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Immobilized enzyme reactors based upon the flavoenzymes monoamine oxidase A and B

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Abstract

Monoamine oxidase (MAO) catalyzes the oxidative deamination of amines. The enzyme exists in two forms, MAO-A and MAO-B, which differ in substrate specificity and sensitivity to various inhibitors. Membrane fractions containing either expressed MAO-A or MAO-B have been non-covalently immobilized in the hydrophobic interface of an immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The MAO-containing stationary phases were packed into glass columns to create on-line immobilized enzyme reactors (IMERs) that retained the enzymatic activity of the MAO. The resulting MAO-IMERs were coupled through a switching valve to analytical high performance liquid chromatographic columns. The multi-dimensional chromatographic system was used to characterize the MAO-A (MAO-A-IMER) and MAO-B (MAO-B-IMER) forms of the enzyme including the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions as well as the determination of IC_{50} values. The results of the study demonstrate that the MAO-A-IMER and the MAO-B-IMER can be used for the on-line screening of substances for MAO-A and MAO-B substrate/inhibitor properties.

Keywords: Immobilized enzyme reactors; Flavoenzymes; Monoamine oxidases

1. Introduction

Monoamine oxidase (MAO), a flavin-adenosine-dinucleotide (FAD)-containing enzyme, is located in the mitochondrial outer membrane and is widely distributed in various tissues. The enzyme is active in extraneuronal cells, nerves and organs such as the heart and liver [1]. MAO is responsible for the oxidative deamination of primary, secondary and tertiary amines. In this process, the amine moiety is oxidized producing an imine that is then hydrolyzed, to creating an aldehyde, Fig. 1. The enzymatic reaction proceeds with the concomitant reduction of FAD. The prosthetic group is then reoxidized by molecular oxygen to generate hydrogen peroxide, Fig. 1.

MAO exists in two different isoforms, MAO-A and MAO-B [2]. The two forms display overlapping distribution in various tissues, but differ in substrate specificity and inhibitor sensitivity; for example, MAO-A inhibitors are used in the treatment of mental disorders such as depression while MAO-B inhibitors are used in the treatment of neurological disorders such as Parkinson's and Alzheimer's [3]. In addition, recent studies have identified a MAO-B inhibitor as a component of tobacco smoke, suggesting that MAO inhibition contributes to the addictive properties of tobacco [4].

The pharmacological and therapeutic importance of MAO inhibitors necessitates the development of methods to rapidly identify these compounds in complex chemical and biological mixtures [5,6]. One approach to the creation of the necessary technology is to immobilize the MAO on a liquid chromatographic support to create an immobilized enzyme reactor (IMER). In the appropriate systems, the immobilized enzymes retain their enzymatic activities and sensitivities to inhibitors. The use of immobilized enzymes in analytical liquid phase separation systems has been extensively reviewed [7].

The immobilization of biopolymers was initially reported in the 1960s and initiated their wide use in industrial and scientific fields [8]. Technological developments in the immobilization of enzymes has allowed for their use in industrial

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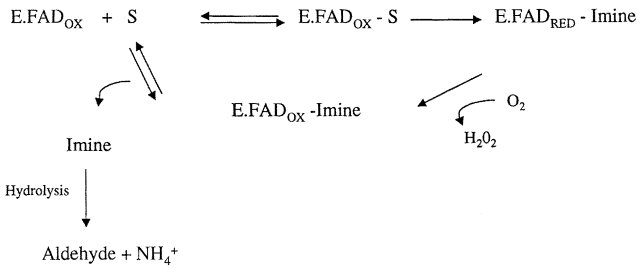

Fig. 1. Mechanism of oxidative deamination by MAO.

processes and in the medical sectors [9]. For example, immobilized lipases have proven useful for the production of biologically active compounds [10] and penicillin acylase reactors are used for the production of 6-aminopenicillanic acid [11]. Many of these enzymes were used in straightforward batch-wise reactions, allowing for the easy detection and production of the target products.

However, most biological systems are complex in nature and present complicated challenges such as enzymes that are cofactor dependent. The use of IMER technology is one approach that can be used to overcome these problems. IMERs have been created using non-cofactor dependent hydrolases such as α-chymotrypsin [12], trypsin [12] and lipase [13], and have been developed using co-factor dependent enzymes such as the NAD/NADH dependent horse liver alcohol dehydrogenase [14], D-glyceraldehyde-3-phosphate dehydrogenase [15] and cytochrome P450s [16], the ATP dependent glutamine synthetase [17], and transferases such as phenylethanolamine *N*-methyltransferase [18] and UDP-glucuronyltransferase [19]. These IMERs have been placed in standard high performance liquid chromatographic systems and used to carryout online synthesis [13,14,16,20,21] as well as standard Michaelis–Menten enzyme kinetic studies for the quantitative determination of enzyme kinetic constants such a K_m and V_{max} [12,15,17–23]. These systems can also be used to identify specific inhibitors, to provide information regarding the mode of inhibition and to calculate the K_i of the inhibitor using both zonal chromatography [12,15] and frontal chromatography techniques [22].

In this study, IMERs were developed using membranes from insect cells expressing human MAO-A and MAO-B [24,25]. The membranes were non-covalently immobilized on an immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The IAM interphase was derived from the covalent immobilization of 1-myristoyl-2-[{(13-carboxyl) tridecanoyl]-sn-3-glycerophospholine on aminopropyl silica, and resembles one-half of a cellular membrane [26]. The resulting IMERs retained their catalytic activities displaying distinct sensitivity to substrates and inhibitors and were used in a multi-dimensional chromatographic system. This represents the initial immobilization and on-line application of FAD-containing enzymes.

2. Experimental

2.1. Materials

Human monoamine oxidase-A (MAO-A) and human monoamine oxidase-B (MAO-B) SUPERSOMES™ were purchased from GenTest (Woburn, MA, USA). The enzymes were expressed from human cDNA using a baculovirus expression system and had representative catalytic activity determined using kynuramine deamination expressed as nmole substrate deamination/(min mg protein) of 142 and 54, respectively [24,25]. Benzylamine, kynuramine, benzaldehyde, 4-hydroxyquinoline, (*R*)-amphetamine, (*R*,*S*)-amphetamine, (1*S*,2*S*)-(+)-ψ-ephedrine, (1*R*,2*R*)-(−)-ψ-ephedrine and other chemicals unless otherwise stated were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC grade methanol and phosphoric acid were manufactured by J.T. Baker (Phillipsburg, NJ, USA) and purchased through Moquin Scientific (Montreal, Que., Canada). The IAM.PC (12 μm, 300 Å) non-endcapped chromatographic support was obtained from Regis Chemical Co. (Morton Grove, IL, USA). The IAM.PC bonded phase, according to the manufacturer, contains a near monolayer of C14 saturated phosphatidylcholine, covalently linked to silica through an amide link.

2.2. Apparatus and chromatographic procedures

Two modular HPLC systems were setup in order to carry out the on-line studies and the systems were connected as depicted in Fig. 2. System 1 consisted of a Thermo Separation Products P1000 pump (ThermoQuest, San Jose, CA, USA), a Rheodyne 7125 injector with a 100 μl sample loop (Rheodyne, Cotati, CA, USA), and the MAO-IMER of interest. System 2 consisted of a Thermo Separation Products P1000 pump, a 5 μm cyano (CN) stationary phase packed in 150 mm × 4.6 mm i.d. column (Regis Chemical Co., Morton

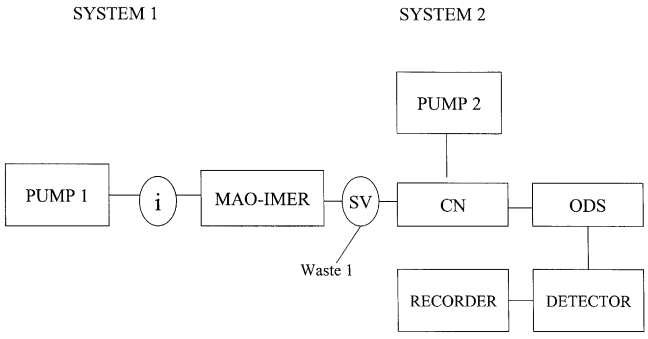

Fig. 2. Schematic representation of the coupled on-line MAO-IMER HPLC system, where systems 1 and 2 can be run independently or connected via a switching valve, see text for details. MAO-IMER: the respective monoamine oxidase immobilized enzyme reactor MAO-A or MAO-B; CN: cyano 150 mm × 4.6 mm i.d. column; ODS: octadecyl 250 mm × 4.6 mm i.d. column; SV: switching valve; i: injector.

Grove, IL), a 5 μm octadecyl (ODS) stationary phase packed in a 250 mm × 4.6 i.d. mm column (Regis Chemical Co., Morton Grove, IL) connected in series, a SpectraSystem UV200 ultraviolet detector, and data collection was carried out using a Thermo Separation Products Chromjet integrator interfaced with a computer equipped with WOW software (ThermoQuest). When desired, the eluent from system 1 was directed onto system 2 through a Rheodyne 7000 switching valve (SV).

System 2 was used independently of system 1 by replacing the latter system with a Rheodyne 7125 injector: (i) in order to analyze the results obtained from incubations involving non-immobilized MAO-A and MAO-B. The temperatures of the MAO-A-IMER and MAO-B-IMER were controlled with a Fiatron System CH-50 Column Heater (Fiatron, Oconomowoc, WI, USA).

2.3. Chromatographic conditions

The mobile phase on system 1 consisted of potassium phosphate buffer (50 mM, pH 7.5) with a flow rate of 0.2 ml/min. The mobile phase used on system 2 consisted of potassium phosphate buffer (50 mM adjusted to pH 2.0 with phosphoric acid). The solutes were quantitated using UV detection set at λ=254 nm. A flow rate of 1.0 ml/min and ambient temperature were used throughout the study.

2.4. Enzyme activity and inhibition studies on non-immobilized MAO-A and MAO-B

MAO-A and MAO-B activities were determined by quantitation of the amount of 4-hydroquinoline formed from the substrate, kynuramine. The individual enzymes were assayed as follows (final concentration): to 25 μl of enzyme solution (62.5 μg) was added 135 μl of phosphate buffer (50 mM, pH 7.5) and the solution was vortexed for 1 min. The resulting solution was preincubated for 20 min at 37 °C. After the preincubation, the reaction was started by the addition of 15 μl of kynuramine (50, 70, 100, 150, 200, 250 and 300 μM) and incubated for an additional 5 min. The reaction was stopped by the addition of 75 μl of 3 M HCl. The resulting mixture was centrifuged at 3000 × *g* for 10 min and the supernatant directly injected onto HPLC system 2 for analysis.

The effect of (*R*,*S*)-amphetamine, (*R*)-amphetamine, (1*S*,2*S*)-(+)-ψ-ephedrine, and (1*R*,2*R*)-(−)-ψ-ephedrine on the enzymatic activities of the non-immobilized enzymes was examined in the following manner: 25 μl of the enzyme solution (62.5 μg) was preincubated for 20 min at 37 °C in the presence of inhibitor in a total volume of 165 μl. After the preincubation, the reaction was started by the addition of kynuramine (150 μM) and incubated for an additional 5 min. The reaction was then stopped and analyzed as described above. Concentrations investigated for both MAO-A and MAO-B, for (1*S*,2*S*)-(+)-ψ-ephedrine and (1*R*,2*R*)-(−)-ψ-ephedrine were 0.1 to 10 mM and (*R*,*S*)-amphetamine and (*R*)-amphetamine the concentrations were 1.85–111 and 1.5–125 μM, respectively.

The kinetic parameters were determined using standard Michaelis–Menten approach utilizing Graph Pad Prism Version 3.02 (Graphpad Software, Inc., San Diego, CA, USA) for analysis. Lineweaver–Burke plots were used to calculate the Michaelis constant (K_m). The rates of reaction (V_{max}) were calculated using nmol/(mg min). The effect of the inhibitors was measured by comparing the remaining percentage of activity to that of an inhibitor-free control. Concentration-inhibition curves (percentage control activity versus log concentration of inhibitor) were drawn and the IC_{50} values for the respective inhibitors were determined.

2.5. Preparation of MAO immobilized enzyme reactors

The MAO-A and MAO-B IMERs were prepared utilizing HR 5/2 columns (5 mm × 25 mm) purchased from Pharmacia Biotech (Uppsala, Sweden). The columns were packed with the IAM stationary phase (100–125 mg) and were washed with the assay buffer, potassium phosphate (50 mM, pH 7.5), at a flow rate of 0.2 ml/min. Following washing, the respective enzyme solutions (125 μg/ml MAO-A and 250 μg/ml MAO-B) were prepared and allowed to recirculate through the columns at a flow rate of 0.2 ml/min for 18 h. The eluent from each IMER was collected and the amount of residual enzyme in the solution was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and by measuring the difference in the absorbance of the eluent before and after immobilization.

2.6. Procedure for on-line analyses using the coupled systems 1 and 2

When the coupled system was used, pump 2 on system 2 was stopped while pump 1 on system 1 was run at a flow rate of 0.2 ml/min, see Fig. 2. A 100 μl aliquot of the substrate was loaded into the injector (i) and the valve switched to the inject position at the same time the SV was switched such that the eluent from the MAO-IMER was directed onto the CN column of system 2. After elution of the substrate and product onto the CN column (2.5 min at a flow rate of 0.2 ml/min), the SV was again rotated to isolate the two systems, pump 2 was started and the separation of substrate and product carried out on the coupled CN and ODS columns.

2.7. Effect of flow rate and contact time

The effect of flow rate and contact time through the MAO-IMERs was investigated. Contact times from 0.17 to 5 min were investigated corresponding to flow rates of 0.1, 0.2, 0.4, 0.6, 1.0, 1.4, 2.0 and 3.0 ml/min. The recoveries of the substrate and product were determined.

2.8. Enzyme activity and inhibition studies on MAO-A-IMER and MAO-B-IMER

Enzymatic activities on the MAO-IMERs were determined by quantification of the amount of 4-hydroxyquinoline formed with a given amount of kynuramine. The temperature of the IMER unless otherwise stated was kept at 37 °C with a column heater. Stock solutions of kynuramine were prepared in potassium phosphate buffer (50 mM, pH 7.5). Enzymatic activity was examined carrying out injections of a series of substrate solutions. Solutions of kynuramine ranging from 50 to 100 μM were injected in duplicate. The solutions were injected onto the MAO-IMERs at a flow rate of 0.2 ml/min for a contact time of 2.5 min. The kinetic parameters were determined as described for the non-immobilized forms of the enzyme (see above). Results are expressed as mean ± standard error of the mean (S.E.M.).

The effect of inhibitors on the enzymatic activities of the MAO-IMERs was also examined. Injecting a series of substrate/inhibitor mixtures carried out the inhibition studies. On the MAO-A-IMER, concentrations investigated for (1*S*,2*S*)-(+)-ψ-ephedrine and (1*R*,2*R*)-(−)-ψ-ephedrine were 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 mM and 1.5, 3.0, 6.0, 12.0, 62.5 to 125 μM for (*R*,*S*)-amphetamine and (*R*)-amphetamine. For the MAO-B-IMER concentrations investigated for (1*S*,2*S*)-(+)-ψ-ephedrine were 4, 8, 16, 32, 64, 128, 200 and 250 mM and (1*R*,2*R*)-(−)-ψ-ephedrine were 4, 8, 16, 20, 32, 40, 80, 115 and 128 mM. Concentrations investigated for (*R*,*S*)-amphetamine were 0.096, 0.192, 1.00, 3.00, 5.00, 8.00 and 10.00 mM and for *R*-amphetamine they were 0.015, 0.095, 0.220, 0.440 and 0.880 mM. Concentration–inhibition curves were prepared and compared to the results obtained for the non-immobilized enzymes.

3. Results and discussion

3.1. Chromatographic separation of kynuramine and 4-hydroxyquinoline

The chromatographic system used to achieve the separation of kynuramine (substrate) and 4-hydroxyquinoline (product) is labeled as system 2 in Fig. 2. The required separation was produced by using columns containing CN and ODS stationary phases, coupled in series, and a mobile phase consisting of potassium phosphate buffer (50 mM, pH 2.0, adjusted with phosphoric acid). Under these conditions, the relative chromatographic retentions (k' values) of kynuramine and 4-hydroxyquinoline were 0.60 and 3.32, respectively, and the chromatography was completed in less than 25 min. Representative chromatograms of the separation are presented in Fig. 3A and B. However, both figures were obtained using the coupled system and a 2.5 min offset was produced by the connection of system 1, i.e. the MAO-A-IMER (Fig. 3A) or MAO-B-IMER (Fig. 3B), to system 2. The retention times and identities of kynuramine and 4-hydroxyquinoline were established by independent chromatography of the two compounds.

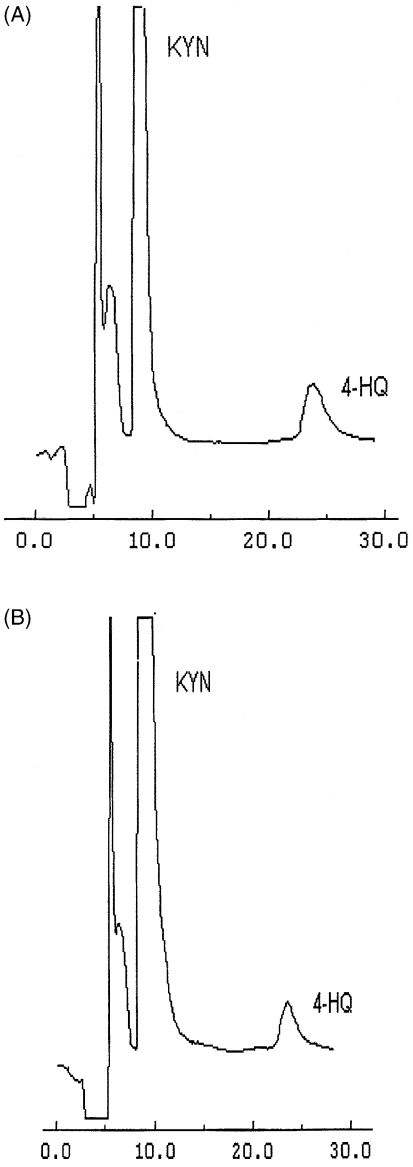

Fig. 3. Representative chromatograms of the on-line deamination of kynuramine (injection of 0.6 mM kynuramine): (A) reaction on MAO-A-IMER; (B) reaction on MAO-B-IMER. KYN: kynuramine; 4-HQ: 4-hydroxyquinoline.

3.2. Synthesis of the MAO-IMERs

After circulation of the solutions containing either MAO-A or MAO-B through the columns containing the IAM stationary phase, the analysis of the protein content of the circulating buffer was used as an indication of the amount of material immobilized on the stationary phase. Using the approach, it was determined that the MAO-A-IMER contained 125 ± 10 μg of protein immobilized onto 127 ± 1.1 mg of IAM (n = 3), while the

MAO-B-IMER contained 225 ± 10 μg of protein immobilized onto 127 ± 0.6 mg IAM ($n = 3$).

The determination of the amount of protein immobilized on the IAM stationary phase is only an indication that MAO had been retained on the stationary phase, not a direct measure of the amount of MAO present in the IMER. The only way to establish that MAO is present and active on the column is to assess the enzymatic activities of the MAO-IMERs. The retained MAO activities were established by injecting the known substrate kynuramine 100 μl of 0.15 mM kynuramine onto the MAO-A-IMER and 0.6 mM kynuramine onto the MAO-B-IMER and the eluent from the IMERs were concentrated onto system 2 containing the coupled analytical columns for separation and analysis. Fig. 3A and B display typical chromatographic profiles achieved on the MAO-A-IMER and MAO-B-IMER respectively, and demonstrate the enzymatic production of 4-hydroxyquinoline under optimal conditions.

3.3. Stability and storage of the MAO-IMERs

Over the 6-month period of this study, both of the MAO-IMERs remained active with a loss of less than 25% of their initial activity. When the MAO-IMERs were not in use they were stored at 4 °C in phosphate buffer (50 mM, pH 7.5) containing 0.01% sodium azide. The stabilities of the IMERs were investigated by washing the columns with pure buffer and injecting controls to compare the overall activity and stability.

3.4. Optimization of IMER activity

The optimal buffer and temperature for the MAO-mediated conversion of kynuramine to 4-hydroquinoline were potassium phosphate buffer (50 mM, pH 7.5) and 37 °C and these conditions were used in the studies with both the non-immobilized and immobilized form of the enzyme. With these conditions set, the key parameter for the optimization of the extent of the enzymatic conversion was the duration of the reaction. Previous studies of the hydrolysis of D-tryptophan methyl ester and L-tryptophan methyl ester on an IMER containing immobilized α-chymotrypsin demonstrated that product production was related to the time that the substrate was in contact with the enzyme and that contact time was a function of the rate of flow of the substrate through the IMER [12]. The fact that the enantiomeric esters had the same physicochemical properties was used to determine that the effect of flow rate on the observed hydrolytic activity of the immobilized α-chymotrypsin was the result of two interrelated processes—the rate of diffusion of the solute into the cavities containing the enzyme and the dissociation rate of the enzyme/product complexes. Studies with IMERs containing co-factor dependent enzymes such as rat liver microsomes [16] and brain glutamine synthetase [17] have confirmed the effect of flow rate on product formation.

Table 1
Effect of flow rate on the production of 4-hydroxyquinoline (4-HQ) for both immobilized enzyme reactor formats (MAO-A-IMER and MAO-B-IMER)

Flow rate (ml/min)	Recovery of 4-HQ on MAO-A-IMER (%)	Recovery of 4-HQ on MAO-B-IMER (%)
0.1	84	79
0.2	95	88
0.4	91	73
0.6	84	65
1.0	50	62
1.4	15	20
2.0	5	0
3.0	0	0

Thus, in this study, the performances of the MAO-A-IMER and MAO-B-IMER were optimized through the determination of the amount of product produced as a function of mobile phase flow rate and the results are presented in Table 1. The maximum conversion of kynuramine to 4-hydroquinoline on both the MAO-A-IMER and MAO-B-IMER systems occurred at a flow rate of 0.2 ml/min, representing a contact time of 2.5 min. This flow rate was used in the rest of the study.

3.5. Determination of the enzyme kinetic parameters K_m and V_{max}

The kinetic parameters, K_m and V_{max}, of MAO-A and MAO-B in the non-immobilized and IMER formats are presented in Table 2. In these studies, immobilization increased the K_m value for the substrate kynuramine relative to the free enzyme for both MAO-A and MAO-B. In the case of MAO-A, there was a two-fold difference and for MAO-B the difference was about five-fold. This is the same trend observed with other IAM-immobilized enzymes. For example, the immobilization of glutamine synthetase on the IAM support increased the observed K_m values for both L-glutamine and D-glutamine by a factor of 2.5 [17].

Table 2
Kinetic parameters (K_m and V_{max}) calculated for MAO-A and MAO-B in non-immobilized (MAO-A and MAO-B) and immobilized enzyme reactor formats (MAO-A-IMER and MAO-B-IMER)

Kinetic parameters	Values	Lineweaver–Burke plot regression
MAO-A		
K_m	204.4 μM	$y = 4.88x + 0.0239$
V_{max}	41.84 nmol/(mg min)	$R^2 = 0.9946$
MAO-A-IMER		
$K_{m,apparent}$	406.5 μM	$y = 13.40x + 0.033$
$V_{max,apparent}$	30.30 nmol/(mg min)	$R^2 = 0.9857$
MAO-B		
K_m	1045.39 μM	$y = 29.062x + 0.0278$
V_{max}	35.97 nmol/(mg min)	$R^2 = 0.9554$
MAO-B-IMER		
$K_{m,apparent}$	5124.44 μM	$y = 253.66x + 0.0495$
$V_{max,apparent}$	20.20 nmol/(mg min)	$R^2 = 0.9622$

The V_{max} values determined using the immobilized MAO-A and MAO-B were reduced by less than two-fold, relative to the results from the non-immobilized enzymes, Table 2. This is also consistent with the results obtained with glutamine synthetase, although in this case immobilization of the enzyme on the IAM support reduced the observed V_{max} values by four- to five-fold for L-glutamine and D-glutamine, respectively [17].

The immobilization of an enzyme on a liquid chromatographic support places the protein in a new microenvironment that can impede the rate at which the substrate reaches the active site of the enzyme. In addition, placing the immobilized enzyme in a flowing system, relative to a non-immobilized enzyme in a static system, affects the kinetics of the distribution of the substrate from the mobile phase to the stationary phase and produces shearing forces that affect the enzyme-substrate complexes. For the MAO-A-IMER and MAO-B-IMER, the K_m and V_{max} parameters were changed by less than 10-fold relative to the non-immobilized enzyme, Table 2. This indicates that although the overall reaction rate was reduced in the IMER format, the placement of MAO-A or MAO-B in the IMER did not significantly alter their activities.

This conclusion is supported by the results from the studies with the IAM immobilized glutamime sythetase. However, while the substrate affinities for the enzyme were reduced, the enantioselectivity of the enzyme was unaltered. This indicated that immobilization produced a quantitative change in the enzyme affinity, rather than a qualitative change in the biochemical properties of the enzyme.

3.6. Determination of IC_{50} values of competitive inhibitors of MAO

Previous studies have demonstrated that (*R*,*S*)-amphetamine, (*R*)-amphetamine, (1*S*,2*S*)-(+)-ψ-ephedrine and (1*R*,2*R*)-(−)-ψ-ephedrine are reversible, concentration-dependant inhibitors of MAO-A and MAO-B [27,28]. In these studies, these compounds also displayed competitive inhibitory effects on the enzymatic activities of non-immobilized MAO-A and MAO-B, Table 3. Representative concentration-inhibition curves obtained with MAO-A are presented in Fig. 4.

Table 3
The effect of known inhibitors on the activity of non-immobilized MAO-A (MAO-A), and MAO-B (MAO-B) on the MAO-A and MAO-B immobilized enzyme reactors (MAO-A-IMER and MAO-B-IMER)

Inhibitor	IC_{50} (μM)	
	MAO-A	MAO-A-IMER
R-Amphetamine	31.1	99.1
(*R*,*S*)-Amphetamine	3.1	24.4
(1*S*,2*S*)-(+)-ψ-Ephedrine	880	1,770
(1*R*,2*R*)-(−)-ψ-Ephedrine	5,350	14,860
	IC_{50}	
	MAO-B	MAO-B-IMER
R-Amphetamine	246	4,030
(*R*,*S*)-Amphetamine	62.5	3,000
(1*S*,2*S*)-(+)-ψ-Ephedrine	10,000	234,000
(1*R*,2*R*)-(−)-ψ-Ephedrine	5,030	88,100

The data represents the average of two experiments.

When the effect of (*R*)-amphetamine was studied, the calculated IC_{50} values for MAO-A and MAO-B were 31.1 and 246 μM, respectively. This is consistent with previous studies of the membrane bound forms of MAO, in which (*R*)-amphetamine was about 10-fold more effective an inhibitor of MAO-A than MAO-B [26]. The IC_{50} values calculated for (*R*,*S*)-amphetamine were 3.1 μM (MAO-A) and 62.5 μM (MAO-B). The results would indicate that (*S*)-amphetamine is a more effective inhibitor than the (*R*)-enantiomer. Previous studies determined K_i values for (*S*)-amphetamine alone and the data from this study cannot be directly compared. However, both studies found that (*S*)-amphetamine was a significantly more potent inhibitor of MAO-A than MAO-B [26,27], which is consistent with the results from this study. In addition, the 10-fold lower IC_{50} value observed for the racemate relative to the

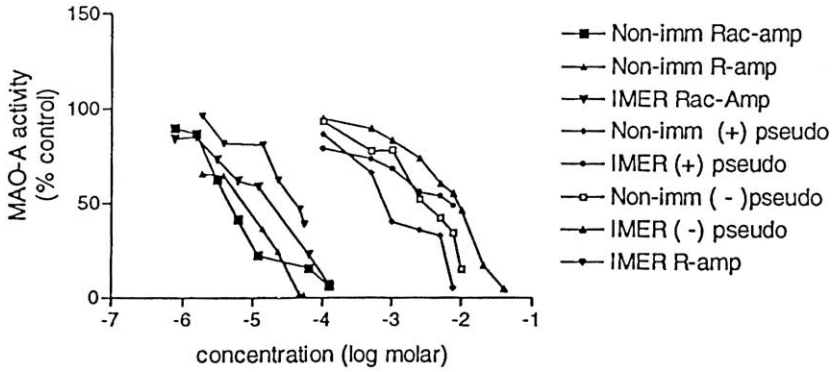

Fig. 4. Concentration Inhibition curves of known MAO-A inhibitors. Each point represents the mean of two experiments. Non-imm: non-immobilized enzyme; Rac-amp: (*R*,*S*)-amphetamine; *R*-amp: (*R*)-amphetamine; (+)-pseudo: (1*S*,2*S*)-(+)-ψ-ephedrine; (−)-pseudo: (1*R*,2*R*)-(−)-ψ-ephedrine, IMER: immobilized enzyme reactor.

(*R*)-enantiomer for MAO-A was consistent with the observed 3.5-fold decrease in the K_i value of (*S*)-amphetamine relative to the (*R*)-enantiomer [26], but the four-fold decrease observed with MAO-B was not consistent with the previous results in which the values were essentially equal [27]. The source of the latter discrepancy has not been identified, but it appears that for the human form of MAO-B, expressed from human cDNA using a baculovirus expression system, the inhibitory activity of (*S*)-amphetamine is greater than the activity of the corresponding (*R*)-enantiomer.

In this study, with MAO-A, the observed IC_{50} value of (1*S*,2*S*)-(+)-ψ-ephedrine was about six-fold lower than that of (1*R*,2*R*)-(−)-ψ-ephedrine, 880 μM versus 5350 μM. The opposite enantioselectivity was observed with MAO-B in which the IC_{50} value of (1*S*,2*S*)-(+)-ψ-ephedrine was two-fold higher than that of (1*R*,2*R*)-(−)-ψ-ephedrine, 10,000 μM versus 5000 μM. The data indicate that with the human isoforms of MAO, there is a significant enantioselectivity between the inhibitory effects of (1*S*,2*S*)-(+)-ψ-ephedrine and (1*R*,2*R*)-(−)-ψ-ephedrine.

IC_{50} values for the MAO-A-IMER and MAO-B-IMER were calculated and compared to those obtained from studies carried out on the non-immobilized enzyme, Table 3. The relative potencies of the tested inhibitors were the same for the non-immobilized MAO-A and the MAO-A-IMER: (*R*,*S*)-amphetamine > (*R*)-amphetamine > (1*S*,2*S*)-(+)-ψ-ephedrine > (1*R*,2*R*)-(−)-ψ-ephedrine (Table 3). The IC_{50} values determined on the MAO-A-IMER were consistently higher than those determined with the non-immobilized enzyme, i.e. the immobilized enzyme was less sensitive to the inhibitor. However, there was a statistically significant linear correlation between the values for the non-immobilized and immobilized enzymes ($R^2 = 0.9978$, $P = 0.0011$).

Similarly, the relative potencies of the tested inhibitors were the same for the non-immobilized MAO-B and the MAO-B-IMER: (*R*,*S*)-amphetamine > (*R*)-amphetamine > (1*R*,2*R*)-(−)-ψ-ephedrine > (1*S*,2*S*)-(+)-ψ-ephedrine (Table 3). While the IC_{50} values found with the MAO-B-IMER were consistently higher than those from the non-immobilized enzyme, as was the case with the MAO-A/MAO-A-IMER, there was a statistically significant linear correlation between the two values ($R^2 = 0.9819$, $P = 0.0091$).

In addition, for the non-immobilized forms of MAO-A and MAO-B, the IC_{50} values of all of the inhibitors tested were lower with MAO-A than MAO-B. This was reflected in seven of the eight IC_{50} values determined on the MAO-A-IMER and MAO-B-IMER. The only exception was (1*R*,2*R*)-(−)-ψ-ephedrine where the IC_{50} values calculated for the non-immobilized isoforms were equivalent while the IC_{50} values calculated for the MAO-A-IMER was approx. six-fold lower than the value observed on the MAO-B-IMER. These results and the correlations established between the non-immobilized enzymes and the IMERs indicate that the MAO-A-IMER and MAO-B-IMER can be utilized to accurately identify inhibitors and predict their IC_{50} values.

4. Conclusions

The data from this study demonstrate that membranes containing human MAO-A and MAO-B can be immobilized on an IAM stationary phase with retention of enzymatic activity. The resulting MAO-A-IMER and MAO-B-IMER can be used to carry out standard Michaelis–Menten enzyme kinetic studies and to quantitatively determine enzyme kinetic constants. In addition, the IMERs can also be used for the identification of specific enzyme inhibitors and the ranking of their inhibitor potencies.

In this study, the production of 4-hydroxyquinoline from kynuramine was followed using UV detection. However, 4-hydroxyquinoline is fluorescent, while the substrate is not, and the enzymatic activity could be directly monitored using a fluorescence detector. This would eliminate the need for a coupled column system and open up the possibility that a single injection of a fixed concentration of substrate and a fixed concentration of a potential inhibitor could be used to qualitatively determine inhibitory activity. This has been previously demonstrated using the brain glutamine synthetase IMER [17]. Thus, this model allows for the rapid screening of multiple compounds and complex mixtures and demonstrates the advantages of coupling the biological and chemical sciences.

In addition, the preliminary findings of these studies are being used to scale down the IMERs to create microscale analyzers in open-tubular formats. The advantages of further developing such IMERs are numerous. Smaller amounts of enzyme and reagents are required, numerous compounds can be screened, and the coupling to sophisticated detection systems allows for qualitative and quantitative determinations. The potential of this approach has been recently demonstrated through the development of open tubular columns containing the *p*-glycoprotein transporter [29].

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