor cross-inhibition to some extent, the monoamine oxidase activity measured in the presence of such inhibitors might be underestimated or not reflect the true value. The current study was conducted to determine the substrate selectivity of amine oxidases using the COS-1 expression systems in the absence of the amine oxidase inhibitors.

At a low concentration of $100 \, \mu M$ that is near to reported K_m values of amine oxidases, serotonin, 1-methylhistamine, and histamine served as a specific substrate for MAO-A, SSAO, and DAO, respectively. In contrast, benzylamine, tyramine, and β-phenylethylamine were oxidized by three amine oxidases of MAO-A, MAO-B, and SSAO even at a low concentration. The results suggest that the metabolism of endogenous biologically active amines might be catalyzed by a specific amine oxidase, whereas that of exogenous amines are done so by several enzymes. As to the metabolism of histamine derivatives, SSAO preferentially catalyzed the oxidation of 1-methylhistamine, but not histamine. MAO-A has been reported to be the specific enzyme responsible for the

oxidation of serotonin[19] and this was confirmed in this study. However, O'Sullivan et al. reported that serotonin served as a substrate for SSAO in pig dental pulp.[23] The discrepancy could be due to the differences in species and/or organs.

Kinemuchi et al. reported that the substrate selectivity of MAO-A and MAO-B changed depending on the substrate concentration.[24] In this study, at a high concentration of 1 mM, DAO and SSAO showed broad substrate selectivities as well as MAO-A and MAO-B. The results suggest that the substrate selectivity of these amine oxidases were lowered when a substrate concentration was high.

The inhibition study using COS-1 expression systems showed that deprenyl, MAO-B inhibitor, and semicarbazide, SSAO inhibitor, significantly inhibited the MAO-A catalyzed oxidation, and that clorgyline, MAO-A inhibitor, and semicarbazide also significantly decreased the oxidation activity catalyzed by MAO-B. More profound cross-inhibition was seen in MAO-A than in MAO-B and cross-inhibition was dependent on the chemical structure of the substrates. However, cross-inhibition by clorgyline and deprenyl was not observed in the oxidation reaction catalyzed by DAO and SSAO. Thus, it should be noted that so-called specific inhibitors of amine oxidases might affect more or less the other amine oxidase.

In conclusion, the substrate selectivity study using a single amine oxidase expressed in COS-1 cells showed that serotonin, histamine, and 1-methylhistamine were preferentially oxidized by MAO-A, DAO, and SSAO, respectively. In contrast, benzylamine, tyramine, and β-phenylethylamine served as a substrate for all of MAO-A, MAO-B, and SSAO. Broadened substrate selectivity was seen at a high substrate concentration. A minor cross-inhibition by so-called specific inhibitors was also observed in MAO-A and MAO-B catalyzed oxidation, especially in MAO-A. Substrate selectivity study of amine oxidases should include consideration of the effects of substrate concentration and specific chemical inhibitors.

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