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On-line synthesis utilizing immobilized enzyme reactors based upon immobilized dopamine beta-hydroxylase

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Abstract

Immobilized enzyme reactors (IMERs) based upon dopamine beta-hydroxylase (DBH) have been developed. Immobilized artificial membrane (IAM) and glutaraldehyde-P (Glut-P) stationary phases have been used to immobilize DBH. When DBH is immobilized on the Glut-P interphase the enzyme is outside the stationary phase whereas with the IAM interphase the enzyme is embedded within the interphase surroundings. The activity of each IMER and their ability for on-line hydroxylation has been investigated. The resulting IMERs are enzymatically active and reproducible. The IMERs can be utilized through the use of coupled chromatography to characterize the cytosolic (DBH-Glut-P-IMER) and membrane-bound (DBH-IAM-IMER) forms of the enzyme. The substrate is injected onto the individual IMERs and the reactants and products are eluted onto a phenylboronic acid column for on-line extraction. The substrates and products are then transported via a switching valve to coupled analytical columns. The results demonstrate that enzyme–substrate and enzyme–inhibitor interactions can be investigated with the on-line system. These IMERs can be utilized for the discovery and characterization of new drug candidates specific for the soluble form and membrane-bound form of DBH. The effects of flow-rate, contact time, pH and temperature have also been investigated.

Keywords: Immobilized enzyme reactors; Enzymes; Dopamine β-hydroxylase

1. Introduction

Dopamine is converted to norepinephrine by dopamine beta-hydroxylase (DBH) (Fig. 1). DBH is a key enzyme involved in the regulation of blood pressure by the nervous system and is a target for antihypertensive drugs [1]. Compounds such as benzylhydrazines, benzyloxyamines, and substances acting by copper chelation such as tropolone are known inhibitors in vivo and in vitro of DBH [1]. DBH is a copper containing protein and does not show a high degree of substrate specificity in that it oxidizes a variety of substrates. The resultant structurally analogous metabolites are capable of replacing norepinephrine at noradrenergic nerve endings therefore functioning as ‘‘false neurotransmitters’’ [2].

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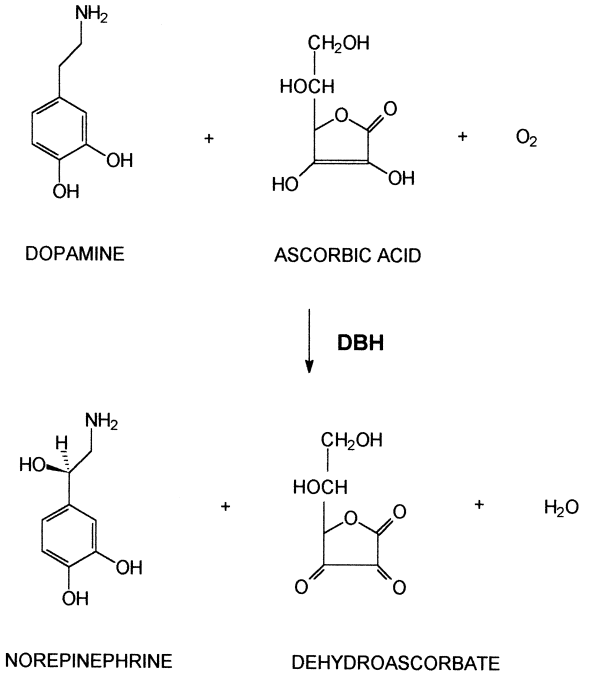

Fig. 1. Reaction of dopamine beta-hydroxylase (DBH).

DBH is situated within catecholamine-containing chromaffin granules, in contrast to the other catecholamine-synthesizing enzymes (tyrosine-hydroxylase, dopa decarboxylase and phenylethanolamine *N*-methyltransferase) that are present in the cytoplasm [3]. DBH exists in two forms, the membrane bound (mDBH) form, which is reinternalized upon exocytosis, and the soluble form (sDBH) that is stored in the granule and secreted [2]. sDBH and mDBH are composed of four subunits [4] and display differences in pH stability and substrate affinity [5].

The majority of information obtained concerning DBH has been through enzymatic assays that have utilized the soluble form of the enzyme. Since the two forms have distinct differences it is important to carry out assays that investigate the effects of substrates and inhibitors on both enzyme forms. We have previously reported the immobilization of DBH onto solid supports that could be used in batch incubations to investigate the qualitative and quantitative aspects of sDBH and mDBH kinetics [6]. The immobilized artificial membrane (IAM) and glutaraldehyde-P (Glut-P) supports were used. The findings of the study demonstrated that through the use of different immobilization procedures and different supports the two enzyme forms could be characterized.

The IAM support is derived from the covalent immobilization of 1-myristoyl-2-[(13-carboxyl) tridecanoyl)]-sn-3-glycerophosphocholine on aminopropyl silica, and resembles one-half of a cellular membrane [7]. In the IAM support, the phosphatidylcholine headgroups form the surface of the support and the hydrocarbon side chains produce a hydrophobic interface that extends from the charged headgroup to the surface of the silica. With the IAM interphase, DBH is embedded within the interphase surroundings.

Glutaraldehyde-P is a wide pore silica that has been covalently clad with a hydrophilic polymer, polyethyleneimine [8]. Immobilization of DBH onto the interphase results in formation of an amine-aldehyde Schiff linkage with Glut-P [6,8]. When DBH is immobilized on the Glut-P support the enzyme is situated outside the phase and is a useful model for the soluble form of the enzyme.

The results of the previous study displayed differences in the kinetic properties of the DBH-Glut-P and DBH-IAM interphases [6]. The study demonstrated the use of the DBH-Glut-P interphase to characterize the enzyme found in the cytosol and information concerning the membrane-bound enzyme could be obtained with the DBH-IAM interphase.

The aim of the present study was to develop immobilized sDBH and mDBH-based liquid chromatographic phases that could be attached on-line to high-performance liquid chromatography (HPLC) analytical columns for screening of DBH inhibitors. The DBH-IAM and DBH-Glut-P stationary phases were prepared and packed into columns. Using switching valves, the individual IMERs were linked to a phenylboronic acid column and coupled analytical columns. The resulting IMERs retained their catalytic activities displaying distinct sensitivity to pH, temperature and inhibitors.

2. Experimental

2.1. Materials

Dopamine-beta-hydroxylase (from bovine adren-

als), catalase (from bovine liver), DL-octopamine hydrochloride, tyramine hydrochloride, (±)-norepinephrine bitartrate salt, (−)-norepinephrine bitartrate salt, dopamine hydrochloride, fumaric acid, fusaric acid, captopril, ascorbic acid, and other chemicals (unless otherwise stated) were obtained from Sigma (St. Louis, MO, USA). Glacial acetic acid, HPLC grade, purchased through Moquin Scientific (Montreal, Canada). The IAM (12 μm, 300 Å) non-endcapped chromatographic support was obtained from Regis (Morton Grove, IL, USA). The IAM bonded phase, according to the manufacturer, contains a near monolayer of C14 saturated phosphatidylcholine, covalently linked to silica through an amide link. Glutaraldehyde-P affinity packing (40 μm, 300 Å) was obtained from J.T. Baker.

2.2. Instrumentation and operating conditions

Three modular HPLC systems were setup in order to carry out on-line hydroxylation of tyramine by the DBH-immobilized enzyme reactors (IMERs). 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 DBH-IMER of interest. System 2 consisted of a Thermo Separation Products P2000 binary pump and a phenylboronic acid (PBA) column. System 3 consisted of a Thermo Separation Products P1000 pump, a 5 μm cyano (CN) stationary phase packed in 150×4.6 mm I.D. column (Regis), a 5 μm octadecyl (ODS) stationary phase packed in a 250×4.6 mm I.D. column (Regis) connected in series, a SpectraSystem FL2000 fluorescence detector, and a Thermo Separation Products Chromjet integrator interfaced with a computer equipped with WOW software for data collection. The eluent from system 1 was directed onto system 2 then onto system 3 through Rheodyne 7000 switching valves (SVs).

System 3 was used independently of systems 1 and 2 by replacing the latter systems with a Rheodyne 7125 injector (i) in order to analyze the results obtained from incubations involving non-immobilized DBH and DBH immobilized onto the loose Glut-P stationary phase. For the temperature studies, the DBH-IMER temperature was controlled with a Fiatron System CH-50 column heater (Fiatron, WI, USA).

2.3. Chromatographic conditions

The mobile phase on system 1 consisted of sodium acetate buffer (10 m*M* at the appropriate pH for each DBH-IMER) with a flow-rate of 0.2 ml/min. System 3 contained two mobile phases A and B. Mobile phase A consisted of sodium phosphate buffer (25 m*M*, pH 8.4) and mobile phase B consisted of sodium phosphate buffer (25 m*M*, pH 4). A mobile phase consisting of potassium phosphate buffer (25 m*M* adjusted to pH 2.0 with trifluoroacetic acid) was utilized for system 3 to achieve the desired chromatographic separation of the products from the substrates. The solutes were quantitated using fluorescence detection with excitation at λ=266 nm and emission at λ=380 nm. A flow-rate of 0.7 ml/min and ambient temperature were used for system 2 throughout the study.

2.4. Enzyme immobilization on loose packing material

2.4.1. Immobilization of DBH onto IAM

DBH was immobilized onto IAM stationary phase utilizing a previously described method [6]. IAM stationary phase (200–250 mg) was washed five times with sodium acetate buffer (0.1 *M*, pH 5.5). The washing was carried out by adding 2 ml of buffer to the packing material, the suspension was centrifuged at 3000 *g* for 5 min and the supernatant decanted. The enzyme solution (1.65 mg in 2 ml 0.1 *M* sodium acetate buffer, pH 5.5) was added to the stationary phase, the mixture was placed in a rotator/stirrer for 12 h at ambient temperature. At the end of 12 h, the suspension was centrifuged for 5 min, the supernatant was collected and the stationary phase was washed an additional five times with buffer. The amount of enzyme immobilized was determined by measuring the amount of residual enzyme present in the supernatant using the Bio-Rad Protein Assay (Bio-Rad Labs., Mississauga, Canada). The difference in the absorbance reading before immobilization and the combined absorbances of the washings after immobilization determined the amount of enzyme bound on the IAM stationary phase.

2.4.2. Immobilization of DBH onto the Glut-P interphase

The immobilization of DBH onto the Glut-P

interphase involved a similar approach to that previously reported [6]. The washing of the stationary phase involved the addition of 2 ml of sodium acetate buffer to the material (0.1 *M*, pH 6.0). The enzyme solution (1.65 mg in 2 ml 0.1 *M* sodium acetate buffer, pH 6.0) was added to the stationary phase, the mixture was placed in a rotator/stirrer for 12 h at ambient temperature. The amount of enzyme immobilized was measured utilizing a similar approach described for immobilization onto IAM material.

2.5. Preparation of DBH immobilized enzyme reactors

DBH immobilized on the IAM or Glut-P stationary phase was packed into a 1 cm×10 mm I.D. column (Regis Technologies) to create the DBH-IMERs. The IMERS were separately connected to a chromatographic system. The DBH-IAM-IMER and the DBH-Glut-P-IMER was washed with sodium acetate buffer (0.1 *M*, pH 5.5) and sodium acetate buffer (0.1 *M*, pH 6.0), respectively. The eluent from both IMERs was collected in order to determine if any of the DBH had been washed off the columns. The Bio-Rad assay was utilized to measure the amount of non-immobilized enzyme. When the columns were not in use they were washed with sodium acetate buffer at the respective pH values and stored at 4°C.

2.6. Procedure for on-line injection

2.6.1. DBH-IAM-IMER and DBH-Glut-P-IMER

A schematic diagram of the coupled HPLC system is presented in Fig. 2. Initially, pumps 2 and 3 on systems 2 and 3 were stopped. With pump 1 running, 100 μl of a substrate–cofactor mixture is loaded into the injector (i) and the valve is switched to the inject position. At the same time, the switching valve 1 (SV1) is switched such that substrate/product are eluted from the DBH-IMERs at a flow-rate of 0.2 ml/min for 20 min, and concentrated onto the PBA column of system 2. The second pump was started and mobile phase A was pumped through the PBA column at a flow-rate of 0.1 ml/min for 30 s in order to elute, ascorbic acid and any other by-products produced from the reaction. The pump was then switched to mobile phase B with a flow-rate of 0.1 ml/min for 2 min and simultaneously switching valve 2 (SV2) was switched such that unreacted substrate and product formed were concentrated on the analytical columns of system 3. When the specific contact time elapses the switching valve 2 (SV2) is switched back to the original position and the pump 3 is started.

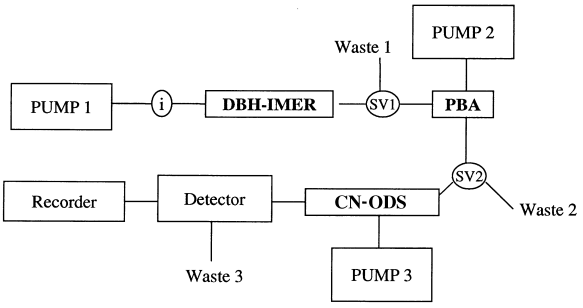

Fig. 2. Schematic representation of on-line DBH-IMER-HPLC system.

2.7. Effect of flow-rate and contact time on on-line system

The effect of flow-rate through the DBH-Glut-P-IMER and DBH-IAM-IMER was investigated at flow-rates ranging from 0.1 to 1.0 ml/min at 0.1 ml increments yielding a fixed elution volume of 3 ml at the respective flow-rates.

The effect of contact time through the DBH-IMERs was also investigated at a fixed flow-rate of 0.2 ml/min. Contact times from 5 to 30 min were investigated at 5 min increments.

The effect of flow-rate and contact time was also examined on the PBA column to determine conditions that would yield maximal recovery of unreacted substrate and product formed. Flow rates ranging from 0.1 to 0.4 ml/min and contact times of 30 s to 3 min were examined.

2.8. Effect of pH and temperature on the DBH-IMERs

The activities of the DBH-IMERs were measured at a series of pH values (with 0.1 *M* buffers) to

determine the optimum pH. The temperature of the IMERs was kept at 37°C.

The effect of temperature was also examined for the individual DBH-IMERs using temperatures ranging from 25 (room temperature) to 60°C.

2.9. Enzyme activity and inhibition studies on DBH-Glut-P-IMER

The enzymatic activity on the DBH-Glut-P-IMER was determined by quantification of the amount of product formed with a given substrate. The temperature of the IMER unless otherwise stated was kept at 37°C with a column heater. Stock solutions of tyramine were prepared in water. The substrate concentrations examined ranged from 0.1 to 10 m*M* and that of the cofactor, ascorbic acid, ranged from 0.1 to 10 m*M*. Enzymatic activity was examined by carrying out injections of a series of substrate–cofactor mixtures. The mixtures were injected onto the DBH-IMER at a flow-rate of 0.3 ml/min for a contact time of 10 min. The kinetic parameters were determined using standard Michaelis–Menten approach [9]. Lineweaver–Burke plots were used to calculate the Michaelis constant (K_m). The rates of reaction (V_{max}) were calculated using μmol/mg/min. Results are expressed as mean±standard error of the mean (S.E.M.).

The effect of known inhibitors, fusaric acid and captopril on the enzymatic activity of the DBH-Glut-P-IMER was also examined. The inhibition of the IMER was carried out using injections of a series of substrate–cofactor–inhibitor mixtures.

3. Results and discussion

DBH was previously reported to be immobilized covalently onto Glut-P silica based chromatographic phase and immobilized by hydrophobic entrapment onto IAM support [6]. In this study, 0.76±0.21 mg of DBH was immobilized onto 320±3.3 mg ($n=3$) of Glut-P support and packed into a column to form the DBH-Glut-P-IMER. The DBH-IAM-IMER was formed in a similar manner with 0.89±0.40 mg of DBH immobilized onto 305±4.7 mg of IAM ($n=3$). These DBH interphases have been formatted into flow systems (see Fig. 2).

Immobilized DBH in the flow system was shown to be active. The two IMERs were shown to be stable and retained over 85% enzymatic activity for over a three-month period of use. Chromatographic studies with the two IMERs are depicted in Fig. 3. A mixture of tyramine and ascorbic acid was injected onto the DBH-IMERs and the eluent from the IMERs were concentrated onto system 2 containing a PBA column for on-line extraction of ascorbic acid and any by-products produced during catalysis. Unreacted substrate and product are then concentrated onto coupled analytical columns for separation and analysis. Fig. 3B and C display typical chromatographic profiles achieved on the DBH-Glut-P-IMER and the DBH-IAM-IMER, respectively. A positive control was carried out by injecting tyramine onto the system without the presence of the cofactor, ascorbic acid. No product formation was observed under these conditions, as seen in Fig. 3A.

Optimal conditions of flow-rate and contact time had to be determined for each subset (i.e. systems 1–3) of the on-line system in order to achieve the maximal productivity. A flow-rate of 0.2 ml/min through both IMERs with a contact time of 20 min resulted in the maximal recovery of the product formed. Extraction of the cofactor and other by-products was achieved on-line through the use of the PBA column. At a flow-rate of 0.1 ml/min and a contact time of 30 s over 95% of the unreacted substrate and product were extracted on-line.

The kinetic parameters, K_m and V_{max} were determined for the DBH-Glut-P-IMER. For the substrate, tyramine, the observed V_{max} was reduced by approximately half and the K_m remained similar to the non-immobilized enzyme, Table 1. Immobilization places the enzyme in a new microenvironment, which can impede the rate at which the substrate reaches the active site of the enzyme. Comparisons of the K_m and V_{max} values obtained with the DBH-Glut-P-IMER and the DBH-Glut-P-SP shows a reduction of the affinity and activity of the DBH-Glut-P-IMER.

The changes seen with the DBH-Glut-P-IMER must be due to the experimental format, i.e., the change from a non-flowing system (non-immobilized DBH and DBH-Glut-P-SP) to a flowing system (DBH-Glut-P-IMER). They can be attributed to the kinetics of the distribution of the substrate from the

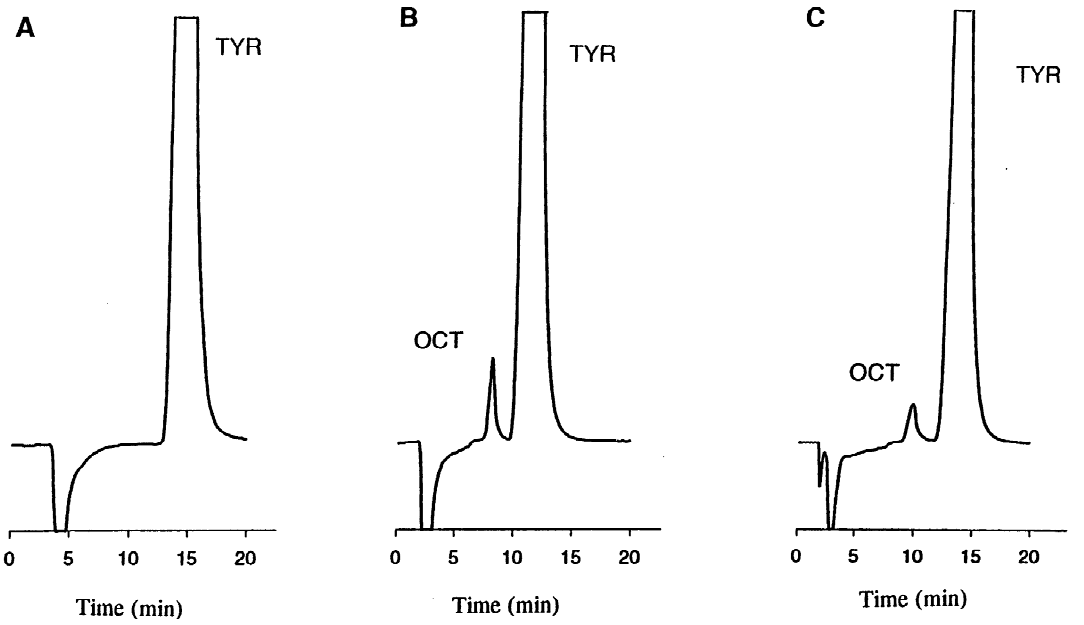

Fig. 3. Representative chromatograms of on-line hydroylation of tyramine. (A) Control (injection of 5 m*M* tyramine with no ascorbic acid present); (B) reaction on DBH-Glut-P-IMER (injection of 5 m*M* tyramine/1 m*M* ascorbic acid); (C) reaction on DBH-IAM-IMER (injection of 5 m*M* tyramine/1 m*M* ascorbic acid).

mobile phase to the stationary phase and the shearing forces produced by the moving phase.

The effect of temperature and pH on the DBH-Glut-P-IMER was also examined. The DBH-Glut-P IMER displayed an optimal activity at 40°C after which the activity decreased with increasing temperature. Similar results were previously reported for the DBH-Glut-P-SP [6]. The activity of the DBH-Glut-P IMER was measured at a series of pHs to determine the optimum pH. A pH optimum of 6.0 was found for the IMER, which is consistent with previous finding for the DBH-Glut-P-SP [6].

The ability to examine DBH activity on an on-line chromatographic system allows for the examination of possible inhibitors of the enzyme. The effect of fusaric acid and captopril, known inhibitors of DBH, was examined on the DBH-Glut-P IMER (Table 2). Fusaric acid and captopril were shown to inhibit the IMER at concentrations as low as 3 n*M* and 50 μ*M*,

Table 1
Kinetic parameters for non-immobilized, immobilized DBH onto the Glut-P interphase (DBH-Glut-P-SP) and DBH immobilized enzyme reactor (DBH-Glut-P-IMER)

	K_m (m*M*)	V_{max} (μmol/mg/min)
Non-immobilized DBH*		
Tyramine	2.85	0.208
Ascorbic acid	0.62	0.185
DBH-Glut-P-SP*		
Tyramine	1.04	0.112
Ascorbic acid	1.10	0.047
DBH-Glut-P-IMER		
Tyramine	2.76±0.46	0.079±0.004
Ascorbic acid	0.54±0.09	0.084±0.002

In the experiments with the DBH-Glut-P-IMER, $n=3$.
*Data obtained from Ref. [6].

Table 2
The effect of known inhibitors on the activity of non-immobilized DBH and the DBH immobilized enzyme reactor (DBH-Glut-P-IMER)

Inhibitor	IC_{50}	
	Non-immobilized DBH	DBH-Glut-P-IMER
Captopril	150 μ*M*	120±1.2 μ*M*
Fusaric acid	7.5 n*M*	5.6±0.3 n*M*

In the inhibition studies with the DBH-Glut-P-IMER $n=3$.

respectively. The IMER can therefore allow for the screening and characterization of potent inhibitors.

Previous findings have demonstrated the DBH-IAM and DBH-Glut-P interphases are representative of the membrane-bound and soluble enzyme [6]. In this study, two individual IMERs, DBH-Glut-P-IMER and DBH-IAM-IMER were prepared and formatted onto an on-line system for the synthesis of octopamine from tyramine. Both IMERs can be used on the system for the generations, separation and identification of inhibitors and substrates. The individual IMERs will prove useful for the screening of substances for their pharmacological properties for membrane bound and soluble forms of DBH.

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