L-DOPA Synthesis Using Tyrosinase Enzyme Precipitate Coatings on the Surface of Electrode

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ABSTRACT

An estimated seven to ten million people worldwide are living with 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. This drug is currently chemically synthesized according to a Monsanto process, but the synthesis of L-DOPA suffers from several disadvantages. L-DOPA was synthesized using a tyrosinase enzyme as a biocatalyst for converting L-tyrosine to L-DOPA and an electrochemical method for reducing L-DOPAquinone and the product resulting from enzymatic synthesis to L-DOPA. In this study, three electrode systems consist of glassy carbon electrode (GCE) as working electrode, platinum and Ag/AgCl electrode as auxiliary and reference electrode, respectively. GCE was modified using enzyme-precipitate coating methods to facilitate the electron transfer process and immobilize tyrosinase. Optimum conditions for enzyme precipitate coatings electrode were found to be 30°C and pH 6.5 resulting in L-DOPA concentration of 0.733 mM. After 40 days, relative activity of enzyme for EPC modified electrode remained 73.26%.

Key words: L-DOPA tyrosinase, enzyme precipitate coatings, Parkinson's disease.

INTRODUCTION

L-DOPA production process was investigated after a fairly massive dose of L-DOPA was used in treating Parkinson’s disease, which created a sizable demand for this rare amino acid (Blaser and Schmidt, 2004).

Nowadays, many researchers are trying to get an alternative way to reduce production cost and make production process more efficient. Some researchers also tried to produce L-DOPA through an enzymatic process.

Tyrosinase (monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme found in micro-organisms, plants and animals. This enzyme has two activities, such as the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity) (Ros et al., 1993).

However, some disadvantages of the enzymatic method are the high cost of the enzyme and the reducing agent, a difficulty separating L-DOPA from the reaction media and low productivity (Ates et al., 2007; Pialis et al., 1996; Min et al., 2010). On the other hand, a difficulty separating L-DOPA from reaction media, low productivity, the high cost of the enzyme and the reducing agent still becomes a problem to be solved (Pialis et al., 1996; Min et al., 2010).

Some of the common approaches were applied to the immobilization of tyrosinase regarding L-DOPA synthesis including entrapment in calcium alginate (Ates et al., 2007), crosslinked methods using glutaraldehyde (Seetharam and Saville, 2002; Tuncagil et al., 2009), membrane (Pialis et al., 1996; Algieri et al., 2012; Donato et al., 2012), cross-linked enzyme aggregates (CLEAs) (Xu et al., 2009), and entrapment in calcium alginate (Tuncagil et al., 2009).
et al., 2012) and polymer (Ho et al., 2003; Erdogan et al., 2010; Yildiz et al., 2013). However, there is still need to find the best immobilization process not only to achieve high activity and stability of enzyme but also a very simple procedure for industrial applications.

The objective of the present work is to immobilize tyrosinase enzyme for application of L-DOPA synthesis. The Enzyme Precipitate Coatings (EPC) method was studied for immobilization to find very simple methods for industrial applications and efficient protocols that allow for great improvement of the enzyme properties as a consequence of immobilization. We also investigated the influence of carbon nanotube as support materials to improve the kinetic electrode activity.

MATERIALS AND METHODS

Materials and equipment

Tyrosinase enzyme, L-Tyrosine, L-DOPA and all the remaining chemicals were purchased from Sigma-Aldrich (St. Louis, USA) at the highest grade available and used without further purification. Equipment for dopamine sensor and L-DOPA synthesis consist of the reactor, temperature control, electrode and potentiostat as shown in Figure 1. Figure 2 shows the electrochemical batch reactor had 30 ml working volume made up 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 measurements were performed using an AUTOLAB potentiostat (PGSTAT302N, Metrohm, Netherlands) with NOVA software.

Methods

For introducing the surface with carboxyl groups, the surface carbon materials were functionalized by the covalent and non-covalent method. Covalent functionalization was conducted oxidizing the materials in concentrated nitric acid (68 wt%) and then sonicated for about 4 h. For non-covalent functionalization, 20 ml of 100 ml 1-pyrenebutyric acid in dimethylformamide (98.9%) was adsorbed onto the hydrophobic surface of 0.5 g of carbon nanopowder for 3 h at room temperature and shaking at 250 rpm. After 1-pyrenebutyric acid treatment, the carbon nanopowder was washed with methanol and then distilled water.

The samples were incubated in 1.5 ml of 200 mM 1-
ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in aqueous buffer (50 mM sodium phosphate buffer, pH 7.2) for 30 min at room temperature and shaking at 250 rpm (Kim et al., 2011). After incubation, the samples were centrifuged and then washed with aqueous buffer.

The functionalized samples and tyrosinase enzyme (2000 units) were incubated in the refrigerator overnight. To prepare EPC (Enzyme Precipitation Coating), 0.55 g/ml of ammonium sulfate was added to the mixture, which precipitates the enzyme molecules in the surface of the functionalized samples (Min et al., 2012).

Glutaraldehyde with the final concentration of 0.5 wt% and pH 8 was added to crosslink the precipitated enzyme molecules. Then, 2.5 wt% of Nafion with pH 7 was added to the solution and the 10 μl of mixing solution was dropped onto the surface of the electrode.

RESULTS AND DISCUSSION

SEM analysis

Figure 3 shows SEM images for EPC methods immobilization of enzyme. Figure 3D shows the surface after precipitation of tyrosinase. There is no significant change, however, the morphology of MWNT changed substantially from smooth to rough surface. This transformation probably correlates with the precipitating agent that precipitates the enzyme to the surface of MWNT.

Cyclic voltammogram of L-DOPA

Figure 4 shows a cyclic voltammogram of 1 mM L-DOPA in 50 mM pH 7 phosphate buffer in the three electrode system to determine the DOPA quinone reduction potential. The cyclic voltammogram was conducted with an Ag/AgCl reference electrode, a platinum wire auxiliary electrode and a tyrosinase enzyme precipitate coatings modified working electrode. As shown in Figure 4, the L-DOPA cyclic voltammogram had an oxidation peak at 206 mV and one reduction peak at -408 mV. The oxidation peak indicated that L-DOPA was oxidized to DOPA quinone and the reduction peak indicated that the DOPA quinone was further reduced to L-DOPA. To convert DOPA quinone to L-DOPA, the working potential was -408 mV for electroenzymatic L-DOPA synthesis. The current also shows a higher peak at 60.6 μA for oxidation peak and 25.8 μA for reduction peak. This result is probably due to the properties of the multi-walled carbon nanotube and as such the peak result showed very good activity in the transfer of the electron. The distance between oxidation peak and reduction peak also became shorter than GCE without multiwalled carbon nanotube modification which is 614 mV.

Comparing the result with the previous work of Min et al. (2010) and Rahman et al. (2012), oxidation and reduction peaks were 376 and -550 mV, respectively. The component and method used in this experiment improved the performance of the electrode. Multi-walled carbon nanotube was used as a supporting material for
immobilization of precipitate enzyme. Its properties could improve the performance of the electrode.

**Effect of pH and temperature on modified EPC electrode**

Figure 5 shows the effect of varying pH on the synthesis of L-DOPA at the surface EPC modified electrode. The enzyme was immobilized using EPC methods attached at the surface of the electrode. As shown in Figure 5, optimum pH for the synthesis of L-DOPA using EPC modified electrode was observed at pH 6.5 producing L-DOPA concentration until 0.733 mM. Relative enzyme activity at pH 7 and 7.5 was also high, producing L-DOPA concentration at 0.51 and 0.588 mM, respectively. We can conclude that synthesis of L-DOPA using EPC modified electrode has Broad effective pH. Xu et al. (2011) showed that the stability of crosslinked tyrosinase aggregates incubated at 60°C has higher stability at pH 6. Xu et al. (2012) further investigated that at 30°C, optimum pH for crosslinked tyrosinase aggregates was also at pH 6. This result is also similar to that of Min et al. 2010. Min et al. (2010) used pH 6 for the experiments and produced 0.7 mM L-DOPA concentration using tyrosinase-carbon nanoparticles-polypyrrole composite electrode.

Relative enzyme activity with respect to temperature was also observed. Figure 6 shows the effect of varying temperature from 10 to 50°C. From Figure 5, the optimum temperature was 30°C. Figure 6 showed that EPC modified electrode has also quite good activity on temperature above 30°C. We can conclude that EPC modified electrode has good thermal stability. This result was similar to the previous work reported by Xu et al. (2011). Xu et al. (2012) showed the same optimum temperature at 30°C and this immobilization method is quite stable at a higher temperature.
Stability of the EPC-modified electrode

The stability of EPC modified electrode was investigated by running enzymatic reaction for 40 days. From Figure 7, it was observed that after 40 days the activity of the enzyme remained at 73.3%. For L-DOPA synthesis, this result indicated very good stability of immobilization. Kim et al. (2011) reported that activity of EPC method was about 40 to 50 times higher than immobilization using the covalent attachment and cross-linked enzyme. The stability of this method was also examined by performing the G0x at room temperature. EPC method exhibited negligible activity loss for 200 days. These results suggest that multi-point covalent linkages of enzyme molecules effectively prevent the enzyme molecules of EPC methods from being structurally denatured and leached (Martinek et al., 1977).

Immobilization of the enzyme through crosslinked tyrosinase aggregates has been proven to be effective in rendering the enzyme more stable against various deactivating conditions including pH, temperature, denaturants, inhibitors and organic solvents (Xu et al., 2011).

The stability of this immobilization in organic media can also be significantly enhanced (Xu et al., 2012, 2011).

Conclusions

The Enzyme Precipitate Coatings (EPC) electrode was used for L-DOPA synthesis. Optimum pH and temperature for EPC electrode were pH 6.5 and 30°C. The concentration of L-DOPA produced using EPC modified electrode achieved 0.733 mM. The stability of EPC modified electrode was investigated by running enzymatic reaction for 40 days. It was observed that after 40 days the activity of the enzyme remained at 73.26%. This result indicated very good...
Figure 5. Effect of pH on L-DOPA synthesis reaction at EPC modified electrode (in phosphate buffer temperature: 30°C).

Figure 6. Effect of temperature on L-DOPA synthesis reaction at EPC modified electrode (in phosphate buffer pH 7).
Figure 7. Shelf life of EPC modified electrode on L-DOPA synthesis reaction (in phosphate buffer pH 7 and temperature: 30°C).

stability of immobilization.

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