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Novel strategy for enhancing productivity in L-DOPA synthesis: The electroenzymatic approach using well-dispersed L-tyrosine

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ABSTRACT

Although L-DOPA (L-3,4-dihydroxyphenylalanine) is widely used as a drug for Parkinson's disease, there are critical drawbacks in the commercial synthetic method such as low conversion rate, poor productivity, and long operational time. In order to overcome these limitations, a novel electroenzymatic system using tyrosinase/carbon nanopowder/polypyrrole composite as a working cathode was reported with the outstanding conversion rate up to 95.9%. However, the productivity was still limited due to a low solubility of the substrate L-tyrosine in aqueous phase. Herein, we demonstrated a novel strategy for enhancing the productivity by employing well-dispersed L-tyrosine as the substrate. When using well-dispersed L-tyrosine, not only the concentration of the substrate was increased to 90.6 gL^{-1} in aqueous phase but also the productivity was enhanced up to 15.3 gL^{-1} h^{-1}. We also determined kinetic parameters in the electroenzymatic system and the kinetic results revealed that the outstanding conversion rate was based on the fast electrical reduction of the by-product to L-DOPA. Thus the electroenzymatic synthesis using well-dispersed L-tyrosine can be a potential candidate as a novel process for L-DOPA synthesis.

1. Introduction

L-DOPA (L-3,4-dihydroxyphenylalanine) is a naturally occurring amino acid made from L-tyrosine in the human body. Because L-DOPA is a precursor of dopamine, can pass across the blood brain barrier while dopamine itself cannot, and thus can increase the dopamine concentration in the brain, it has been widely used as drug for Parkinson's disease since 1960's [1].

Since Mosanto developed the asymmetric hydrogenation process for L-DOPA synthesis, about hundreds tons of L-DOPA have been produced per year by the asymmetric hydrogenation. However, the asymmetric processes exhibit critical limitations including a poor conversion rate and a low enantiomeric excess [2,3]. Besides Mosanto process, Ajinomoto industrialized the asymmetric synthesis of L-DOPA from catechol, pyruvic acid and ammonia, catalyzed by tyrosine phenol lyase (E.C. 4.1.99.2) [4]. However, tyrosine phenol lyase is only synthesized under L-tyrosine-induced condition in cells and then addition of L-tyrosine to culture medium is unavoidable for preparing tyrosine phenol lyase [5]. Therefore, researches have focused on developing alternatives for L-DOPA synthesis not only to economize the production cost but also to improve the conversion rate and the enantioselectivity.

As one of the alternatives, L-DOPA has been enzymatically produced from isolated tyrosinase using L-tyrosine as a substrate. Tyrosinase (E.C.1.14.18.1) is a multifunctional copper-containing oxidoreductase and has two enzymatic activites; a cresolase activity for *o*-hydroxylation of monophenols and a catecholase activity for oxidation of diphenols to quinones. L-DOPA is produced from L-tyrosine by the cresolase activity and then L-DOPA is sequentially oxidized to DOPAquinone by the catecholase activity. To reduce the by-product DOPAquinone to L-DOPA again, chemical reducer has been usually employed. However, this enzymatic system also has several drawbacks: (1) the high cost of the reducing reagent; (2) the difficulty in separating L-DOPA from the reaction media including unreacted L-tyrosine and the reducing reagent; and (3) the low conversion rate and the poor productivity in a batch reactor [6–8].

In order to overcome the drawbacks in the enzymatic system and achieve a higher conversion rate, a novel synthetic method for L-DOPA using electrical reducing power was suggested as shown in Scheme 1 with an outstanding a conversion rate of 95.9% [9]. However, there is still limitation in the electroenzymatic system for improving the productivity because of the low solubility of substrate L-tyrosine in aqueous phase. For resolving the solubility limitation, we perceived that emulsification often enforced for poor soluble or insoluble material in aqueous phase and thus choose dispersed substrate by emulsification, not solution.

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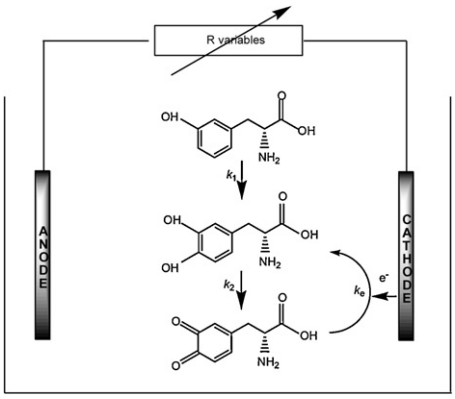

Scheme 1. Schematic diagram of electroenzymatic L-DOPA synthesis (anode: ELAT with 20% Pt wire, cathode: tyrosinase/carbon nanopowder/polypyrrole composite electrode).

Herein, we used well-dispersed L-tyrosine in order to increase the substrate concentration in aqueous phase and then achieved the improving productivity. Also we reported the reason for the high conversion rate achieved in the electro-enzymatic system by determining the kinetic parameters.

2. Materials and methods

Tyrosinase (E.C. 1.14.18.1) from mushroom purchased from Sigma–Aldrich. Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich at the highest grade available and were used without further purification. ELAT with 20% Pt and Ag/AgCl electrode were purchased from WonATech (Korea). All electrochemical experiments were controlled by Autolab PGSTAT 302 N potentiostat/galvanostat (Ecochimie, Netherland).

2.1. L-DOPA synthesis using well-dispersed L-tyrosine

Well-dispersed L-tyrosine was prepared by the wet-milling method referred to in the US patent 5145684. Zirconium oxide and polyvinylpyrrolidinone K-30 were used as milling media and surfactant, respectively. The reaction mixture including the milling media, the surfactant, and L-tyrosine was incubated for 72 h at ambient temperature, while shaking at 20 rpm. After the reaction had reached completion, the milling media was removed by filtration. The concentration of the prepared well-dispersed L-tyrosine solution was determined after filtration using Bradford reagent.

The 3-dimensional tyrosinase/carbon nanopowder/polypyrrole composite electrode was prepared in accordance with the previous report [9]. All L-DOPA synthesis were conducted in batch reactor with 60 mL of well-dispersed L-tyrosine in phosphate buffer (pH 6.5). The 3-dimensional tyrosinase/carbon nanopowder/polypyrrole composite electrode, ELAT with 20% Pt, and Ag/AgCl were used as the working, counter, and reference electrode, respectively. The cathodic potential was maintained at −530 mV vs. Ag/AgCl during the reaction. The synthesized L-DOPA was analyzed by modified Arnow method as previously reported [9].

2.2. Kinetic study

To determine the reaction constants of tyrosinase catalysis by Lineweaver–Burk plot, L-tyrosine (0.1–1.0 mM) and of L-DOPA (0.1–1.0 mM) in phosphate buffer (pH 6.5) were used as a substrate for cresolase and catecholase activity, respectively.

The reaction constant for the electrical reduction of DOPAquinone to L-DOPA was calculated by following equations, where F is Faraday constant, S is surface area of electrode, C is concentration of L-DOPA, D is diffusion coefficient, v is scan rate in cyclic voltammogram, R is ideal gas constant, T is temperature, $n = 2$ meant charge transfer number, and $\varphi = 0.1$ is calculated by Nicolson method [10,11].

$$i_p = 0.496 * F * S * C * D^{1/2} * \left(\frac{Fv}{RT}\right)^{1/2} \quad (1)$$

$$k_e = \left(\frac{n\pi FD}{RT}\right)^{1/2} \varphi v^{1/2} \quad (2)$$

2.3. Current response

The current response was investigated using 3-electrode system. Glassy carbon, ELAT, and Ag/AgCl were used as the working, counter, and reference electrode, respectively. 1 mM of L-DOPA was used as the substrate. When the current in the system was stabilized, tyrosinase was added and then the current response was investigated.

3. Results and discussion

3.1. Electroenzymatic L-DOPA synthesis using well-dispersed L-tyrosine as a substrate

The electro-enzymatic system achieved the excellent conversion rate in L-DOPA synthesis [9], but the productivity in the system is still limited due to the low solubility of the substrate, L-tyrosine, <2.5 mM. In order to increase the solubility, various water-miscible organic solvents were tested, but the solubility of L-tyrosine was not significantly changed (data not shown). So, as a solution for the low solubility of L-tyrosine, we perceived that emulsification can sometimes be an alternative for the insoluble or poor soluble chemical in the reaction phase [12,13]. For example, phytosterols, which can significantly reduce cholesterol levels in human beings, is insoluble in water and poorly soluble in oil. To increase the amount of the phytosterol in functional foods, an emulsification was formed by esterifying phytosterol [13,14]. Herein, we prepared well-dispersed L-tyrosine solution by using the wet-milling method to aim for overcoming the solubility limitation of L-tyrosine and then improving the productivity in the electroenzymatic L-DOPA synthesis.

The overall results of L-DOPA productions for various concentrations of well-dispersed L-tyrosine are summarized in Table 1. Initially, the electroenzymatic L-DOPA synthesis was carried out using 1 mM solution of well-dispersed L-tyrosine. As a result, the substrate was almost fully (99.8%) converted to L-DOPA within 30 min at the composite electrode of size (1 cm × 1 cm × 0.6 cm). This implies that the well-dispersed L-tyrosine sample, which can supply the concentrated substrate in aqueous phase, is converted quickly to L-DOPA with a productivity of 0.39 g L^{-1} h^{-1} and thus our electroenzymatic system was expected to be used at various concentrations of well dispersed L-tyrosine by suitably increasing the electrode size and enzyme concentration. So, we gradually increased concentration of the well-dispersed L-tyrosine up to 500 mM by suitably increasing electrode size. As shown in Table 1, the process resulted in 77.7% conversion rate and the productivity also dramatically increased to 15.3 g L^{-1} h^{-1} due to the rapid reduction of DOPAquine to L-DOPA again by the steady electron supply from the working cathode as discussed in Section 3.2. Also, to the best of our knowledge, there has been no report on L-DOPA production with such outstanding productivity (Table 2). For all the different concentrations of well-dispersed L-tyrosine, there was no brown precipitate, formed by spontaneous oxidation of DOPAquinone, indicating that the modified electrode was efficient in electron transfer.

Table 1
Electroenzymatic L-DOPA synthesis using well-dispersed L-tyrosine as a substrate.

L-Tyrosine (mM)	L-DOPA (g L^{-1})	Conversionrate (%)	Productivity (g L^{-1} h^{-1})	Reaction time (h)	Electrodesize (cm)
1	0.20	99.8	0.39	0.5	1 × 1 × 0.6
7	1.37	99.4	1.37	1	2 × 1 × 0.6
10	1.94	98.5	1.94	1	2 × 1 × 0.6
50	8.92	90.4	4.46	2	4 × 2 × 0.6
125	22.2	90.2	7.41	3	4 × 3 × 0.6
250	39.8	80.8	9.95	4	4 × 3 × 1
500	76.6	77.7	15.3	5	4 × 3 × 2

Table 2
L-DOPA production by different reactions.

Reaction type	Productivity (mg L^{-1} h^{-1})	Conversion (%)	Reference
Erwiniaherbicola culture	1800	7.34	[14]
Immobilized tyrosinase (batch reactor)	209	53.0	[15]
Free tyrosinase (electroenzymatic batch reactor)	97.6	49.5	[7]
Immobilized tyrosinase (electroenzymatic batch reactor)	47.3	95.9	[7]
Immobilized tyrosinase-well-dispersed L-tyrosine (electroenzymatic batch reactor)	15,300	77.7	This study

We also observed that the productivity of L-DOPA increased substantially with a decrease in the conversion upon increasing the concentration of well-dispersed L-tyrosine. The increase in productivity is obviously attributed to the high concentration of the substrate in aqueous phase and the ability of the modified electrode to instantaneously reduce DOPAquinone back to L-DOPA by continuous electron supply. The decline in conversion rate may be due to the mass transfer limitation associated with highly densed substrate.

3.2. Kinetic study

Related the outstanding conversion rate in the electro-enzymatic system, we perceived that the electrical reducing power was sufficiently and steadily provided to the batch reactor in the electro-enzymatic system while the chemical reducer, which was initially added in the previous enzymatic system as same as the substrate concentration, is insufficient as time passed in the batch reactor. Thus we hypothesized that the electrical reduction of DOPAquinone to L-DOPA was faster than the oxidation of L-DOPA to DOPAquinone by the catecholase activity and then DOPAquinone was not accumulated in the batch reactor. To verify the hypothesis, reaction constants of the electro-enzymatic system were determined. The reaction constant of the enzymatic catalysis and the electrical reduction were calculated from the Lineweaver–Burk plot (data not shown) and a relationship between the reduction peak current and the root mean square of scan rate with different concentration of L-DOPA as shown in Fig. 1, respectively. From the slope of the Fig. 1, the diffusion coefficient (D) was determined using the equation (1) and the kinetic constant of electrical reduction (k_e) was calculated from the equation (2). A spontaneous oxidation of DOPAquinone was much slower than the reduction of DOPAquinone to L-DOPA and hence ignored in this study. The determined kinetic constants were summarized in Table 3. As already known [15], the oxidation of L-DOPA to DOPAquinone by the catecholase activity (k_2) was faster than the hydroxylation of L-tyrosine to L-DOPA by the cresolase activity (k_1), as shown in Table 3(a). The reaction constant by the catecholase activity (k_2) is also lower than those of the electrical reduction of DOPAquinone (k_e) at all experimental L-DOPA concentrations as shown in Table 3(b). Therefore, the kinetic parameters afforded one of the evidences to validate our hypothesis for explaining the outstanding conversion rate in the electro-enzymatic system.

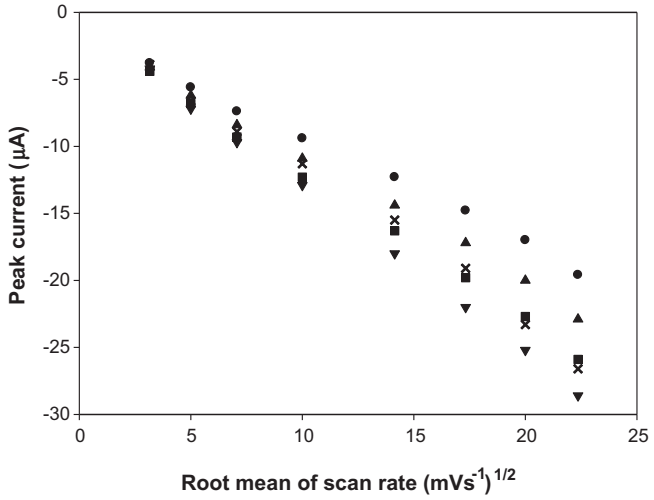

Fig. 1. Relationship between the root mean square of scan rates and peak currents with different concentrations of L-DOPA: (●) 0.05 mM, (▲) 0.10 mM, (■) 0.25 mM, (×) 0.50 mM, (▼) 0.75 mM L-DOPA in 200 mM phosphate buffer (pH 6.5) at 20 °C and −530 mV (working electrode: glassy carbon electrode, counter electrode: ELAT with 20% Pt, reference electrode: Ag/AgCl).

3.3. Current response

As another verification of our hypothesis, current response of L-DOPA during the enzymatic catalysis at the reduction potential of DOPAquinone was investigated with a different amount of

Table 3
Reaction constants of (a) enzyme catalysis and (b) electrical reduction of DOPAquinone to L-DOPA.

(a) Substrate	k_{cat} (min^{-1})	K_m (mM)
L-tyrosine	0.10[a]	0.21
L-DOPA	0.25[b]	0.39

(b) L-DOPA (mM)	k_e (min^{-1})
0.05	2.65
0.10	1.54
0.25	0.70
0.50	0.35
0.75	0.26

[a] Represent for k_1.
[b] Represent for k_2.

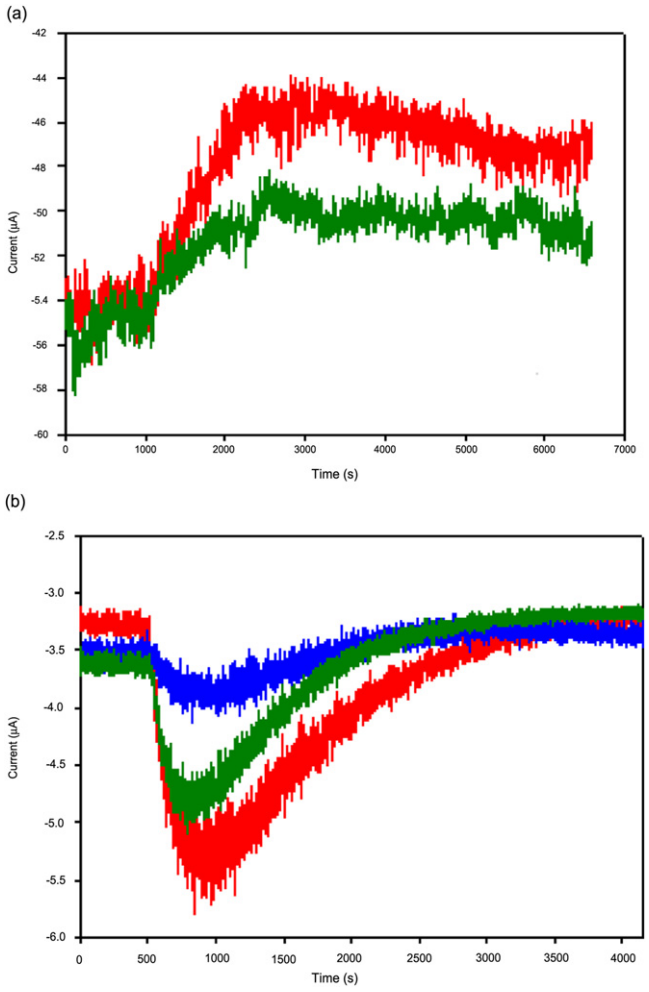

Fig. 2. Current response of L-DOPA during tyrosinase catalysis and electrical reduction (a) 20 unit and (b) 200 unit tyrosinase in 200 mM phosphate buffer (pH 6.5) at 20 °C and −530 mV (working electrode: glassy carbon electrode, counter electrode: ELAT with 20% Pt, reference electrode: Ag/AgCl) blue line: 0.1 mM L-tyrosine, green line: 0.5 mM L-tyrosine, red line: 1 mM L-tyrosine. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

tyrosinase as shown in Fig. 2. The current decreased with addition of 20 unit of tyrosinase while it increased with addition of 200 unit tyrosinase. In the presence of 20 unit of tyrosinase, the working cathode was quite capable for supplying enough electrons to reduce DOPAquinone which was produced from L-DOPA by the catecholase activity. However, current increased with the addition of 200 units tyrosinase because (1) L-DOPA was rapidly oxidized to DOPAquinone in the presence of large amount of tyrosinase, (2) the cathode could not afford to supply electrons in plenty for the reduction of rapidly produced DOPAquinone, and then (3) DOPAquinone, which is an electrically active molecule being used as an electron mediator, is accumulated in the reactor consequently. In summary, the outstanding conversion rate could be achieved in the electro-enzymatic system when the cathode is able to supply sufficient electrons for the reduction of the by-product DOPAquinone to L-DOPA in the batch reactor.

4. Conclusion

In this work, in order to improve the productivity in the electroenzymatic system for L-DOPA synthesis, well-dispersed L-tyrosine was employed as the substrate and the concentration of the substrate has been increased to 500 mM in aqueous solution, which is 200 times higher compared with the commercial tyrosinase (2.5 mM). When using 500 mM substrate, we achieved not only the outstanding conversion rate but also the highly improved productivity, 77.7% and 15.3 gL^{-1} h^{-1}, respectively. Also we determined kinetic parameters in the electroenzymatic system for L-DOPA synthesis. The kinetic data revealed that the outstanding conversion rate in the electroenzymatic system was based on the difference of reaction rate; the electrical reduction of by-product to L-DOPA was much faster than the oxidation of L-DOPA to the by-product by catecholase activity of tyrosinase. The results discussed here are promising for applying our technology toward commercial production of L-DOPA in industrial scale.

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