Published in final edited form as:

Protein Expr Purif. 2010 April ; 70(2): 211–217. doi:10.1016/j.pep.2009.10.013.

High-level Expression and Purification of Rat Monoamine Oxidase A (MAO A) in *Pichia pastoris*: Comparison with Human MAO A

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Abstract

The high-level heterologous expression in *Pichia pastoris*, purification and characterization of recombinant membrane-bound rat liver monoamine oxidase A (MAO A)¹ are described. A one liter culture of cells produces ~700 units of rat MAO A activity. The rat MAO A activity is found in outer mitochondrial membrane of the cell. Using a modification of the human MAO A purification procedure, ~200 mg of recombinant rat MAO A is purified in a 43% yield and exhibits a molecular weight of ~60,000 kD on SDS-PAGE. The purified enzyme contains a covalently bound FAD and forms a N(5) flavocyaneine adduct on inhibition by clorgyline. Edman sequencing shows that the amino terminus of rat MAO A is blocked at an N-terminal threonyl residue. The purified rat enzyme exhibits a higher thermal stability than does purified human MAO A. Compared with human MAO A, rat MAO A oxidizes serotonin or kynuramine with 2-fold higher k_{cat}/K_m values, oxidizes phenethylamine with a 6.7 fold higher catalytic efficiency and benzylamine with a ~40-fold higher catalytic efficiency. Although ~90% identical in sequence to human MAO A, rat MAO A is a more efficient catalyst for amine neurotransmitter oxidation.

Keywords

Monoamine Oxidase A; Rat Liver MAO A; *Pichia pastoris*; Flavoenzyme; Thermal Stability

Introduction

The pharmacologically important membrane-bound flavoenzyme monoamine oxidase A (MAO A)¹ functions in the oxidative degradation of the neurotransmitters dopamine, norepinephrine and serotonin [1]. Human MAO A is found to be ~90% identical in sequence with rat MAO A with each enzyme containing an FAD cofactor covalently attached to conserved cysteinyl residues via an 8- α -S-thioether linkage. These similarities constitute the premise for using rat MAO A as a model for the human enzyme in drug development studies. X-ray structural analysis on both enzymes demonstrate that crystalline human MAO A is monomeric [2,3] and that rat MAO A is dimeric [4]. Recent pulsed EPR data show both human and rat MAO A's exist as dimers in their membrane-bound forms while only partially (50%) as dimers in their detergent solubilized forms. A number of studies have shown rat MAO A exhibits differing behavior from human MAO A demonstrating caution must be exercised in the use of the rat model in the development of highly specific MAO A inhibitors for use in the

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human. A previous study of alkylthio-substituted amphetamine analogs as MAO A inhibitors shows that the human exhibits differing sensitivities to *para*-alkyl chain length than the rat enzyme [5].

Therefore, to further investigate any functional consequences of the differences between these two enzymes, it would be valuable to develop an efficient expression system for the rat enzyme to obtain sufficient quantities of functional enzyme so that the catalytic and the inhibitor binding properties of the human with the rat can be directly compared.

Recombinant human liver MAO A has been successfully overexpressed in the *P. pastoris* expression system and purified in a high yield [6]. Ma *et al.* have expressed N-terminal His-tagged full-length rat MAO A in *Saccharomyces cerevisiae* [4] and purified 10 mg of rat MAO A per liter of culture. In our laboratory, taking advantage of the *P. pastoris* expression system, rat MAO A is expressed at a high level with ~200 mg of purified enzyme obtained from a 1-L fermentation culture. Previous studies have shown that the binding properties of rat MAO A to ion-exchange columns differs from that of human MAO A [7] which suggests that, in spite of the high degree of sequence homology, human MAO A and rat MAO A exhibit different surface electrostatic properties. In this work, rat MAO A is purified using a modified procedure from the human MAO A purification. The basic functional properties of purified rat MAO A are characterized and compared with those of the human enzyme.

Materials and Methods

Materials

A male Fisher 344 (F344) rat liver cDNA library was the generous gift from Dr. Edward Morgan, Emory University, Department of Pharmacology. The plasmid (pPIC3.5K) and yeast strain KM71 were purchased from Invitrogen Corp. All media for spheroplast transformation and cell growth were prepared as described in the Invitrogen Multi-Copy *Pichia* Expression Kit manual. The antibiotic G418 was purchased from Mediatech (Herndon, VA). β -Octylglucopyranoside was from Anatrace Inc. Reduced Triton X-100 was from Fluka. All the other chemicals were purchased from Sigma-Aldrich.

cDNA cloning of rat MAO A

A 1581 bp cDNA sequence encoding the full-length rat MAO A was amplified by PCR using the rat liver cDNA library as the template and the following oligonucleotides as the primers: rMAOA-F: 5'-GCGGGATCCATGACGGATCTGGAGAAG-3'; rMAOA-R: 5'-CGCGAATTCTCAGCATGGGAGCTTCTTAAT-3'. Underlining in the rMAOA-F and rMAOA-R denotes the restriction sites of *Bam*HI and *Eco*RI, respectively. The isolated fragment was subcloned into a *Bam*HI-*Eco*RI-digested pPIC3.5K vector. Gene sequence determinations were carried out by Agencourt Bioscience Corporation.

Transformation of rat MAO A gene into *P. pastoris*

The pPIC3.5K/rMAO A construct was linearized with *Sal*I to target integration at the *HIS4* locus in the *Pichia* genome. Spheroplast transformation of the rat MAO A gene into the yeast strain KM71 was performed by the method described previously [6].

Expression of rat MAO A

Several G418-resistant transformants were chosen to perform shake-flask expression screening as instructed in the Invitrogen *Pichia* manual. After examination by activity assay and Western blot analysis, the clone which produces the largest quantity of rat MAO A was chosen for fermentation growth and expression. Fermentation cultures expressing rat MAO A was carried out according to the same protocol as for human MAO A [6]. The cells were harvested by

centrifugation at $1500 \times g$ for 10 min at 4°C . After washing with breakage buffer (50 mM sodium phosphate, 5% glycerol, 1 mM EDTA, 1 mM PMSF, pH 7.2), the cells were stored at -80°C .

Isolation of mitochondria and OMM from *P. pastoris*

Intact mitochondria from *P. pastoris* cells expressing rat MAO A were isolated by enzymatic disruption of the yeast cell walls using zymolyase followed by suspension in a glass homogenizer and sequential centrifugations [8]. The outer mitochondrial membrane (OMM) was isolated by osmotic shock and mild sonication of intact mitochondria followed by 30–55% (w/v) sucrose density gradient centrifugation in 10 mM Tris-HCl buffer (pH 7.4) at 20,000 rpm (Beckman SW-28 rotor) for 15 hours. The OMM pellets were suspended in 10 mM Tris-HCl buffer containing 10% (v/v) glycerol at a protein concentration of 10 mg/mL and kept frozen in small aliquots at -80°C until used.

Purification of rat MAO A

Cell paste from a 1 liter of culture was suspended in 1 liter of breakage buffer with an equal volume of silica-zirconia beads (0.5 mm in diameter) and then disrupted in a Biospec Beadbeater (Bartlesville, OK) with 6 cycles of 2-min beating and 5-min chilling on the ice. After removal of the glass beads by filtration through a layer of Miracloth (Calbiochem), the cell lysate was separated from unbroken cells and large cell debris by centrifugation at $1500 \times g$ for 10 min at 4°C . The supernatant, which is termed the cell lysate, was centrifuged at $100,000 \times g$ for 25 min at 4°C to isolate the membrane fraction. The pellet was resuspended to a protein concentration of 25 mg/mL in 10 mM potassium phosphate, pH 7.2 and then digested by addition of 1 mg of phospholipase C and 6700 units of phospholipase A_2 per 500 mg of protein with the addition of CaCl_2 to 25 mM final concentration. The digestion reaction was stirred at room temperature in the dark for 1 hour with the pH maintained at 7.0–7.2 by the addition of dilute NH_4OH . The digestion mixture was centrifuged at $100,000 \times g$ for 15 min at 4°C and the pellet was resuspended into 10 mM potassium phosphate at pH 7.2 to a protein concentration of approximately 15 mg/mL. Triton X-100 was added to a final concentration of 0.5% (w/v) and the mixture was stirred at room temperature in the dark for 30 min. After centrifugation at $100,000 \times g$ for 15 min at 4°C , the Triton X-100 extract was collected and loaded onto a fast flow DEAE-Sephacrose column (25×600 mm) which was charged with 5 column volumes of 250 mM potassium phosphate (pH 7.2) and pre-equilibrated with 10 column volumes of 10 mM potassium phosphate containing 20% glycerol (pH 7.2). After washing with 10 column volumes of 10 mM potassium phosphate containing 20% glycerol and 1 mM PMSF (pH 7.2) to remove any unbound protein, the enzyme was eluted with a linear gradient of 10 to 200 mM potassium phosphate buffer containing 20% glycerol, 30 μM DTT, 0.8% (w/v) β -octyl-glucopyranoside, 0.5 mM d-amphetamine and 1 mM PMSF (pH 7.2). The enzyme fractions were pooled according to $A_{280/456}$ ratios and specific activities. After concentration to 30 μM using an Amicon Ultra 30K centrifugal filter (Millipore), the salt concentration of the enzyme was adjusted to 50 mM potassium phosphate and the purified rat MAO A preparation was stored at 4°C in 50% (v/v) glycerol.

The protein concentrations of all samples were determined using the Biuret method [9] except for the purified protein in which the Bearden method [10] was used.

Mass spectral analysis

A sample for MALDI-TOF-MS experiments was prepared as follows: the rat MAO A band was excised from a 2-D gel, destained (10 mM ammonium bicarbonate/50% acetonitrile/water) and dried in a Speed Vacuum. The gel slices were treated with a freshly prepared solution of trypsin (0.05 $\mu\text{g}/\mu\text{l}$ of trypsin in 10 mM ammonium bicarbonate/water) and digestion buffer (10 mM ammonium bicarbonate) and incubated at 37°C overnight. The reaction was stopped by adding 10% (v/v) trifluoroacetic acid, the digestion mixture was removed, and stored at

–20°C. Peptides were extracted from gel slices using 150 µL of 10 mM ammonium bicarbonate/60% acetonitrile/water. All digestion mixtures were collected, concentrated and reconstituted in 10 µL of 10 mM ammonium bicarbonate/60% acetonitrile/water. The aliquots were then desalted using ZipTip_{C18} treatment equilibrated with 0.1% trifluoroacetic acid, and eluted with 70% acetonitrile/0.1% trifluoroacetic acid/water. The in-gel trypsin digested rat MAO A peptide sample was analyzed on a Bruker Daltonics Ultraflex II MALDI-TOF/TOF-MS (Billerica, MA). The matrix used for MALDI-TOF/TOF-MS was α -cyano-4-hydroxycinnamic acid and peptide samples were analyzed in the positive ionization mode.

Amino-terminal sequence analysis

Purified recombinant rat MAO A (40µg) was electrophoresed on SDS-PAGE gel and blotted to a polyvinylidene fluoride (PVDF) membrane stained with Coomassie Blue. Edman degradation analysis is carried out by the Emory University Microchemical Facility.

Thermal stability

Human MAO A (10 µM), and rat MAO A (10 µM) in 50 mM potassium phosphate containing 20% glycerol, 30 µM DTT, 0.8% (w/v) β -octyl-glucopyranoside, (pH 7.2).were incubated at three different temperatures: 0°C, 25°C, 30°C. The enzyme activity was determined over a 2-hour period. Every 10 minutes, 5 µL aliquots were removed for the determination of catalytic activity. For both human MAO A and rat MAO A, the rate of 1 mM kynuramine oxidation in 50 mM HEPES with 0.5% reduced Triton X-100 (pH 7.5) was monitored at 316 nm ($\epsilon=11,800 \text{ M}^{-1}\text{cm}^{-1}$).

Steady-state kinetic studies

All rat MAO A activity assays were performed in 50 mM potassium phosphate assay buffer (pH 7.5) with 0.5% (w/v) reduced Triton X-100 and the rate of product formation was spectrophotometrically determined using a Perkin-Elmer Lambda 2 UV-Vis spectrophotometer at 25°C. d-Amphetamine, added for enzyme stabilization, was removed by gel filtration (Sephadex G-25) before performing any steady-state kinetic studies. Units of enzyme activity are defined as the amount of enzyme catalyzing the oxidation of 1 µmole kynuramine/min.

Determination of $K_m(\text{O}_2)$

Assays at 25°C in 50 mM potassium phosphate, pH 7.5, containing 0.5% reduced Triton X-100 were followed spectrophotometrically by the oxidation of kynuramine. Oxygen concentrations were varied by equilibration of the buffer with 10%, 50% oxygen/nitrogen mixtures in stoppered cuvettes. The final concentrations of O_2 were measured polarographically using a Model 782 Oxygen Meter (Strathkelvin Instruments Limited).

Results

Cloning and expression of rat MAO A

For integration into the KM71 strain, the rat MAO A gene (cloned into pPIC3.5K vector) was linearized with *Sall* for insertion at *HIS4* and His^+ Mut^s recombinants in KM71 strain. The His^+ transformants was initially selected on minimal media plates without histidine followed by screening for varying levels of Geneticin resistance. Ten transformants were randomly selected from Geneticin-resistant colonies for small scale expression analysis. From determinations of enzymatic activity and western blot analysis, the transformant which exhibited the highest level of expression of rat MAO A was chosen for scale-up expression. PCR analysis of the selected *Pichia* transformant confirmed integration of the rat MAO A gene

into the *Pichia* genome. Primers targeting the rat MAO A gene were used and resulted in a 1.5 kb band corresponding to the gene encoding rat MAO A was observed on a 0.7% agarose gel.

Properties of rat MAO A in intact mitochondria and OMM

Monoamine oxidases have been found to be localized to the outer mitochondrial membrane in all mammals. To investigate if rat MAO A overexpressed in *P. pastoris* is also situated in the outer mitochondrial membrane, both intact mitochondria and outer mitochondrial membrane were isolated and catalytic activities were determined. Due to light scattering in the UV region resulted from the turbidity of membrane particles, Amplex Red-peroxidase coupled assays, which detects H₂O₂ produced by MAO reacting amine substrates (e.g. kynuramine or benzylamine) at 560 nm ($\epsilon_{560} = 54,000 \text{ M}^{-1} \text{ cm}^{-1}$), was used to determine MAO activity. Specific activities of rat MAO A have been found to be 0.026 U/mg and 0.180 U/mg in intact mitochondria and OMM, respectively, suggesting expression of rat MAO A in *Pichia* retains its natural organelle localization.

Purification and characterization of rat MAO A

The purification scheme for recombinant rat MAO A (Table 1) shows that cells from a 1 liter fermentation culture contain 687 units of rat MAO A which is purified in a 43% yield from the crude cell extract. Rat MAO A binds to the DEAE-Sepharose less tightly than does wild-type human MAO A since 25 mM potassium phosphate buffer, (used for purification of wild-type human MAO A) interferes with enzyme binding. Therefore, a lower salt concentration (10 mM potassium phosphate) was used for all steps prior to column fractionation, and resulted in reproducible binding behavior to the Sepharose matrix. The specific activity (kynuramine assay) of purified rat MAO A (1.42 U/mg) is similar to that of human MAO A (1.7 U/mg) [6]. These data show that the expressed rat MAO A constitutes 12.6% of the *Pichia* OMM protein and 1.8% of total mitochondrial protein.

The purity of rat MAO A at each purification step using SDS-polyacrylamide gel electrophoresis is shown in Figure 1A. The main bands of each lane have the same molecular weights (60 kD) which represent MAO A. The purified rat enzyme cross-reacts with antisera raised against human MAO A by Western blot analysis (Figure 1B). Rat MAO A expressed in *P. pastoris* contains a covalent flavin cofactor as detected by an antisera specific for the covalent flavin moiety (Figure 1C) [11]. From the known X-ray data [4], this covalent flavin is an 8 α -S-cysteiny FAD.

Mass spectral analysis

The amino acid sequence of purified rat MAO A was verified by in-gel digestion and MALDI-TOF-MS analyses of tryptic peptide fragments. Figure 2A and 2B show the peptide mass profile of the trypsin-digested rat MAO A in two different intensity scales (Y). Figure 2B shows the peaks with lower intensities that are not obvious in Figure 2A. The observed matched and calculated mass peaks of trypsin-digested peptides of rat MAO A are listed in Table 2. The missing peptides in the mass spectrum might be due to either incomplete digestion or to inefficient elution of peptides from the de-salting C18 Zip-Tip column.

Previous studies on human MAO A have shown that the methionine at the N-terminus is acetylated resulting in resistance to standard Edman sequencing [6]. To identify whether an N-terminal modification exists on recombinant rat MAO A, the enzyme was subjected to Edman analysis. The following sequence: X-Asp-Leu-Glu-Lys-Pro-Asn-Leu-Ala (X was not identified in sequencing analysis, but is at the position of threonine in the coding sequence) is found. These data suggest that the N-terminal methionine residue of rat MAO A is cleaved on protein processing and that the amino group of the penultimate residue (threonine) in the

sequence is modified and possibly acetylated. This situation is similar to that found in *Pichia*-expressed human MAO B (an acetylated N-terminal seryl residue) [12].

UV/Vis spectral properties and functional characterization

To estimate the functionality of rat MAO A after purification, the level of anaerobic reduction of the flavin cofactor by the substrate was determined by the spectral changes of the flavin absorbance (456 nm) on addition of an excess of tyramine. As shown in Figure 3, after addition of the excess substrate tyramine, the absorption maximum of the flavin (the solid line, 1) is significantly reduced corresponding to its reduction to the hydroquinone form (the dashed line, 2). To further confirm if the flavin cofactor was completely reduced, an excess reducing reagent (sodium dithionite) was added to the protein solution with only a small further reduction of flavin cofactor observed (the dotted line, 3). Comparison of the spectral changes of flavin cofactor after the sequential additions of tyramine and sodium dithionite shows the purified rat preparation is ~96% functional.

As shown in Figure 4, addition of the acetylenic mechanism-based irreversible inhibitor clorgyline to purified enzyme results in the stoichiometric conversion to its covalent flavin cofactor to an N(5) flavocyanine adduct ($\epsilon_{410} = 23,400 \text{ M}^{-1} \text{ cm}^{-1}$), further confirming that the preparation of purified rat MAO A is nearly fully functional.

Thermal stability

As reported previously, purified dimeric human MAO B [13], exhibits a higher thermal stability than the partially dimeric human MAO A in detergent solutions [2]. Therefore, thermal stability experiments were performed to investigate if purified rat MAO A is thermally more stable than human MAO A. The data in Figure 5 show that both purified human and rat MAO A remain fully active when incubated at 0°C for 120 minutes. At 25°C, only 10% of rat MAO A activity is lost after the 120-minute incubation, whereas human MAO A loses ~60% of its catalytic activity. Rat MAO A retains ~80% catalytic activity after incubation at 30°C for 120 minutes; while more than ~80% of human MAO A activity is lost.

Catalytic properties

The steady-state kinetic parameters for rat MAO A compared with the published values for human MAO A are shown in Table 3. Rat MAO A exhibits a much higher catalytic efficiency (k_{cat}/K_m) (40-fold increase) for oxidation of benzylamine relative to human MAO A. For the substrates kynuramine and serotonin, the turnover numbers and K_m values of rat MAO A do not differ significantly from those of human MAO A. Rat MAO A catalyzes the oxidation of phenylethylamine, a MAO B specific substrate, with a ~7 fold higher catalytic efficiency than does human MAO A.

The K_i values exhibited by rat MAO A for the reversible inhibitors, isatin, phentermine and d-amphetamine, are essentially identical with those found with human MAO A. The MAO B-specific reversible inhibitors, such as farnesol, chlorostyrylcaffeine and 1,4-diphenyl-2-butene show no observable inhibitory activity with rat MAO A as shown previously with human MAO A [14].

Determination of K_m (O_2)

The activity of rat MAO A was measured at different concentrations of oxygen with kynuramine as the amine substrate. As shown in Figure 6, the Hanes-Woolf plots of rat MAO A with oxygen as the variable substrate intersect either on or very close to the y axis which obeys the following equation, indicating rat MAO A follows a ternary-complex mechanism which is similar to that of human MAO A [15].

$$v = \frac{Vab}{K_{ia}K_{mb} + K_{mb}a + K_{ma}b + ab}$$

where v is observed velocity, V is maximum velocity, a is the concentration of amine, b is the concentration of oxygen, and K_{ma} and K_{mb} are the Michaelis constants for a and b , respectively. K_{ia} is the dissociation constant for a .

The calculated value for $K_m(\text{O}_2)$ of rat MAO A is 9.3 μM ; a value similar to that exhibited by human MAO A (~6 μM) [15].

Discussion

This study demonstrates that, as shown previously for human MAO A, the rat MAO A gene can be incorporated into the *Pichia* genome to result in the high level expression of the enzyme and localization to the outer mitochondrial membrane of this methylotrophic yeast. The amount of rat MAO A produced in the *Pichia* expression system is 50-fold higher than the level reportedly expressed in *S. cerevisiae*. Similar differences are observed on comparison of human MAO A expression in *Saccharomyces* with that in *Pichia*. The most likely explanation for this differential expression is that *Pichia* is grown with glycerol as carbon source resulting in a very aerobic metabolism and therefore a higher level of mitochondria are expected whereas *Saccharomyces* is grown on a glucose containing medium resulting in a more anerobic metabolism and therefore fewer mitochondria.

It is of interest that, as observed in purification profile of recombinant rat liver MAO A, a low concentration of potassium phosphate buffer is required for efficient enzyme binding to the DEAE-Sepharose anion exchange column. The interaction of proteins with the column resin depends upon the distribution of charge residues on the surface of the proteins. Since DEAE-Sepharose is a weak anion exchanger, the negative surface charges on MAO should facilitate the binding of the enzyme. Electrostatic studies (from Pymol structures) show that sums of formal charges for both enzymes are identical (+2). However, as shown in Supplementary Material (Figure S1), negative patches (in red) on the surface of human MAO A are more concentrated in the region that is expected to protrude into the aqueous milieu from the detergent micelle than in rat MAO A. These considerations probably account for the stronger binding of human MAO A to the positively charged DEAE-Sepharose resin. The salt concentration that allows binding of human MAO A (25 mM potassium phosphate) to the resin must be decreased to 10 mM for the efficient binding of rat MAO A.

Cleavage of N-terminal methionine residue and N-terminal acetylation are the two post-translational processes occurring in eukaryotes and of biological significance [16,17]. Previous study has shown that the N-terminal methionine is not cleaved with human MAO A expressed in *P. pastoris* but is acetylated [6] with its N-terminal sequence (N-Met-Asn-Gln-Glu-Lys-Ala-Ser-Ile-Ala) corresponding to the consensus sequence required for the NatB class of N-terminal acetyltransferase [18]. Sherman et. al. have shown that methionine at N-termini are cleaved from the penultimate residues that exhibit radii of gyration of 1.29 Å or less (Gly, Ala, Ser, Cys, Thr, Pro and Val) [19]. With regard to rat MAO A, the second residue in its N-terminal sequence (N-Met-Thr-Asp-Leu-Glu-Lys-Pro-Asn-Leu-Ala) is Thr which correlates with the sequence for methionine cleavage, further validating the suggestion from Edman degradation analysis suggests that the amino terminal residue threonine is blocked probably by acetylation and that the coded methionine residue is cleaved on protein processing.

Rat MAO A exhibits a higher thermal stability than does human MAO A. Since MAOs are outer mitochondrial membrane proteins, most biochemical studies on these purified enzymes

must be performed in detergent solution. The oligomeric states of the two MAOs would be key factors affecting their relative stabilities. Therefore, the different thermal stabilities observed between human and rat MAO A's might be explained by the fact that in their detergent-solubilized form, the human MAO A dimer exhibits a weaker formation constant than does rat MAO A.

Different catalytic efficiencies observed on comparison of rat MAO A and human MAO A show that rat MAO A is a more efficient catalyst than human MAO A. The rat enzyme is more efficient in catalyzing the oxidation of traditional "MAO B" specific substrates, such as benzylamine and phenylethylamine. Analysis of steady state kinetic data show rat MAO A catalysis follows a ternary-complex mechanism (as does human MAO A). No differences are observed for reversible inhibitor affinities between the two enzymes. Although the two enzymes exhibit ~90% identity, their comparative functional and structural differences demonstrated in this study suggests caution should be used in the extrapolation of results obtained with the rat enzyme to the human.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the grant from the National Institutes of Health (GM29433). We thank Dr. Jan Pohl from Emory University Microchemical Facility for performing N-terminal sequencing analysis and mass spectrometry of the tryptic peptide digestion.

Abbreviation

MAO A	monoamine oxidase A
OMM	outer mitochondrial membrane
FAD	flavin adenine dinucleotide

References

1. Weyler W, Hsu YP, Breakefield XO. Biochemistry and genetics of monoamine oxidase. *Pharmacol Ther* 1990;47:391–417. [PubMed: 2290855]
2. De Colibus L, Li M, Binda C, Lustig A, Edmondson DE, Mattevi A. Three-dimensional structure of human monoamine oxidase A (MAO A): Relation to the structures of rat MAO A and human MAO B. *Proc Natl Acad Sci U S A* 2005;102:12684–12689. [PubMed: 16129825]
3. Son SY, Ma J, Kondou Y, Yoshimura M, Yamashita E, Tsukihara T. Structure of human monoamine oxidase A at 2.2-angstrom resolution: the control of opening the entry for substrates/inhibitors. *Proc Natl Acad Sci U S A* 2008;105:5739–5744. [PubMed: 18391214]
4. Ma J, Yoshimura M, Yamashita E, Nakagawa A, Ito A, Tsukihara T. Structure of rat monoamine oxidase A and its specific recognitions for substrates and inhibitors. *J Mol Biol* 2004;338:103–114. [PubMed: 15050826]
5. Fierro A, Osorio-Olivares M, Cassels BK, Edmondson DE, Sepulveda-Boza S, Reyes-Parada M. Human and rat monoamine oxidase-A are differentially inhibited by (S)-4-alkylthioamphetamine derivatives: Insights from molecular modeling studies. *Bioorg Med Chem* 2007;15:5198–5206. [PubMed: 17521909]
6. Li M, Hubalek F, Newton-Vinson P, Edmondson DE. High-level expression of human liver monoamine oxidase A in *Pichia pastoris*: comparison with the enzyme expressed in *Saccharomyces cerevisiae*. *Protein Expr Purif* 2002;24:152–162. [PubMed: 11812236]

7. Dennick RG, Mayer RJ. Purification and immunochemical characterization of monoamine-oxidase from rat and human liver. *Biochem J* 1977;161:167–174. [PubMed: 851417]
8. Daum G, Bohni PC, Schatz G. Import of proteins into mitochondria - cytochrome-b₂ and cytochrome-c peroxidase are located in the intermembrane space of yeast mitochondria. *J Biol Chem* 1982;257:13028–13033. [PubMed: 6290489]
9. Layne E. Spectrophotometric and turbidimetric methods for measuring proteins. *Method Enzymol* 1957;3:447–454.
10. Bearden JC. Quantitation of sub-microgram quantities of protein by an improved protein-dye binding assay. *Biochim Biophys Acta* 1978;533:525–529. [PubMed: 647023]
11. Barber MJ, Eichler DC, Solomonson LP, Ackrell BA. Anti-Flavin Antibodies. *Biochem J* 1987;242:89–95. [PubMed: 3109386]
12. Newton-Vinson P, Hubalek F, Edmondson DE. High-level expression of human liver monoamine oxidase B in *Pichia pastoris*. *Protein Expr Purif* 2000;20:334–345. [PubMed: 11049757]
13. Binda C, Newton-Vinson P, Hubalek F, Edmondson DE, Mattevi A. Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. *Nat Struct Biol* 2002;9:22–26. [PubMed: 11753429]
14. Hubalek F, Binda C, Khalil A, Li M, Mattevi A, Castagnoli N, Edmondson DE. Demonstration of isoleucine 199 as a structural determinant for the selective inhibition of human monoamine oxidase B by specific reversible inhibitors. *J Biol Chem* 2005;280:15761–15766. [PubMed: 15710600]
15. Ramsay RR. Kinetic mechanism of monoamine oxidase A. *Biochemistry* 1991;30:4624–4629. [PubMed: 2021654]
16. Bradshaw RA, Brickey WW, Walker KW. N-terminal processing: the methionine aminopeptidase and N-alpha-acetyl transferase families. *Trends Biochem Sci* 1998;23:263–267. [PubMed: 9697417]
17. Arfin SM, Bradshaw RA. Cotranslational processing and protein turnover in eukaryotic cells. *Biochemistry* 1988;27:7979–7984. [PubMed: 3069123]
18. Tercero JC, Wickner RB. Mak3 Encodes an N-Acetyltransferase Whose Modification of the L-a Gag NH₂ Terminus Is Necessary for Virus Particle Assembly. *J Biol Chem* 1992;267:20277–20281. [PubMed: 1400344]
19. Sherman F, Stewart JW, Tsunasawa S. Methionine or Not Methionine at the Beginning of a Protein. *Bioessays* 1985;3:27–31. [PubMed: 3024631]
20. Miller JR, Edmondson DE. Structure-activity relationships in the oxidation of para-substituted benzylamine analogues by recombinant human liver monoamine oxidase A. *Biochemistry* 1999;38:13670–13683. [PubMed: 10521274]
21. Nandigama RK, Miller JR, Edmondson DE. Loss of serotonin oxidation as a component of the altered substrate specificity in the Y444F mutant of recombinant human liver MAO A. *Biochemistry* 2001;40:14839–14846. [PubMed: 11732903]
22. Nandigama RK, Newton-Vinson P, Edmondson DE. Phentermine inhibition of recombinant human liver monoamine oxidases A and B. *Biochem Pharmacol* 2002;63:865–869. [PubMed: 11911838]

Figure 1. SDS-polyacrylamide gel electrophoresis of rat MAO A. (A) Coomassie-stained gel (7.5%) of rat MAO A at different purification steps: lane 1: protein MW standards; lane 2: wild-type human MAO A (control); lane 3: cell lysate; lane 4: membrane fraction; lane 5: after phospholipase digestion; lane 6: after Triton X-100 extraction; lane 7: purified rat MAO A. (B) Western blot of rat MAO A detected by antisera raised against human MAO A. (C) Western blot of rat MAO A detected by antisera specific for the covalent flavin cofactor.

Figure 2. MALDI-TOF/TOF-MS analysis of the trypsin digested rat MAO A expressed in *P. pastoris*. (A) Peptide mass profile shown in original intensity scale. (B) The same peptide mass spectrum as shown in panel A in an expanded intensity scale to show the peaks with weaker intensities.

Figure 3.

UV-Vis absorption spectral changes of purified rat MAO A upon anaerobic reduction by tyramine in 50 mM potassium phosphate containing 20% glycerol and 0.8% (w/v) β -octylglucopyranoside, pH 7.2. Line 1 (—) is the spectrum of the purified rat MAO A before the addition of tyramine. Line 2 (---) is the spectrum after the addition of 2-fold molar excess tyramine. Line 3 (·····) is the spectrum after the addition of a few crystals of sodium dithionite.

Figure 4.

UV-Vis spectrum of purified rat MAO A before (spectrum 1, solid line) and after (spectrum 2, dashed line) the addition of 10-fold molar excess of clorgyline. The reaction was performed in 50 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol and 0.8% (w/v) β -octylglucopyranoside.

Figure 5. Comparison of thermal stabilities of human MAO A (A) and rat MAO A (B). Profiles of remaining enzyme activities versus incubation time at 0°C, 25°C and 30°C, respectively, are shown.

Figure 6. Effect of the oxygen concentration on the rates of oxidation of various kynuramine concentrations by rat MAO A. The oxygen concentrations were 38 μM (—■), 146.5 μM (—○) and 245 μM (—▲), respectively. The inset is a Hanes-Woolf plot of effect of oxygen concentration on V_{max} values at saturated kynuramine concentrations.

Table 1Purification scheme for recombinant rat liver MAO-A expressed in *P. pastoris*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
cell lysate	27,010	687	0.025	100
membrane fraction	12,464	596	0.048	87
phospholipase digestion	11,938	472	0.040	69
Triton X-100 extraction	2,441	432	0.177	63
DEAE sepharose elution pooled fractions	207	294	1.42	43

Table 2

Observed and computed monoisotopic masses of the tryptic peptides of recombinant rat MAO A expressed in *P. pastoris*.

Position of cleavage site	Resulting peptide sequence	Observed Peptide mass (Da)	Calculated Peptide mass (Da)
45	INVLVLEAR	1026.846	1026.244
76	WVDVGGAYVGPTQNR	1619.061	1618.769
129	GAFPVWNPLAYLDYNNLWR	2406.965	2406.728
147	EIPVDAPWQAR	1281.904	1281.434
237	FVGGSGQVSEQIMGLLGDK	1922.368	1922.183
291	IHFKEPPEPPER	1362.997	1362.594
395	VLGSQEALYPVHYEEK	1862.219	1862.069
421	NWCEEQYSGGCYTAYFPPGIMTQYGR	3021.116	3022.331
454	IYFAGTETATQWSGYMEGAVEAGER	2725.423	2724.938
493	DVPAIEITHFLER	1641.162	1640.857

Table 3
Comparison of substrate and inhibitor specificities for purified rat and human MAO A

	Rat MAO A				Human MAO A			
	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)		
Substrate	Benzylamine	19.5±0.2	0.21±0.02	92.9±8.9	1.04±0.15 ^a	2.4±0.4 ^a		
	Kynuramine	149.1±1.7	0.09±0.01	1656.7±185.0	0.13±0.01 ^b	964.6±98.9 ^b		
	Phenylethylamine	85.0±0.7	0.35±0.01	242.9±7.2	1.48±0.08 ^c	36.4±2.1 ^c		
	Serotonin	358.7±6.7	0.30±0.02	1195.7±82.8	0.30±0.05 ^c	583.7±97.5 ^c		
Reversible Inhibitor				K_i (μM)				
	Isatin		10.5±1.8		15 ^d			
	Phentermine		625.6±81.9		498 ^e			
	d-Amphetamine		6.0±0.5		3.7±0.5			
	Farnesol		No Inhibition		No Inhibition			
	Chlorostyrylcaffeine		No Inhibition		No Inhibition			
	1,4-Diphenyl-2- butene		No Inhibition		No Inhibition			

^afrom Miller *et al.* [20]

^bfrom Nandigama *et al.* [21]

^cfrom Li *et al.* [6]

^dMin Li, Ph.D. dissertation, Emory University, 2006.

^efrom Nandigama *et al.* [22]