Korean Chem. Eng. Res., 54(6), 817-821 (2016)
http://dx.doi.org/10.9713/kcer.2016.54.6.817
PISSN 0304-128X, EISSN 2233-9558

L-DOPA Synthesis Using Tyrosinase-immobilized on Electrode Surfaces

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(Received 21 September 2016; Received in revised form 17 October 2016; accepted 25 October 2016)

Abstract – Levodopa or L-3,4-dihydroxyphenylalanine (L-DOPA) is the direct precursor of the neurotransmitter dopamine. L-DOPA is a well-known neuroprotective agent for the treatment of Parkinson's disease symptoms. L-DOPA was synthesized using the enzyme, tyrosinase, as a biocatalyst for the conversion of L-tyrosine to L-DOPA and an electrochemical method for reducing L-DOPAquinone, the product resulting from enzymatic synthesis, to L-DOPA. In this study, three electrode systems were used: A glassy carbon electrode (GCE) as working electrode, a platinum, and a Ag/AgCl electrode as auxiliary and reference electrodes, respectively. GCE has been modified using electropolymerization of pyrrole to facilitate the electron transfer process and immobilize tyrosinase. Optimum conditions for the electropolymerization modified electrode were a temperature of 30 °C and a pH of 7 producing L-DOPA concentration 0.315 mM. After 40 days, the relative activity of an enzyme for electropolymerization remained 38.6%, respectively.

Key words: L-DOPA synthesis, Tyrosinase-immobilized, Electrode surface, Parkinson's disease

1. Introduction

More than ten million people in the world suffer from Parkinson's disease. Parkinson's disease is characterized by a decreased level of the neurotransmitter dopamine. Levodopa (L-DOPA) is the drug most often prescribed for Parkinson's disease. Synthesis of this drug by a proprietary Monsanto process suffers from several disadvantages [1]. The Monsanto Process was the first commercialized catalytic asymmetric hydrogenation synthesis employing a chiral transition metal complex, and it has been in operation since 1974 [2,3]. However, this method suffers from disadvantages such as low overall yields, the need for separation of diastereomers, and the need for expensive chiral catalysts [4].

Many studies have been conducted to limit production costs, improve the conversion rate, and improve the enantioselectivity of L-DOPA. Microbial production of L-DOPA from *Erwinia herbicola*, *Stizolobium hassjoo*, and *Escherichia coli* has also been investigated. However, microbial production of L-DOPA can be expensive due to the need to remove proteins and hormones produced by microbial cells. These processes are time-consuming and result in low conversion rates [5-9]. One promising approach is production of L-DOPA from the enzymatic conversion of the L-tyrosine by using tyrosinase. The high cost of enzymes becomes one of the disadvantages of the enzyme-based process. Current biotechnological needs are enhanced enzyme productivity and development of techniques to increase the shelf-life of enzymes for various applications [10]. These requirements are essential to facilitate large-scale and economical formulation. Enzymatic immobilization provides an excellent base for increasing the availability of the enzyme to the substrate with greater turnover over a considerable period of time [11]. The ability to stop the reaction and easily separate the enzyme and product are added advantages of enzyme immobilization. Although there are many published immobilization methods, few studies improve the properties of the enzyme and none of the methods can be scaled to industrial levels. However, there is an unmet need for a simple and efficient protocol to immobilize an enzyme with good activity and stability at an industrial scale [12].

In this study, the tyrosinase enzyme was immobilized using electropolymerization methods for the purpose of synthesizing L-DOPA.

2. Experimental

2-1. Materials and equipment

Tyrosinase enzyme, L-Tyrosine, L-DOPA, and all remaining chemicals were purchased from Sigma-Aldrich (St. Louis, USA) at the highest grade available and were used without further purification. Equipment for dopamine sensor and L-DOPA synthesis consist of the reactor, temperature control, electrode, and potentiostat as shown in photo 1. Photo 2 shows the electrochemical batch reactor had 30 mL working

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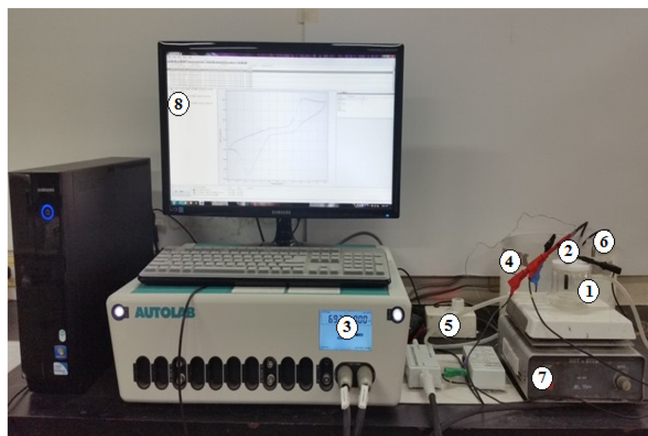

Photo 1. Experimental equipment for L-DOPA synthesis.
1. Reactor
2. Electrode
3. Electrochemical analyzer
4. Water bath
5. Temperature controller
6. Heating coil
7. Magnetic Stirrer
8. Monitor

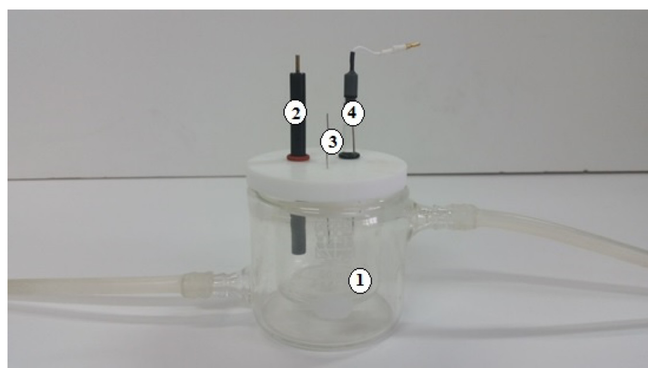

Photo 2. Reactor system for L-DOPA synthesis.
1. Reactor, 2. Glassy carbon working electrode, 3. Platinum wire auxiliary electrode, 4. Ag/AgCl reference electrode

volume and was made of glass. The electroenzymatic synthesis was carried out in three electrode cells, comprising Ag/AgCl (WonATech, Seoul, Korea) reference electrode, the platinum wire auxiliary electrode (Dongsun Science Co. Ltd, Ansan, Korea), and glassy carbon working electrode (WonATech, Seoul, Korea). Cyclic voltammetry and amperometric measurement were performed using an AUTOLAB potentiostat (PGSTAT302N, Metrohm, Netherlands) with NOVA software.

2-2. Methods

2-2-1. Preparation of modified electrode

The bare GCE was polished with an aluminum slurry and then ultrasonically washed with distilled water to remove adsorbed impurities. A hundred milligrams of multi-walled Carbon nano tube (MWNT) was treated with 10 mL of a nitric acid and sulfuric acid mixture (3:1, v/v%) for four hours, washed with distilled water until the mixture had a neutral pH (pH=7), and dried at ambient temperature to introduce carboxyl groups [13]. Acid-treated MWNTs and tyrosinase (2000 units) were incubated in a refrigerator for one hour. Electropolymerization was carried out in an aqueous solution containing 0.01 M pyrrole and MWNT modified tyrosinase in a three-electrode cell with a Pt-wire auxiliary and Ag/AgCl electrodes. The electrode was rinsed thoroughly with Milli-Q water after electropolymerization to remove any loosely-bound enzyme. Electropolymerization of pyrrole was achieved by applying 1.0 V for 40 min [14]. After polymerization, a thin layer of dark polypyrrole was formed on the electrode surface. Entrapment of tyrosinase was achieved during polymerization. The electrode was rinsed with deionized water to remove any unreacted pyrrole and enzymes.

2-2-2. Electroenzymatic synthesis of L-DOPA

L-DOPA was electroenzymatically synthesized in a 50 mL batch reactor with three electrode system [15]. A total of 30 mL variation concentration of L-tyrosine (in 50 mM phosphate buffer) was used as the substrate in experiments. The reaction was conducted under the reduction potential of DOPAquinone. The pH and temperature also were varied to get the best result for the condition of the reaction.

2-2-3. Quantitative Analysis

The L-DOPA content of the samples was determined in the following manner: to each sample (1 mL), in this sequence, 1 mL of 2 M hydrochloric acid, 1 mL sodium hydroxide and 1 mL of a solution containing 15% (w/v) sodium molybdate and 15% (w/v) sodium nitrite were added. The hydrochloric acid inactivated free residual tyrosinase, stopping the formation of L-DOPA, and prevented the subsequent conversion of L-DOPA to melanin. Sodium hydroxide was added to neutralize the medium. Sodium nitrite reacts with L-DOPA to yield a yellow solution which may be detected spectrophotometrically. Sodium molybdate was added to prevent decomposition of the sample. The sample treated in this manner was shaken briefly in a vortex mixer. Since formation of L-DOPA complex is time dependent, substantial L-DOPA concentration was determined after one hour spectroscopically at 414 nm [15,16].

3. Results and Discussion

3-1. SEM Analysis

To observe the surface morphology of electropolymerization of pyrrole, the surface of working electrode was examined by scanning electron microscope. The SEM images as shown in Fig. 1A showed the morphology of electropolymerization of polypyrrole. Fig. 1B and 1C show the MWNT/PPy without and with tyrosinase enzyme, respectively. From these results, we can conclude that after immobilization of the enzyme, the surface of MWNT/PPy has a smoother surface and well dispersed polymer.

3-2. Cyclic voltammogram of L-DOPA

Cyclic voltammetry is an electrochemical tool to study electrochemical reactions and determine the performance of an electrode [17]. Fig. 2 shows a cyclic voltammogram of 1 mM L-DOPA in 50 mM

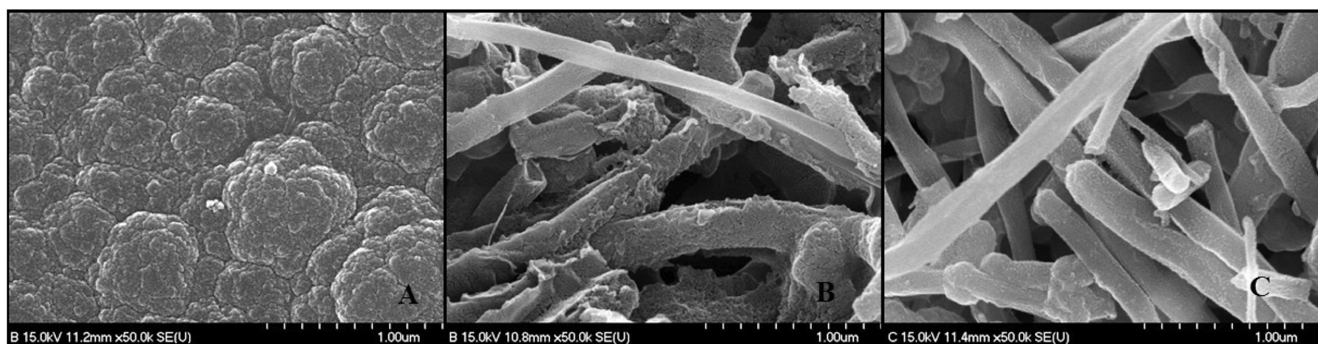

Fig. 1. Scanning electron microscope images for (A) Electropolymerization of Pyrrole (B) Electropolymerization of MWNT/PPy (C) Electropolymerization of MWNT/Tyr/PPy.

pH 7 phosphate buffer in the three-electrode system to determine the reduction potential of DOPAquinone. The cyclic voltammogram was conducted with a Ag/AgCl reference electrode, a platinum wire auxiliary electrode, and an electropolymerized carbon working electrode. In an electrochemical polymerization, the monomer (pyrrole), dissolved in an appropriate solvent containing the desired anionic doping salt, $LiClO_3$, is oxidized at the surface of the electrode by application of an anodic potential (oxidation). The choice of the solvent and electrolyte is of particular importance in electrochemistry, since both solvent and electrolyte should be stable at the oxidation potential of the monomer and provide an ionically conductive medium [18]. As shown in Fig. 2, the L-DOPA cyclic voltammogram had an oxidation peak at 218 mV and one reduction peak at 129 mV. The oxidation peak indicates that L-DOPA was oxidized to DOPAquinone, and the reduction peak indicates that the DOPAquinone was reduced to L-DOPA. To convert DOPAquinone to L-DOPA again, the working potential was 129 mV for electroenzymatic L-DOPA synthesis. Liu et al. [19] tested the electrochemical behavior of L-DOPA and reported the cathode and anode potential values as 576 mV and 610 mV, respectively. The authors did the experiment at an acidic pH. This might be the cause for the difference in cathode and anode potential for the electrochemical reaction. Rahman et al. [16] conducted the optimization of an electrochemical L-DOPA synthesis and reported that L-DOPA has and oxidation peak at 376 mV and a reduction peak at −550 mV. This peak difference also might be from different working electrode material. Polypyrrole is one of the most extensively used conducting polymers in the design of bioanalytical sensors. Of all conducting polymers, polypyrrole might be easily formed from neutral-pH aqueous solutions containing pyrrole monomers [20].

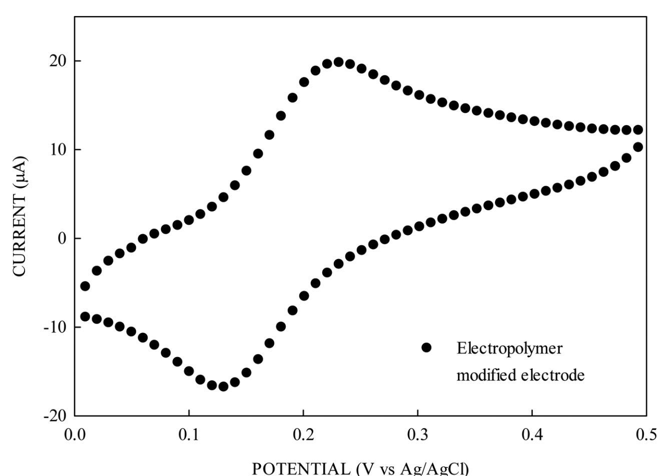

Fig. 2. Cyclic voltammogram of 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in 50 mM phosphate buffer (pH 7, temperature: 30 °C, and scan rate: 50 mV).

3-3. Effect of pH and temperature on electroenzymatic reaction

The effects of pH and temperature were determined by changing pH between pH 6 to 8 and temperature between 10~50 °C. The optimum pH and temperature (Figs. 3 and 4) for the electroenzymatic synthesis of L-DOPA using polypyrrole modified electrode was at pH 7 and 30 °C producing L-DOPA with a concentration of 0.315 mM. The entrapped enzyme was negatively charged at pH 7 phosphate buffer that protects enzyme against denaturation. This polymer matrix has a suitable environment for enzyme and electrode reaction.

Our result confirmed the findings of Rahman et al., 2012 [16] that the optimum conditions for the electroenzymatic synthesis of L-DOPA and/or free enzyme were at pH 7 and 30 °C. Erdogan et al. [21] and Yildiz et al. [14] reported the optimum pH and temperature for L-

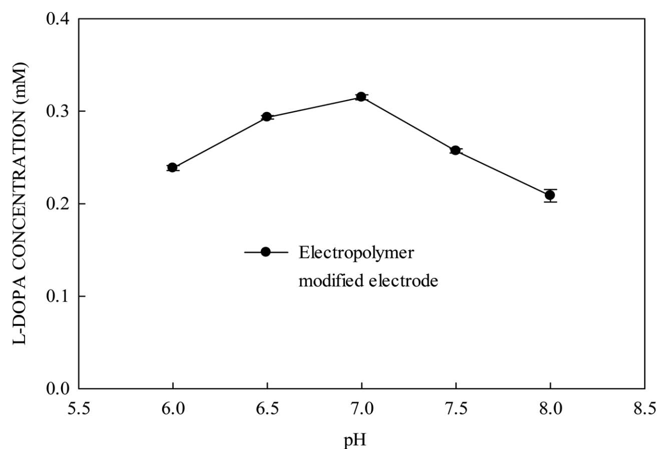

Fig. 3. Effect of pH on L-DOPA synthesis reaction at the polypyrrole-modified electrode (in phosphate buffer temperature: 30 °C).

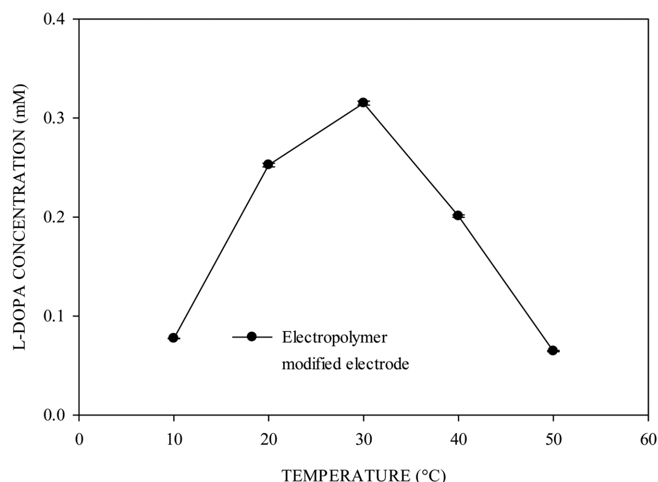

Fig. 4. Effect of temperature on L-DOPA synthesis reaction at the polypyrrole-modified electrode (in phosphate buffer pH 7).

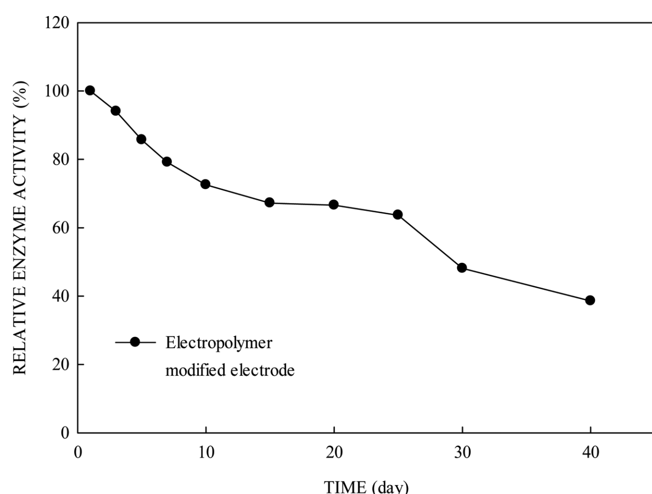

Fig. 5. Shelf-life of the polypyrrole-modified electrode on L-DOPA synthesis reaction (in phosphate buffer pH 7 and temperature: 30 ^{o}C).

DOPA synthesis using conducting polymers. Erdogan et al. [21] changed pH between pH 6 and 7.5 and for all experiments using a free enzyme, polypyrrole, and Poly(3,4-ethylenedioxythiophene) (PEDOT) matrices; maximum activity was observed at pH 7. For maximum temperature, the maximum activity was found at 30 ^{o}C and polypyrrole matrix showed a reasonable activity in a wide temperature range compared to PEDOT matrix.

Yildiz et al. [14] investigated the synthesis of L-DOPA by immobilizing tyrosinase on conducting polymers: thiophene-capped poly (ethyleneoxide)/polypyrrole (PEO-co-PPy) and 3-methylthienyl methacrylate-co-p-vinylbenzyloxy poly(ethyleneoxide)/pyrrole (CP-co-PPy). The optimum pH for both the conducting polymers was around pH 7. However, maximum activity for CP-co-PPy and PEO-co-PPy matrices was at 50 and 60 ^{o}C, respectively. PEO and PPO polymer modifications may have contributed to higher stability at higher temperatures, while some researchers reported that higher temperature can induce protein denaturation and deactivation [22,23].

3-4. Stability of polypyrrole-modified electrode

The stability of the polypyrrole-modified electrode was investigated by running enzymatic reaction until 40 days. It is obvious that 25 days after storage under specified condition, the relative activity decreased to around 60%. And after 40 days, it showed further decrease to around 40% and 30%. The electrode was stored in the phosphate buffer while not in use.

Erdogan et al. [21] reported that immobilization of tyrosinase, the activity of polypyrrole electrode was checked every 5 days for 65 days and after 50 days, the activity of polypyrrole matrix was decreased by 40%.

4. Conclusions

L-DOPA was synthesized by using electropolymerization. Electropolymerization of tyrosinase on the surface electrode was observed the effect of pH and temperature for L-DOPA synthesis. Optimum pH and temperature for electropolymerization of the electrode were pH 7 and 30 ^{o}C, respectively. It produced L-DOPA concentration 0.315 mM. The stability of electrode also showed good activity with the concentration of L-DOPA after 40 days, the relative activity of enzyme remained 38.60%.

Acknowledgment

This study was financially supported by Chonnam National University (2012-2825).

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