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Purification and Characterization of the Active Form of Tyrosine Hydroxylase From Mesangial Cells in Culture

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Abstract The capacity of mesangial cells (MC) to produce catecholamines (CAs) has been investigated in our laboratory. To study the CA cascade, it is necessary to examine some steps in their metabolic pathway. Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in the biosynthesis of these biogenic amines (dopamine (DA), norepinephrine (NE), and epinephrine (EPI)). Since the glomerular mesangium is their target in the regulation of renal sodium transport and renin secretion, the aim of the study was to determine the presence of TH in these cells in culture. The CA levels were detected in immortalized MC by high-performance liquid chromatography with electrochemical detection. The following concentrations were found in the intracellular region and in the medium, respectively: NE = 284 ± 31 and 134 ± 22, EPI = 75 ± 14 and 22 ± 5, and DA = 42 ± 14, 40 ± 20 pg/mg cell protein. The enzymatic activity of the cell lysate and medium was measured based on L-dopa formation. In the presence of *o*-phenanthroline, both samples presented 39% inhibition. The biopterin was detected in the intracellular and in the medium (64.87 and 631.99 pmol/mg protein, respectively) using high-performance liquid chromatography with ultraviolet detection. The cell lysate was submitted to a DEAE-Sephacel column, followed by gel filtration, and Heparin-Sepharose. TH was purified 613.16-fold with a specific activity of 466.0 pg/mg cell protein. Immunoblotting using monoclonal antibody revealed the presence of TH in the different purification steps. Purified TH was sequenced, presenting an alignment with amino-terminal sequence of mouse enzyme. Our results demonstrated the presence of active TH in MC, suggesting that these cells are able to produce CA ''in vivo'', and establishing a convenient purification method for TH that can be applied to the study of the molecular properties of the enzyme modified ''in vivo'' by different physiological and pathophysiological stimuli. J. Cell. Biochem. 87: 58–64, 2002.

Key words: mesangial cells; catecholamines; tyrosine hydroxylase; purification; kidney

Catecholamines (CA) (norepinephrine, NE, epinephrine, EPI, and dopamine, DA) are neurotransmitters/hormones that may perform functions unrelated to the nervous system. In the kidney, these amines activate α- and β-adrenoceptors and DA receptors, regulating renal hemodynamics, Na^+ and water balance, and renin secretion [Garg, 1992]. Some investigators have postulated that DA is a physiologically significant natriuretic factor and NE a significant antinatriuretic factor [Sthephenson et al., 1982]. CAs might also be important in cell physiology and development [Pendlenton et al., 1998].

Di Marco et al. [1999] detected significant primary CA levels in the intracellular and extracellular compartments of mesangial cells (MC) and demonstrated that MC express the enzymes and a biopterin cofactor necessary for the production of the monoamines. In these cells, CA may exert autocrine, paracrine, or endocrine effects. Among the enzymes detected by Di Marco et al. [1999], tyrosine hydroxylase (TH) is a monooxygenase which catalyzes the formation of L-3,4-dihydroxyphenylalanine (L-dopa) from L-tyrosine, the initial and rate-limiting step in the biosynthesis of CAs. TH requires biopterin as a cofactor; and the simple presence of TH in cells indicates the potential for CA biosynthesis. L-dopa, in turn, is converted to DA and eventually to NE and EPI if the necessary enzymes are expressed.

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In recent studies, Kumai et al. [2000] suggested that TH production plays an important role in the mechanism of hypertension, and that gene therapy using antisense TH induces hypotensive effects in spontaneously hypertensive rats (SHR). Analyzing "in utero" differences in CA biosynthesis in the adrenals of SHR, Teitelman et al. [1981] showed that although the timing of the first expression of adrenergic phenotypes was similar in SHR and normotensive controls, the differences in TH activity in the adrenals suggested an enhanced biosynthetic capacity for CAs in this strain before birth. They concluded that SHR differ from normotensive rats from the first expression of some of the genes controlling CA biosynthesis.

There are well established methods for purification of TH from the adrenal glands [Togari et al., 1983], but there are no references about TH in the kidney, principally in MC in culture.

Since cultured MC provide a convenient model for the study of renal synthesis and release of CA lacking neuronal contribution, cell culture can be a useful tool for the "in vivo" study of the molecular properties of the enzyme modified or not physiologically or pharmacologically.

The aim of this study was to isolate for the first time an active form of TH from MC and to establish a convenient method for its purification.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Federal University of São Paulo, São Paulo, Brazil.

Immortalized Mesangial Cell Culture (IMC)

The cells were purchased from the American Type Culture Collection (ATCC: CRL-1927). The immortalized cells were plated onto Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5,000 U penicillin, 50 mg streptomycin, and 2.6 acid HEPES. The cultures were allowed to develop in a CO_2 incubator (5% CO_2, 95% air) at 37°C and the medium was replaced every 36 h. At the time of 90% confluence, the cultures were submitted to trypsinization and subcultured in flasks (25 or 75 cm^2) under the same culture conditions, being incubated with 5 or 10 ml of DMEM. Before the experiments, IMC were incubated with DMEM without FBS for 24 h. The culture flasks were rinsed twice with 5 or 10 ml of phosphate-buffered saline (PBS) to eliminate all serum residues and scraped in the presence of 1 or 2 ml of PBS. The medium was collected and placed on ice or transferred to a freezer for storage at −80°C. The scraped cells were placed in a centrifuge conical tube and centrifuged at 1,250*g* for 2 min. The supernatant was poured off and the cell pellet was also stored at −80°C.

The cells were plated onto 25 cm^2 flasks for CA and biopterin quantification and each analyzed sample corresponds to each flask. The pellet was lysed with 1 ml ice water under vigorous vortexing (1 min) and a 0.2 ml aliquot was reserved for protein quantification. For purification, 10–20 (75 cm^2) flasks were plated together to provide a large amount of cell protein.

CA Quantification by High Performance Liquid Chromatography (HPLC-ED)

CAs released into the medium and intracellularly were measured by ion-pair reverse phase chromatography coupled with electrochemical detection (0.5 V) as described by Naffah-Mazzacoratti et al. [1992]. Fast isocratic separation was obtained using an RP 18 Brownlee column (4.6 × 250 mm, Millipore Co.) eluted with the following mobile phase: 20 mM sodium dibasic phosphate, 20 mM citric acid, pH 2.64, containing 10% methanol, 0.12 mM Na_2EDTA, and 566 mg/L heptanesulfonic acid. The total time for sample analysis was 30 min. The concentrations were directly determined by the following equation:

$$\text{pgA/ml} = \frac{\text{PsA/PsDHBA}}{\text{PstA/PstDHBA}} \times \text{Standard quantify} \times \text{Dilution factor}$$

where: A = amine; Ps = peak area of amine in the sample; PstA = peak area of amine in the standard; PsDHBA = peak area of DHBA (dihydroxybenzylamine) in the sample; and PstDHBA = peak area of DHBA in the standard. DHBA is the internal standard of extraction. Results were expressed as pg amine/mg cell protein.

The cell lysate (0.8 ml) and the incubates (2 ml) were previously submitted to the following purification steps: 50 mg Al_2O_5 was weighed out in centrifuge tubes and the samples were added in Tris-buffer, pH 8.8, plus 40 μl (8 ng) of DHBA. The suspension was vortex-mixed for 10 min. The precipitated alumina was washed three times and vortex-mixed with 1 ml of water and the CAs were eluted with 400 μl of 100 mM perchloric acid after 3 min of vortex mixing.

After centrifugation for 3 min at 2,500*g*, the supernatant was filtered and 100 μl was injected into the reverse phase column. The concentration of L-dopa was expressed as ρg/mg cell protein.

TH Activity Assay

TH activity was measured based on assay of L-dopa formed from L-tyrosine by high-performance liquid chromatography with electrochemical detection [Naffah-Mazzacoratti et al., 1992]. TH activity in MC homogenate or in the medium was expressed as percent remaining activity. The cells were lysed with 1.0 ml of 200 mM sodium acetate buffer, pH 6.0, and shaken for 1 min. For the assay, the incubation mixture containing 1 mM L-tyrosine and 400 μl of the cell lysate or the corresponding medium, 0.04 mM *o*-phenanthroline in 200 mM sodium acetate buffer, pH 6.0, in a total volume of 1.0 ml, was incubated at 37°C for 60 min. In the control incubation, the TH inhibitor, *o*-phenanthroline, was omitted in order to obtain total L-dopa formation. The reaction was started by the addition of tyrosine and was stopped by adding 100 μl of 100 mM perchloric acid. L-dopa was quantified as described above and the results were obtained from the comparison of percentage of released product in presence and absence of the inhibitor.

Biopterin Assay

The biopterin cofactor was quantified by HPLC with UV detection (365 nm) using two coupled Aquapore OD 300 columns, 5 μ (4.6 × 250 mm), and an RP 18 Licrospher 100 pre-column, 5 μm (4 × 4 mm) (Brownlee). The columns were equilibrated with 20 mM potassium phosphate buffer, pH 3.0, at a flow of 0.8 ml/min and eluted isocratically with the same buffer for 20 min. The cell lysate and the medium were filtered and injected directly into the HPLC apparatus. The values were obtained from a standard curve for biopterin. Results were expressed as pmol/mg cell protein.

Purification of TH Enzyme

The IMC pellet was homogenized in four volumes of 50 mM Tris-HCl, pH 7.3, containing 320 mM sucrose in an ultrasonic bath and vortexed and centrifuged for 10 min at 2,500*g*. The supernatant was applied to an ion exchange DEAE-Sephacel column (10 ml) equilibrated with 20 mM Tris-HCl buffer, pH 7.3, containing 8% sucrose and 1 mM dithiothreitol (DTT). The column was washed with the same buffer and eluted with 50 and 250 mM NaCl at a flow rate of 20 ml/h. The pooled fractions containing TH activity were submitted to gel filtration on a Superdex 200 HR 10/30 column (AP Bioscience, Sweden) equilibrated with 20 mM potassium phosphate buffer, pH 7.3, containing 8% sucrose and 1 mM DTT and eluted isocratically at a flow rate of 30 ml/h. Finally, the pooled enzyme obtained in the last step was purified by affinity chromatography on Heparin–Sepharose CL-6B, equilibrated with 20 mM Tris-HCl buffer, pH 7.3, containing 8% sucrose, 1 mM DTT, and 100 mM KCl. After the enzyme solution had sunk into the bed, the column was washed with the following buffers: ten bed volumes of the equilibrium buffer, 20 bed volumes of 20 mM Tris-HCl buffer, pH 8.3, containing 8% sucrose, 1 mM DTT and 100 mM KCl, and five bed volumes of the equilibration buffer. The flow rate of the column was adjusted to 20 ml/h. The enzyme was eluted with the equilibration buffer containing 400 mM KCl [Togari et al., 1983]. All procedures were carried out at 4°C. During all chromatographic steps, 1 ml fractions were collected, the profile was monitored by absorbance at 280 nm and by dot blot using monoclonal antibody; and the enzymatic activity was determined based on assay of L-dopa formed from L-tyrosine. One aliquot (400 μl) of the collected fractions was incubated with 1 mM L-tyrosine, 0.04 mM *o*-phenanthroline and 200 mM sodium acetate buffer, pH 6.0, to make up a total volume of 2.0 ml. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine. The reaction was stopped by adding 100 μl of 100 mM perchloric acid. The L-dopa formed was measured as described in the section of L-dopa quantification by HPLC-ED.

Protein Determination

Protein concentration was determined by the method of Bradford [1976] using bovine albumin as standard (Bio-Rad Protein Assay kit, Bio-Rad Laboratories Inc., USA), except when absorbance at 280 nm was used for the chromatographic elution profile.

Dot Blot and Western Blot Analysis

One aliquot (50 μl) of the fractions collected from each chromatography procedure was filtered through the membrane by gravity flow for 30 min in the dot blot system. A wash step

was performed by adding 50 μl of TBS (20 mM Tris, 500 mM NaCl, pH 7.5) that was also passively filtered. The nitrocellulose membrane (Amersham Pharmacia Biotech, Sweden), the Bio-Dot Microfiltration Apparatus and all subsequent steps were prepared as recommended by the manufacturer (Bio-Rad Laboratories Inc., USA).

The cell homogenate was analyzed by sodium dodecyl sulfate–polyacrylamide gel (7.5%) electrophoresis (SDS–PAGE) performed by the method of Laemmli [1970] under reducing conditions. The proteins separated by SDS–PAGE (100 μg) were submitted to Western blotting analysis using a nitrocellulose membrane.

Both membranes were blocked with 5% non-fat milk (Molico, Nestlé, Brasil) in TBST (20 mM Tris, 500 mM NaCl, 500 μl Tween,[20] pH 7.5) for 6 h. The membranes were washed twice with TBS and once with TBST (10 min each time) and incubated with mouse anti-TH-1 monoclonal antibody (1:250, 1% albumin in TBS) (Sigma Co., USA) for 8 h. The antibody bound to the enzyme was detected by the secondary antibody anti-mouse IgG-biotin conjugate (1:1000, 1% albumin in TBS) for 1 h. The membranes were incubated with streptavidin/alkaline phosphatase (1:3000, 1% albumin in TBS) for 30 min. The wash step was repeated between incubations. The protein bands were finally developed using the substrates BCIP/NBT, as recommended by the manufacturer (Bio-Rad Laboratories Inc., USA).

TH Sequence

The amino-terminal sequence was deduced by amino acid identification using the Protein Sequencer PPSQ-23 system (Shimadzu Corporation, Tokyo). Sequencing was kindly performed by Dr. Isaura Yoshico Hirata, Department of Biophysics, Escola Paulista de Medicina, Federal University of São Paulo, São Paulo, Brazil.

Statistical Analysis

Results are presented as mean ± SEM and data were analyzed statistically by the Student *t*-test.

RESULTS

Figure 1 shows the CA levels expressed as pg/mg cell protein. There were significant differences in NOR and EPI levels between the intracellular compartment and the culture medium, but no differences in the production/release of DA.

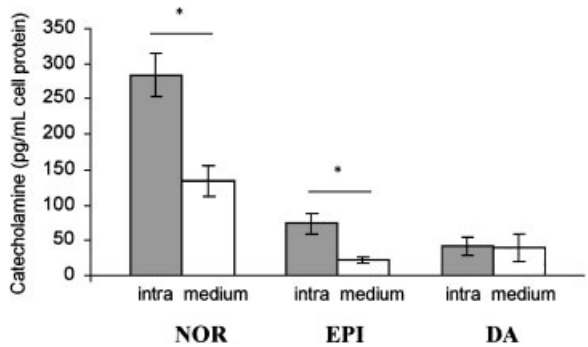

Fig. 1. Catecholamine distribution in the intracellular (intra) and extracellular compartment of IMC after 24 h of incubation with no-FBS culture medium. All data are expressed as means ± SEM, n = 8, based on number of culture flasks used, $^*P < 0.001$ (Student *t*-test).

Figure 2 shows the percentage of remaining enzymatic activity after incubation of IMC lysate and culture medium with L-tyrosine and the inhibitor *o*-phenanthroline. This activity was measured based on HPLC-ED assay of L-dopa formed from L-tyrosine. Inhibition of 39% of the formation of L-dopa was obtained for both samples (39% ± 1.47, n = 4).

The cofactor of enzymatic activity, biopterin, was detected in the intracellular compartment and in the culture medium (n = 8) by HPLC with UV detection, as shown in Figure 3.

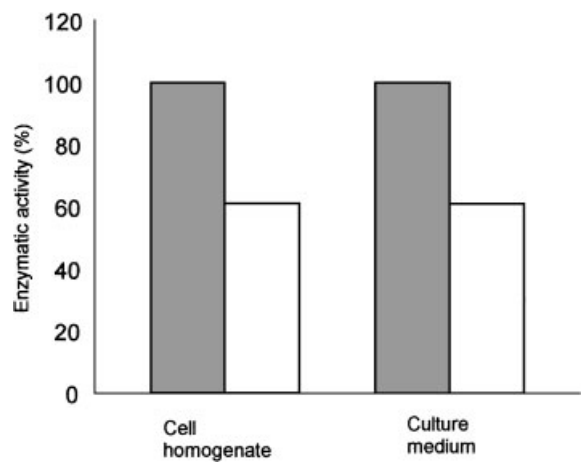

Fig. 2. Enzymatic activity was determined measuring the formation of L-dopa from L-tyrosine. The samples were incubated with L-tyrosine (1 mM) with or without the inhibitor OPhe (0.04 mM). ■ assay control; □ with the inhibitor.

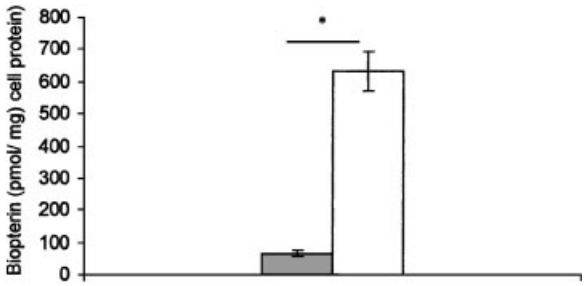

Fig. 3. Distribution of biopterin cofactor in the intracellular compartment and in the culture medium of IMC. All data are expressed as means ± SEM, n = 8, based on number of culture flasks used, * $P < 0.001$ (Student *t*-test). ■ intracellular; □ culture medium.

The IMC homogenate was chromatographed on a DEAE–Sephacel column followed by gel filtration on a Superdex 200 column and affinity chromatography. In the different chromatography steps, the fractions that presented TH activity were pooled and the enzymatic activity was determined based on assay of L-dopa formed from L-tyrosine by HPLC–ED. One peak with TH activity was eluted in each purification step. The enzyme was purified 613.16-fold with a specific activity of 466.0 pg/mg cell protein (Table 1). Figure 4 shows the chromatographic pattern of TH of IMC eluted as a single peak after affinity chromatography.

Western blotting analysis of IMC homogenate and pooled fractions containing TH activity from the different chromatography steps revealed the presence of TH with the mouse anti-TH-1 monoclonal antibody (Fig. 5). The pooled fractions from DEAE–Sephacel and gel filtration showed bands with different molecular masses. In the final purification step, Heparin–Sepharose, TH was obtained as a single band of 60 kDa.

The partial N-terminal sequence of purified TH is shown in Figure 6. The first 20 amino acids were analyzed and the sequence obtained was similar to that described for mouse and rat TH.

DISCUSSION

CAs leaving the kidney in urine, lymph, and renal venous plasma can come from four sources: arterial plasma, renal nerves, non-neuronal renal tissue, and direct adrenal–renal vascular anastomoses [Katholi et al., 1977].

Literature reports show that the kidney may be the organ most involved in extra-adrenal CA production in bi-adrenalectomized patients [Schwab and Krause, 1992]. Previous studies from our laboratory have detected the presence of TH in the MC [Di Marco et al., 1999]. Considering the fact that primary MC and IMC present the same profile, it is possible to suggest that "in vivo" in the basal state these cells are able to synthetize and store cathecolamines that could participate in the regulation of glomerular and tubular functions. These cells in culture could be a useful tool to study alterations in the synthesis and levels of renal CAs in experimental physiological and pathophysiological models of renal diseases. Based on this fact and considering that these hormones participate in the hemodynamic regulation of the kidney, we decided to study the production of these monoamines by IMC. Then, because of the importance of the presence of TH, the rate-limiting enzyme in the CA cascade formation in these cells, we decided to purify it.

In the present study, using HPLC-ED, we detected significant CA levels in the intracellular compartment and in the culture medium of immortalized MC. In these cells, we detected a higher concentration of NOR and EPI in the intracellular than in the extracellular compartment. An interesting fact is that the primary MC present the same hormonal profile as found for IMC, differing only by a lower production and release of NOR [Di Marco et al., 1999]. The comparison study of MC in primary culture and IMC demonstrated that the dose of TH activity able to liberate L-Dopa in IMC lysate is 0.46 pg/ml and in the mesangial cell in primary

TABLE 1. Purification of TH From IMC in Culture

	Volume	Protein		L-dopa	Specific activity	Purification	Recovery
Description	(ml)	(mg/ml)	Total (mg)	(pg/ml)	(pg/mg)	(Fold)	(%)
Cell lysate	46	0.6	27.9	0.46	0.76	1	100
DEAE-Sephacel TH	105	0.064	6.72	6.83	106.7	140.39	24.1
Superdex 200 TH	14	0.29	4.06	32.48	112	147.37	14.55
Heparin-sepharose TH	46	0.044	2.03	20.5	466.0	613.16	7.25

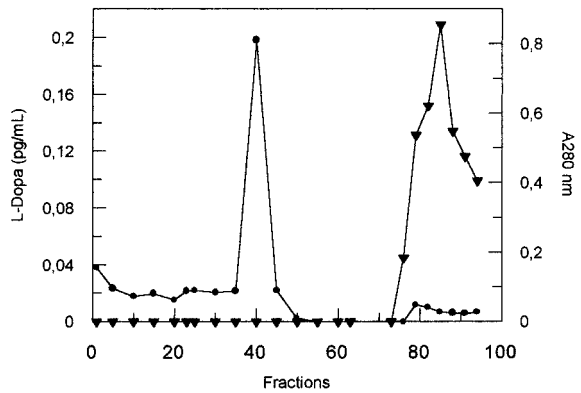

Fig. 4. Affinity chromatography using Heparin-Sepharose CL-6B resin, equilibrated with 20 mM Tris-HCl buffer, pH 7.3, containing 8% sucrose/1 mM DTT/100 mM KCl. The TH enzyme was eluted with 20 mM Tris-HCl buffer, pH 7.3, containing 8% sucrose/1 mM DTT/400 mM KCl. ◆; A 280 nm; ▼ L-dopa (pg/ml).

culture is 0.48 pg/ml. Based on this profile and considering that the growing of MC in primary culture until confluence is around 3 months and the preparation of these cells involves an ample protocol, we decided to use in this study the IMC that is available commercially and the growing is obtained in 2 days. Considering that the mean protein value of IMC was 0.517 mg/ml and that the CA concentration (pg/ml) was corrected according to these values, we obtained monoamine levels ranging from 20 (EPI in the culture medium of IMC) to 275 pg/ml (NOR in the

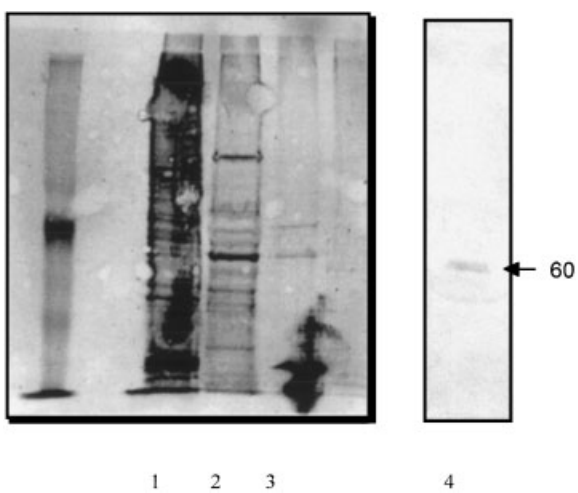

Fig. 5. Western blotting using mouse anti-TH-1 monoclonal antibody. **Lane 1**, IMC homogenate; **lanes 2**, **3**, and **4** correspond to the pooled fractions containing TH activity eluted by DEAE-Sephacel, gel filtration and Heparin-Sepharose, respectively. The arrow indicates the estimated molecular mass.

	2 5 10 15
TH IMC	MPT PSAPS PQPKG FXXAV
TH Rat	MPT PSAPS PQPKG FRRAV
TH Mouse	MPT PSASS PQPKG FRRAV

Fig. 6. Amino-terminal sequence of TH of IMC. Alignment with amino-terminal sequences of rat and mouse TH. Bold amino acids indicate non-conserved regions in the respective species.

intracellular of IMC). These values are significant when compared with plasma of normal young rats, which have about 70 pg/ml of EPI and 260 pg/ml of NOR, and with the erythrocytes of these rats that present about 13 pg/ml of EPI and 10 pg/ ml of NOR [Gupta et al., 1997].

Although we did not evaluate factors that are able to affect TH activity, consequently influencing CA production/release, we observed that IMC express the enzyme in its active form, which is inhibited by 39% by *o*-phenantroline ($L_{50} = 0.04 \times 10^{-3}$). Moreover, the single fact that the cell homogenate converted L-tyrosine to L-dopa demonstrates that these cells also present the cofactor biopterin. This evidence was confirmed using HPLC with UV detection. The results showed that the concentration released in the medium was significantly higher than the intracellular value.

The method for TH purification would be extremely useful to study the biochemistry and molecular properties of the enzyme and seems to be applicable to any cells,and can produce highly purified TH with high specific activity.

Attempts to purify this enzyme have been made for a long time, with high difficulty, since the enzyme easily aggregates during the chromatographic steps and is extremely unstable, losing 50% of its activity within 5 h at 4°C, even with the presence of 8% sucrose and 1 mM DTT [Togari et al., 1983]. In our laboratory, we succeeded in developing a method of TH purification, which has been applied to isolate the enzyme from IMC.

The purification steps consisted of DEAE–Sephacel, Superdex 200, and Heparin–Sepharose. TH was purified 613.16-fold with a 7.25% yield and the specific activity was 466.0 pg/mg cell protein. In the previous methods described for TH from adrenal [Oka et al., 1982], the authors used as a first step of purification, centrifugation of homogenate followed by DEAE–Sephacel, Bio Gel A-5m, Heparin–Sepharose

CL-6B, and Bio Gel A 1.5m. In our protocol we used only three steps: DEAE–Sephacel followed by gel filtration using Sephadex 200 and Heparin–Sepharose. The elimination of one step resulted in an increase of enzyme stability and sensibility in purification requiring less protein.

The enzyme from each purification step was recognized by a monoclonal antibody against TH-1. In the final step the enzyme was purified to homogeneity and was obtained as a single band with a molecular mass of 60 kDa.

In the literature, the meaning of the different molecular masses detected can be elucidated by two enzyme fractions (I and II) with different kinetic properties from bovine adrenal medulla and caudate nucleus purified on a DEAE–Sephacel column. The molecular mass of the subunit of Fraction I was estimated to be 60 kDa by SDS–polyacrylamide slab gel electrophoresis. Fraction II was judged to be composed of several enzyme forms with different molecular weights. In summary, Oka et al. [1982] demonstrated that TH from the adrenal medulla is composed of at least two forms, and each enzyme has a different apparent K_m value for the biopterin cofactor.

In addition, IMC TH was also sequenced, showing similarity to the aminoterminal sequences of rat [Grima et al., 1985] and mouse TH [Ichikawa et al., 1991].

The isolation, purification, and characterization of TH from IMC demonstrated for the first time that this enzyme is synthesized and expressed with activity in these cells, suggesting that the kidney is an extraneuronal and adrenal site of CA production. Elucidation of the physiological importance of these results requires additional studies. From here we cannot reach a conclusion regarding TH function at the kidney level, our study suggests that the model of MC in culture, which is free of neuronal contribution, can be useful for the study of renal CA synthesis.

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