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

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Abstract

Tyrosine hydroxylase (TH) is the rate-limiting step in dopamine (DA) synthesis, oxidizing tyrosine to L-DOPA, which is 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 L-DOPA is followed using sodium periodate to oxidize 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.

Keywords

Tyrosine hydroxylase; Real-time assay; Plate reader; Dopachrome; High-throughput screening

Tyrosine hydroxylase (TH,² EC 1.14.16.2) catalyzes the rate-limiting step in dopamine (DA) synthesis, oxidizing tyrosine to L-DOPA, which is then further metabolized to DA [1,2]. Currently, a number of assays are used to assess TH activity, including the tritium release method, high-performance liquid chromatography (HPLC) analysis for L-DOPA production, and ¹⁴CO₂ trapping [3-7].

These methods are viable options for the measurement of TH activity; however, they present considerable disadvantages. First, these methods are time-consuming; for example, HPLC analysis requires an average of 20 min per sample, leading to considerable lag time between experiment and data analysis [4,6]. Second, common methods for TH activity involve the isolation of radiolabeled products, necessitating purchase of radiochemicals that are costly and entail special handling and disposal. Tritium release assays require extensive post-run

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²*Abbreviations used:* TH, tyrosine hydroxylase; DA, dopamine; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; MAO, monoamine oxidase; MBP-TH, pMALc2H1; hTH, recombinant human tyrosine hydroxylase; DOPAL, 3,4-dihydroxyphenylacetaldehyde; PD, Parkinson's; oT-TH; LB/Amp, LB medium supplemented with ampicillin; BH₄, tetrahydrobiopterin; 3IT, 3-iodo-tyrosine; CoCl₂, cobalt chloride; DMPH₄, 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride.

work-up in order to separate tritiated water from the reaction mixture [3], and the alternative method of measuring release of $^{14}\text{CO}_2$ necessitates trapping of this compound; both assays are slow and involve liquid scintillation counting [8,9]. Another disadvantage to each of these methods is that measurement of product formation occurs at fixed time points, which does not allow for observation of real-time TH activity.

This study employed recombinant human tyrosine hydroxylase (hTH) to develop a novel real-time assay that measures TH activity by monitoring the production of L-DOPA spectrophotometrically. Sodium periodate was used to oxidize and cyclize L-DOPA to a chromophore (dopachrome), which absorbs at 475 nm and is easily detected using a plate reader. The specific activity of TH activity using the plate reader was compared with previously obtained data acquired using an HPLC instrument [4], with results for the two assays being comparable. A number of conditions were examined, including inhibition of hTH activity using 3-iodo-tyrosine [10] and cobalt chloride [11], which act as competitive inhibitors of tyrosine and iron binding, respectively. In addition, inhibition of TH by the reactive dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), previously characterized in our laboratory and implicated in the pathogenesis of Parkinson's disease (PD) [12,13], was assessed using this newly developed assay.

The advantages to this procedure include quicker sample work-up, shorter reaction time, and (most significant) real-time monitoring of TH activity. Furthermore, this assay demonstrates great promise for translation to high-throughput screening (HTS), which could prove to be useful in future studies involving therapeutic modulation of hTH activity.

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 flasks were cooled to 25 °C and monitored until growth reached OD₆₀₀ = 0.6. Then, concentrated solutions in water of isopropyl β-D-1-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 100,000g, 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, BH_4 , 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 (CoCl_2), was used as a second negative control [11]. Immediately prior to the addition of mixture B, 50 μM 3IT or 100 μM CoCl_2 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 $\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$ [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 CoCl_2 , background absorbance was assessed in wells containing all components (i.e., sodium periodate, Fe, BH_4 , CoCl_2 , 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, BH_4 , 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 BH_4 , 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride (DMPH_4) was used. DMPH_4 is a non-natural cofactor, and although it is less active than the natural cofactor BH_4 , 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 DMPH_4 (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, CoCl_2 (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:

$$Z_{\text{factor}} = 1 - \frac{3x(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|},$$

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 dopachrome production with $\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$ [9]. For HPLC analysis of L-DOPA production, conversion of peak area into concentration units was achieved using a calibration curve of standards. Data for the plate reader assays was compared with the control (i.e., no DOPAL or 3IT), and significant differences ($P < 0.01$) were determined using an analysis of variance with a Tukey posttest.

Results

Novel activity assay follows L-DOPA production in real time

Scheme 1 depicts the oxidation of tyrosine to L-DOPA by TH, followed by further oxidation of L-DOPA to the chromophore by sodium periodate. This cyclized product absorbs at 475 nm and was followed using the plate reader over the course of 30 min. Furthermore, a representative spectrum is depicted in Fig. 1, demonstrating the linear increase in absorbance as L-DOPA is produced and oxidized by sodium periodate over the course of the 30-min assay.

As described previously, DOPAL potently inhibits TH activity, leading to a significant decrease in both L-DOPA and DA [4]. Furthermore, 3IT and CoCl₂ are known competitive inhibitors of substrate and iron binding, respectively, and were used as control studies [10,11]. To measure TH inhibition by DOPAL, 3IT, and CoCl₂ using the plate reader assay, the inhibitors were added to mixture A immediately prior to the addition of mixture B. Absorbance readings were taken over the course of 30 min, and the concentration of L-DOPA produced was measured using $\epsilon = 3700 \text{ M}^{-1} \text{ cm}^{-1}$ [9]. Fig. 2 displays the data for the control, DOPAL (5, 10, and 20 μM), 50 μM 3IT, and 100 μM CoCl₂. On the addition of DOPAL (5 μM), enzyme activity decreased by more than 30%. TH inhibition was concentration dependent, with DOPAL at 10 and 20 μM causing 66% and 75% inhibition, respectively. 3IT and CoCl₂ also inhibited enzyme activity, with 50 μM 3IT leading to 50% inhibition of L-DOPA production and 100 μM CoCl₂ resulting in more than 80% inhibition.

Comparison of TH specific activities obtained via HPLC and plate reader

When the specific activity of TH determined using the plate reader assay is compared with the previously published value (HPLC method) [4], the findings are closely matched. Table 1 lists the average TH specific activities for the control and DOPAL (10 and 20 μM)- and

3IT-containing assays and compares them with values obtained via HPLC analysis. Comparison of data obtained using the two methods revealed no difference, indicating that the plate reader assay is a reliable method for measuring the production of L-DOPA over time.

Plate reader assay can be used in HTS studies

To determine whether the real-time hTH activity method could be applied to HTS, the plate reader assay was miniaturized in order to use a 384-well plate. In total, 48 wells were used for both positive (hTH with no inhibitor present) and negative (CoCl₂, competitive inhibitor for Fe binding) controls and were used to calculate the *Z*-factor. Reads were carried out for 3 h, and the *Z*-factor ranged from 0.825 to 0.925, well over the accepted threshold of 0.5 [18].

Furthermore, the excellent *Z*-factor was maintained over the course of the assay. Fig. 3A contains the spectra obtained from HTS analysis, and such findings demonstrate a high signal-to-noise ratio and a large stable screening window that could be used in future studies. Fig. 3B shows the consistency of the *Z*-factor over the course of the 3 h and also demonstrates the robustness of this method, with *Z*-factor values being well over 0.5. Combined, these results indicate the strong potential for this assay to be used in an HTS format. A variety of conditions and possible inhibitors of hTH activity could be investigated easily and quickly, possibly resulting in data currently unknown about the structure and function of hTH and how it is affected by both endogenous and exogenous toxins that humans may encounter throughout their lives. Furthermore, this assay could be used to investigate possible activators of TH activity, which could prove to be useful in PD patients who exhibit low DA levels.

Discussion

The work described here demonstrates a novel, real-time colorimetric assay for monitoring TH activity. This assay uses sodium periodate and leads to the formation of dopachrome, which can be monitored using a plate reader set to an absorbance of 475 nm.

It is important to note that TH activities measured in a variety of conditions (control, DOPAL, and 3IT) are closely matched to results from the previously published HPLC method used in our lab [4]. Such findings are outlined in Table 1 and demonstrate continuity between the two assays. Of note, the HPLC method does not use sodium periodate, and therefore the finding that both the HPLC and plate reader assays produce similar values for TH activity demonstrates that sodium periodate does not adversely affect TH. It is also important to note that incubation of each assay component with sodium periodate in Hepes buffer exhibited no significant change in absorbance at 475 nm (data not shown). Such results indicate that sodium periodate is reacting with L-DOPA using conditions described for the plate reader assay, making sodium periodate an acceptable agent for use in these studies.

Use of the plate reader to assess TH activity has a variety of advantages over other methods. First, it is significantly faster; there is no major lag time between sample preparation and data analysis. By employing a 96-well plate, it is easy to do multiples of an assay at once, thereby decreasing variability. Sodium periodate does not interfere with TH activity or interact with other components in the assay, making it an ideal reagent for converting L-DOPA to the dopachrome chromophore. Furthermore, this assay allows for real-time observation of TH activity as the reaction proceeds. The potential for the use of HTS was also investigated, with the assay showing promise for translation in screening high volumes of compounds in the future. As mentioned previously, the *Z*-factor was found to be between 0.825 and 0.925, which is well above the accepted threshold of 0.5. The HTS assay also

exhibited a high signal-to-noise ratio, and the Z-factor demonstrated constancy over the course of the assay, giving a large stable screening window that could be used to assess hTH activity. This assay could be used to investigate activators of enzyme activity [19], which could prove to be useful in PD patients who exhibit low levels of DA. Although the assay was developed for purified enzyme, at this point its utility for measuring TH activity in unpurified or partially purified tissue is conceivable but not known. Adapting the activity assay for tissue samples would require further optimization to minimize confounders such as turbidity, background absorbance, and tissue reactivity with assay components.

As our lab has shown previously, TH is potently inhibited by the endogenous neurotoxin DOPAL [4], with implications for PD such as decreased L-DOPA production [20]. The plate reader assay, as well as HTS studies, could be employed to further study the inhibition of hTH or, conversely, the activation of the enzyme, which could prove to be useful in discovering better treatments for PD patients.

Overall, this novel assay for TH activity can be performed for a variety of conditions with ease and speed using a plate reader to make sample analysis faster. The described assay may prove to be valuable given the disadvantages of other methods for measuring TH activity such as long sample analysis time, use of radiolabeled substrates, and extensive post-run work-up.

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Fig.1.

Representative spectra demonstrating the measurement of dopachrome production at 475 nm using the plate reader assay. TH activity exhibits a linear phase over the course of a 30-min assay, indicating the turnover of tyrosine to L-DOPA, which is further oxidized by sodium periodate to form dopachrome.

Fig.2.

Dopachrome production over the course of a 30-min assay. Treatment of hTH with DOPAL yielded concentration-dependent inhibition of activity. Furthermore, both 3IT and CoCl₂ led to significant inhibition of activity as compared with controls. All values shown represent the mean production of dopachrome $\pm$ standard errors between 0 and 30 min ($n = 5$ for control, DOPAL, and 3IT; $n = 3$ for CoCl₂). An asterisk (*) indicates significance from control cells (i.e., no DOPAL, 3IT, or CoCl₂ present) where $P < 0.01$.

Fig.3.

HTS assay for hTH activity in positive (maximum signal, no hTH inhibition) and negative (+ CoCl_2 , minimum signal) controls. (A) Absorbance values as L-DOPA is produced over time in the presence of no inhibitor (control) and 100 μM CoCl_2 over the course of 3 h with 90-s read intervals. Dotted lines demonstrate a stable screening time from 75 to 180 min and a high signal-to-noise ratio of 59. (B) Z-Factor over the course of the assay demonstrates a range from 0.825 to 0.925 (well above the accepted 0.5 threshold) and exhibits stability over the course of the assay.

Scheme 1.

L-DOPA production from tyrosine via TH. Oxidation of L-DOPA by sodium periodate (NaIO₄) with subsequent cyclization to dopachrome is the basis for the real-time kinetic assay, as dopachrome production can be measured at 475 nm via a plate reader.

Table 1

Comparison of specific activities determined via the HPLC assay [4] and the plate reader assay.

Assay	Control (nmol/min/mg)	10 μ M DOPAL (nmol/min/mg)	20 μ M DOPAL (nmol/min/mg)	50 μ M 3IT (nmol/min/mg)
HPLC	16.2 $\pm$ 1.42	5.06 $\pm$ 0.891	3.31 $\pm$ 0.542	2.13 $\pm$ 1.32
Plate reader	15.4 $\pm$ 1.13	3.55 $\pm$ 0.670	2.51 $\pm$ 0.776	2.63 $\pm$ 1.48

Note: There are no significant differences in results obtained using the two assays, indicating that the plate reader is a viable method for determining L-DOPA production by hTH. All values shown represent the means $\pm$ standard errors ($n = 5$ for plate reader assay, $n = 3$ for HPLC assay).