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Short Communication

Post-column immobilized tyrosinase reactor for determination of L-3,4-dihydroxyphenylalanine and L-tyrosine by high-performance liquid chromatography with fluorescence detection

Nobutoshi Kiba*, Masatoshi Itoi and Motohisa Furusawa

Department of Applied Chemistry and Biotechnology, Faculty of Engineering, Yamanashi University, Kofu 400 (Japan)

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ABSTRACT

Immobilized tyrosinase was used as a reactor in a liquid chromatographic system for the selective detection of L-3,4-dihydroxyphenylalanine (L-DOPA) and L-tyrosine. Tyrosinase was immobilized on controlled-pore glass beads. The compounds were separated on an ODS column with 0.1 *M* phosphate buffer (pH 7.2) as mobile phase. The fluorescent dihydroxyindol formed was detected at 490 nm (excitation at 360 nm). For L-DOPA the linear working range was 0.005–15 μM, with a detection limit of 1 n*M* (6 pg in a 30-μl injection); for L-tyrosine the range was 0.01–30 μM, with a detection limit of 5 n*M* (27 pg in a 30-μl injection). The reactor was stable for at least 2400 injections. Its usefulness for simultaneous determination of L-DOPA and L-tyrosine in serum is described.

INTRODUCTION

L-3,4-Dihydroxyphenylalanine (L-DOPA) is synthesized by the hydration of L-tyrosine with tyrosine hydroxylase in peripheral and central catecholaminergic neutrons and chromaffin cells of the adrenal medulla [1–3]. Usually, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) is used successfully for simultaneous determination of catechols and phenols [4,5]. Several papers have discussed the use of immobilized enzyme in post-column reactors for the selective determination of L-tyrosine and several amino acids [6–8], but there is no paper on the determination of catechols.

Tyrosinase (EC 1.14.18.1) catalyses the oxidation of catechols and phenols to *o*-quinones [9]. Immobilized tyrosinase was used in a flow-injection system for the determination of L-tyrosine in serum with fluorescence detection [10]. This paper describes the use of immobilized tyrosinase as a post-column reactor in a liquid chromatographic system for the simultaneous determination of L-DOPA and L-tyrosine. The compounds eluted from the separation column are oxidized to dopaquinone in a reactor. The quinone formed is rearranged to fluorescent 2,3-

* Corresponding author.

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dihydro-5,6-dihydroxyindole-2-carboxylic acid (indol) in a strongly alkaline solution. The indol is monitored by fluorescence detection.

EXPERIMENTAL

Reagents

Tyrosinase (2400 U mg^{-1}, from mushrooms), L-DOPA and L-tyrosine were obtained from Sigma (St. Louis, MO, USA). Aminopropyl-controlled-pore glass (CPG) (mean pore diameter 59 nm, amine concentration 76 μmol g^{-1}, particle size 55 ± 20 μm) was supplied by CPG (NJ, USA). TSKgel ODS-80T_M (5 μm) was obtained from Tosoh (Tokyo, Japan). All other reagents were of analytical-reagent grade. The preparation of the immobilized enzyme was similar to that described previously [9]. The immobilized enzyme was packed into a stainless-steel column (2 cm × 4 mm I.D.).

HPLC

Chromatography was carried out with a Model L-6000 pump (Hitachi, Tokyo), a Model 7125 injector valve (Rheodyne, CA, USA) with a 30-μl loop, a separation column (15 cm × 4 mm I.D.) and an immobilized tyrosinase reactor. A Model KHU-W-52 pump (Kyowa Seimitsu, Tokyo, Japan) was linked, via a T-piece junction, into the system after the reactor. Peaks were detected using a Model FP-210 spectrofluorimeter (Jasco, Tokyo, Japan), at excitatory and emission wavelengths of 360 nm and 490 nm, respectively, and peaks were integrated using a Chromatocorder II (System Instrument, Tokyo, Japan). The immobilized tyrosinase reactor was installed between the separation column and the T-piece junction. The separation of L-DOPA and L-tyrosine was achieved using a mobile phase of 0.1 *M* phosphate buffer (pH 7.2). The flow-rate was 0.6 ml min^{-1}. The reactor eluate was made basic by mixing with 3 *M* sodium hydroxide, which was pumped at 0.6 ml min^{-1} into the system via the T-piece junction using the KHU-W-52 pump. The mixing coil tubing was 2 m × 0.5 mm I.D. PTFE.

Procedure

Serum (500 μl) was deproteinated by adding 0.60 *M* perchloric acid (500 μl) and mixing on ice for 10 min. The mixture was filtered and the filtrate (50 μl) was neutralized with 0.15 *M* dipotassium hydrogenphosphate (50 μl). The neutralized solution was cooled at 4°C for 10 min and the supernatant (40 μl) was diluted with 0.1 *M* phosphate buffer (pH 7.2) to 100 μl. By this treatment, samples are diluted 10-fold. Aliquots of 30 μl were injected for HPLC determination.

RESULTS AND DISCUSSION

Reactor performance

To evaluate the properties of immobilized tyrosinase, the system was used in flow-injection mode by omitting the analytical column. The flow-rate of phosphate buffer was 0.6 ml min^{-1}. The influence of pH on the enzymatic reaction was studied over the pH range 6.5–7.8 by injecting 0.1 μM L-DOPA (30 μl). The optimum pH for the enzymatic reaction was from 7.2 to 7.4, as shown in Fig. 1. The buffer of pH 7.2 was selected for further work. The reactor was placed in a water bath and the temperature was varied between 20 and 40°C. The reactor exhibited the highest activity at 30°C, although the activity was not strongly influenced by the temperature (Fig. 1). The reactor was used by repeated injections of L-DOPA solution (5 μM) at a sample speed of 5 h^{-1}. The activity decreased gradually and remained at 80% of the initial value after 2400 injections. The responses to L-DOPA, L-tyrosine and other related compounds were as follows: L-DOPA (100), L-tyrosine (85), D-tyrosine (26), epinephrine (23), D,L-synephrine (4), L-tyrosyl-L-alanine (9), L-

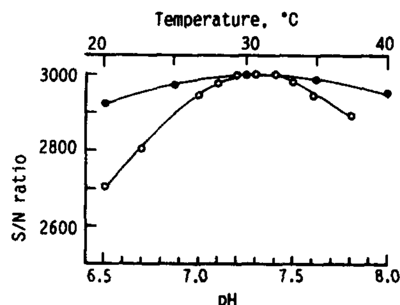

Fig. 1. Effects of (○) pH and (●) temperature on the signal-to-noise (*S/N*) ratio for L-DOPA. The *S/N* ratio was obtained by dividing the peak height (mm) by the baseline noise (mm).

tyrosyl-L-leucine (13), L-tyrosyl-L-glycine (15), L-tyrosyl-L-glutamic acid (9) and L-tyrosyl-L-glycyl-L-glycine (6).

Indol formation reaction

The eluate from the reactor was mixed with various concentrations of sodium hydroxide solution. The concentration was varied from 2 to 4 *M*. The maximum response was obtained at 3 *M* sodium hydroxide. The effect of flow-rate was studied by changing the flow-rate of 3 *M* sodium hydroxide from 0.3 to 1.0 ml min^{-1}. Maximum response was obtained from 0.6 to 0.8 ml min^{-1}. With increasing flow-rate of sodium hydroxide the baseline stability became poor because of the incomplete mixing. A flow-rate of 0.6 ml min^{-1} was chosen.

Chromatogram

A representative chromatogram illustrating resolution of standard mixtures of L-DOPA and L-tyrosine is shown in Fig. 2. The ratio of peak heights for L-DOPA and L-tyrosine was 100:67. The peak height was plotted against the concentration of the compounds. The concentration ranges of linear response were from 0.005 to 15 μM for L-DOPA and from 0.01 to 30 μM for L-tyrosine. The detection limits (signal-to-noise ratio 3) for L-DOPA and L-tyrosine were 1 n*M* (6 pg) and 5 n*M* (27 pg in a 30-μl injection), respectively. Repeated determination ($n = 7$) of standard mixtures at a concentration of 0.1 μM gave relative standard deviation (R.S.D.) of 1.8% for L-DOPA and 2.1% for L-tyrosine.

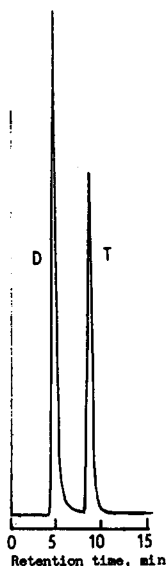

Fig. 2. Chromatogram of a standard mixture of L-DOPA and L-tyrosine. The 30-μl injection consisted of aqueous mixture containing 100 n*M* each. D = L-DOPA; T = L-tyrosine; mobile phase, 0.1 *M* phosphate buffer (pH 7.2) (0.6 ml min^{-1}); separation column, TSKgel ODS-80T_M (5 μm) (15 cm × 4 mm I.D.); reactor, immobilized tyrosinase (2 cm × 4 mm I.D.); basifier, 3 *M* sodium hydroxide (0.6 ml min^{-1}); detection, fluorimetry ($\lambda_{ex.}$ 360 nm, $\lambda_{em.}$ 490 nm).

Application

This system was used to determine the amounts of L-DOPA and L-tyrosine in serum. A sample of human serum containing known L-DOPA and L-tyrosine concentrations was supplemented with L-DOPA and L-tyrosine to give final concentrations of 0.1–6 μM L-DOPA and 65–255 μM L-tyrosine. The recoveries were 98–103% for L-DOPA and 99–101% for L-tyrosine. The detection limit (signal-to-noise = 3) for L-DOPA for serum samples was 10 n*M*. This method was used for the determination of L-DOPA and L-tyrosine in the serum obtained from a Parkinsonian patient treated with Sinemet. L-DOPA and L-tyrosine in the serum were 4.5 μM [R.S.D. ($n = 5$) 2.4%] for L-DOPA and 67.6 μM (2.6%) for L-tyrosine. The detection limits (signal-to-noise ratio = 3) for L-DOPA and L-tyrosine for serum samples were 10 n*M* and 50 n*M*, respectively.

CONCLUSIONS

We have shown that an immobilized tyrosinase reactor is useful for the simultaneous detection of L-DOPA and L-tyrosine using reversed-phase HPLC and fluorimetric detection. The reactor in a post-column system is stable enough to permit the measurement of more than 2400 samples for 20 days. Although this method can be easily used routinely for the determination of L-DOPA and L-tyrosine in the serum of Parkinsonian patients on DOPA therapy, endogenous L-DOPA (*ca.* 15 n*M* [11]) in the serum of non-treated and/or normal subject cannot be determined by this method. The HPLC–ED methods are sensitive but require rather complicated sample clean-up procedures and maintenance for electrodes [12,13]. This method proved to be simple and

selective for the simultaneous determination of therapeutic L-DOPA and L-tyrosine in serum.

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