Real-time monitoring of tyrosine hydroxylase activity using a plate reader assay

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**Abstract**

Tyrosine hydroxylase (TH, EC 1.14.16.2) catalyzes the rate-limiting step in dopamine (DA) synthesis, oxidizing tyrosine to \(\text{L-DOPA}\), which is then further metabolized to DA. Current assays for monitoring activity of this enzyme require extensive work-up, require long analysis time, and measure end points, thereby lacking real-time kinetics. This work presents the development of the first real-time colorimetric assay for determining the activity of TH using a plate reader. The production of \(\text{L-DOPA}\) is followed using sodium periodate to oxidize \(\text{L-DOPA}\) to the chromophore dopachrome, which can be monitored at 475 nm. Advantages to this method include decreased sample analysis time, shorter assay work-up, and the ability to run a large number of samples at one time. Furthermore, the assay was adapted for high-throughput screening and demonstrated an excellent \(Z\)-factor (>0.8), indicating suitability of this assay for high-throughput analysis. Overall, this novel assay reduces analysis time, increases sample number, and allows for the study of activity using real-time kinetics.

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Materials and methods

Materials

DOPAL was biosynthesized as described previously using enzyme-catalyzed conversion of DA to DOPAL by rat liver monoamine oxidase (MAO) [14], and the concentration was determined via an aldehyde dehydrogenase assay [15] and HPLC analysis as described below. Tyrosine, l-DOPA, sodium periodate, and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted.

hTH was purified as described previously [16]. Briefly, hTH was purchased from Harvard PlasmID (clone ID: HsCD00378692), and the TH open reading frame was subcloned into the pMALc2H10T vector. The pMALc2H10T-TH (MBP-TH) plasmid was used to transform BL21(DE3) Escherichia coli. A single colony was grown overnight in 10 ml of LB medium supplemented with 100 μg/ml ampicillin (LB/Amp) at 37 °C, expanded to 4 L of LB/Amp, and grown to OD600 = 0.4. The flask were cooled to 25 °C and monitored until growth reached OD600 = 0.6. Then, concentrated solutions in water of isopropyl β-D-thiogalactopyranoside, FeSO4, and glucose were added to final concentrations of 250 μM, 100 μM, and 0.2% (w/v), respectively, to induce MBP-TH expression, for enzyme stability, and to prevent the production of bacterial amylases, respectively. After 20 h at 25 °C, the culture was centrifuged and lysis was performed using lysozyme and DNase I. The lysate was centrifuged for 1 h at 100,000 g, and the supernatant was retained. MBP-TH fusion protein was captured from the prefiltered supernatant with an amylose resin column and digested with TEV protease for 8 h at 4 °C and hTH was separated using a HiPrep Q FF 16/10 column and NaCl gradient. MBP eluted first, with 99% pure TH following.

TH plate reader activity assay

TH, tetrahydrobiopterin (BH4), and iron(II) sulfate were pre-mixed (mixture A) and allowed to incubate for 5 to 10 min on ice to facilitate binding of the iron and cofactor to the enzyme. During this incubation, a second mixture was made containing 10 mM HEPES, tyrosine, and sodium periodate (mixture B). To a 96-well plate, A and B were combined in a 1:1 ratio, with final concentrations for tyrosine, BH4, iron, tyrosine, and sodium periodate being 10 μg, 0.25 mM, 2.5 μM, 50 μM, and 100 μM, respectively. The plate was immediately placed in a Molecular Devices SpectraMax plate reader with absorbance set to 475 nm. After an initial mix for 3 s, the plate was read every 10 s for 30 min at 37 °C. A known competitive inhibitor of hTH activity, 3-iodo-tyrosine (3IT), was used as a negative control to determine inhibition of TH activity [10]. Furthermore, a competitive inhibitor of Fe binding, cobalt chloride (CoCl2), was used as a second negative control [11]. Immediately prior to the addition of mixture B, 50 μM 3IT or 100 μM CoCl2 was added to mixture A. DOPAL was added to A, with the addition of B following immediately at a final concentration of 5, 10, or 20 μM. Production of l-DOPA was determined using a molar extinction coefficient for dopachrome of ε = 3700 M–1 cm–1 [9].

A number of control experiments were performed to determine background absorbance and to ensure minimal reactivity with assay components such as sodium periodate during the specified time frame. Exclusion of tyrosine or hTH demonstrated background absorbance was due to assay components and the enzyme. Individual components of the assay were incubated with sodium periodate and HEPES to ensure that there was no reaction that would affect absorbance readings. Furthermore, due to the color of CoCl2, background absorbance was assessed in wells containing all components (i.e., sodium periodate, Fe, BH4, CoCl2, and HEPES [concentrations described previously]) minus hTH. All experiments were done at a final volume of 200 μl in 10 mM HEPES buffer (pH 6.8) at 37 °C.

HPLC analysis of TH activity

HPLC assays and analysis were carried out as described previously [4] in order to directly compare with plate reader results. Briefly, all concentrations of the assay are the same as described above with the omission of sodium periodate (i.e., TH, tyrosine, BH4, and iron sulfate). On the addition of 50 μM tyrosine (final concentration), samples were incubated at 37 °C for 20 min and time points were taken at 5-min intervals. Aliquots were acidified with 5% (v/v) perchloric acid to stop the reaction, and an Agilent 1200 Series capillary HPLC system with a photodiode array detector measuring absorbance at 202 and 280 nm was used for separation. A Phenomenex Luna C18 column was employed, and peaks were separated using an isocratic flow of mobile phase consisting of 97% water, 0.1% trifluoroacetic acid and 3% acetonitrile (v/v). Using standards, retention times for l-DOPA, tyrosine, DOPAL, and 3IT were 6.4, 9.5, 10.5, and 21.1 min, respectively.

HTS assay and Z-factor calculation

To determine whether the plate reader assay could be applied to HTS for future assessment of possible inhibitors or activators of hTH activity, the real-time method was modified to be performed in Corning 384-well, clear flat-bottom plates (Corning, New York, NY, USA). HTS experimental plates were read using an EnVision 2104 Multilabel Plate Reader (PerkinElmer), with data collection being performed using the Wallac EnVision Manager (version 1.12, PerkinElmer). The final sample volume was 100 μl, and in place of BH4, 6,7-dimethyl-5,6,7,8-tetrahydropyrimidine hydrochloride (DHMP4) was used. DMHP4 is a non-natural cofactor, and although it is less active than the natural cofactor BH4, it allows for higher tyrosine concentrations to be used in the assay [17], which is important to observe the maximum difference between positive and negative controls. This assay structure (above) was used in the experiments involving HTS; therefore, the same concentrations of hTH (10 μg), iron (2.5 μM), and DMHP4 (0.25 mM) were preincubated (mixture A) for 5 to 10 min on ice. Mixture B contained tyrosine (200 μM), sodium periodate (400 μM), and HEPES. Mixture A was added to the plate, and in order to achieve inhibition in HTS assays, CoCl2 (100 μM) was used as a competitive inhibitor of Fe binding [11]. In total, 48 wells were used for control experiments (maximum signal, no hTH inhibition), and 48 wells were used for negative controls (minimum signal, CoCl2 inhibition). CoCl2 was added to the wells immediately prior to the addition of mixture B, and plates were then read for 3 h at 90-s intervals. Background absorbance at 475 nm for CoCl2 was determined as described above in the plate reader activity assay, and absorbance was corrected after the conclusion of the experiment. The Z-factor was determined using the following equation:

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Z\text{-factor} = 1 - \frac{3x(σp + σn)}{|μp - μn|}
\]

where σ is the standard deviation for the positive (p, no inhibition) and negative (n, CoCl2 inhibition) and μ is the mean of each control population.

Statistical analysis

All linear regression and statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). For plate reader assays, TH activity was measured via