

Amperometric Detection of Hydroquinone and Homogentisic Acid with Laccase Immobilized Platinum Electrode

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Received February 18, 2004

DeniLite™ laccase immobilized platinum electrode was used for amperometric detection of hydroquinone (HQ) and homogentisic acid (HGA) by means of substrate recycling. In case of HQ, the obtained sensitivity is 280 nA/μM with linear range of 0.2-35 μM ($r^2 = 0.998$) and detection limit (S/N = 3) of 50 nM. This high sensitivity can be attributed to chemical amplification due to the cycling of the substrate caused by enzymatic oxidation and following electrochemical regeneration. In case of HGA, the obtained sensitivity is 53 nA/μM with linear range of 1-50 μM ($r^2 = 0.999$) and detection limit of 0.3 μM. The response times ($t_{90\%}$) are about 2 seconds for the two substrates and the long-term stability is 60 days for HQ and around 40-50 days for HGA with retaining 80% of initial activities. The very fast response and the durable long-term stability are the principal advantages of this sensor. pH studies show that optimal pH of the sensor for HQ is 6.0 and that for HGA is 4.5-5.0. This shift of optimal pH towards acidic range for HGA can be attributed to the balance between enzyme activity and accessibility of the substrate to the active site of the enzyme.

Key Words : Laccase, Amperometric biosensor, Hydroquinone, Homogentisic acid, Substrate recycling

Introduction

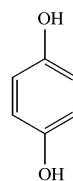
Hydroquinone (HQ, 1,4-benzenediol) is important in a wide number of biological and industrial processes such as coal-tar production, paper manufacturing, etc.¹ HQ has become one of the most widely used organic-reducing agents, especially in photographic developers, the production of polymerization inhibitors, rubber and food anti-oxidants.² HQ and its reaction products have been determined in various fields such as cosmetics, hair products and pharmaceutical preparations in both air samples and biological fluids.¹ Several methods for HQ detection were reported, such as GC-MS,³ MS,⁴ chromatography,^{5,6} HPLC,⁷⁻¹⁰ etc.

Human metabolic disorders often cause the accumulation of specific metabolites in patients serum and urine.¹¹ For example, the presence of homogentisic acid (HGA, 2,5-dihydroxyphenylacetic acid) in urine is a diagnostic for alcaptonuria.¹² A massive increase of HGA level in plasma will result primarily in arthropathies, but also other manifestations including cardiovascular and renal disease.¹³ Therefore, determination of HGA is important in clinical and pharmaceutical research. Several methods for HGA detection were reported, such as CE (capillary electrophoresis)-MS,¹¹ GC-MS,¹³ IR,¹⁴ paper chromatography,¹⁵ HPLC,^{16,17} etc.

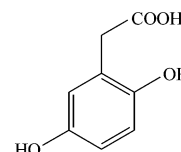
Besides above instrumental analysis methods for HQ or HGA, biosensor methods were also reported. In contrast of easy fabrication, fast analysis, low-cost, etc. for biosensor method, although the instrumental analysis is accurate, specific and sensitive it often requires delicate and time-

consuming sample pretreatment prior to the analysis and requires utilization of heavy equipment, therefore, they are inappropriate for massive screening.^{12,18} In reported biosensor methods, for detection of HQ enzymes such as laccase (*T. versicolor*),^{19,20} glucose dehydrogenase (GDH, *A. calcoaceticus*),²¹ glucose oxidase (GOD, *Aspergillus*)²² and cellobiose dehydrogenase (CDH, *P. chrysosporium*),²³ etc. were used, and for detection of HGA enzymes such as laccase (*T. versicolor*),¹⁹ homogentisate dioxygenase (*A. nidulans*),¹² etc. were used. Bacterial sensor based on Zucchini (*C. pepo*)² or sweet potato tissue modified graphite electrode²⁴ for HQ detection was also reported.

Recently, we reported that laccase purified from DeniLite™ could be successfully covalently immobilized on platinum electrode²⁵ and the electrode could be used as a biosensor for detection of aromatic diamine and aminophenol,²⁶ catechol and catecholamines²⁷ by means of substrate recycling. Laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) is a blue multi-copper containing enzyme, which catalyzes the oxidation of a variety of organic substrates such as phenols coupled to the reduction of molecular oxygen to water.²⁸⁻³¹ In this paper, we describe the application of the sensor for amperometric detection of hydroquinone (HQ) and homogentisic acid (HGA) (Figure 1).



1,4-Benzenediol
(Hydroquinone, HQ)



2,5-Dihydroxyphenylacetic acid
(Homogentisic acid, HGA)

Figure 1. Structures of hydroquinone and homogentisic acid.

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Experimental Section

Hydroquinone (99%), homogentisic acid (97%), *p*-phenylenediamine (PPD, 98%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 98%), 3-aminopropyltriethoxysilane (APTES, 99%), glutaraldehyde (25% aqueous solution) and bovine serum albumin (BSA, 98%) were purchased from Sigma & Aldrich Co. and used without further purification. Other chemicals were of analytical grade. Deionized water (18 M Ω ·cm) from Milli Q water purification system was used for preparing buffer and stock solutions. Stock solutions of the substrates were prepared just before use and kept in the dark enclosed by aluminum foil under argon until the measurements.

McIlvaine buffer (0.05 M citric acid/0.1 M Na₂HPO₄, pH 2.5-8.0) was used for pH dependence studies. 0.05 M phosphate buffer solution (PBS, Na₂HPO₄/KH₂PO₄) or McIlvaine buffer was used for amperometric sensor experiments.

Pt disk working (ϕ 4 mm), Pt wire counter (spiral), and Ag/AgCl (3 M KCl) reference electrodes were used for electrochemical measurements. BAS 50W or cDAQ-1604 (Elbio Co., Korea) potentiostat was used to run CVs and measure current-time responses. The electrochemical cell (5 mL in volume) was equipped in a thermostatic water bath (25 \pm 0.2 $^{\circ}$ C) and buffer solution was continuously stirred by magnetic bar during amperometric experiments.

Laccase was isolated and purified from DeniLiteTM, which is a commercial product for decolorization of indigo dye from Novo Nordisk Co., according to the published method.^{25,32} MES buffer (8 mM, pH 5.3) was used for the activity measurement of the purified laccase. The activity is 65 units/mg for ABTS oxidation at room temperature.³³

The covalent immobilization of laccase was done according to the method we previously reported.^{25,27} The method consists of oxidation of Pt electrode surface, introduction of amine functional group by silanization with APTES, and immobilization of laccase by glutaraldehyde. The amount of immobilized enzyme was fixed by dropping 5 μ L of 6 mg/mL enzyme solution to the modified electrode surface.²⁰ The laccase immobilized electrode was stored in 0.05 M PBS, pH 6.0 at 4 $^{\circ}$ C when not in use.

BSA was also immobilized by the same method to test the substrate recycling efficiency.¹⁹ The working potentials in amperometric experiments with laccase immobilized electrode were applied at 100 mV negative (for HQ) or 60 mV negative (for HGA) than reduction peak potentials of respective substrates at corresponding pHs. The sensor responses were normalized by those for PPD to compensate the variations resulted from different fabrications of the enzyme electrodes.

Results and Discussion

HQ detection. Figure 2 shows the typical steady state current response of the laccase immobilized Pt electrode to successive 5.0 μ M increments in HQ concentrations at pH 6.0. This pH is optimal for the substrate detection and will be

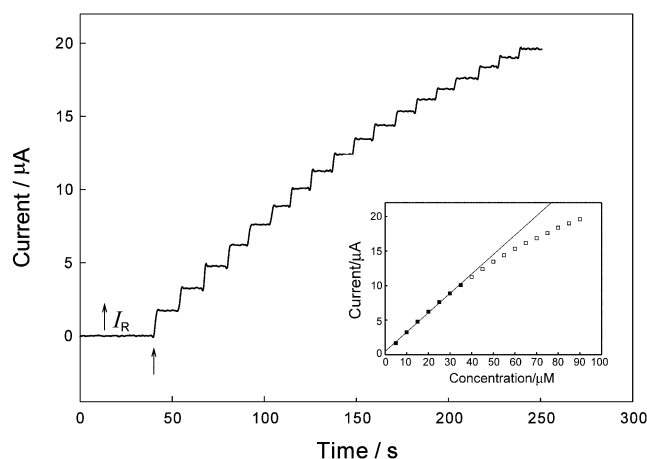


Figure 2. Steady state current response of the laccase immobilized Pt electrode to successive 5.0 μ M increments for hydroquinone in phosphate buffer at pH 6.0. The applied potential is -130 mV vs. Ag/AgCl. Inset: Calibration curve.

discussed in the following section. Substrate concentrations were changed by stepwise addition of a concentrated solution to a stirred buffer. As can be seen in Figure 2, the current response is very stable and fast with around 2 seconds response time ($t_{90\%}$). The inset of Figure 2 shows the sensitivity of the sensor for HQ is 280 nA/ μ M, the linear response range is 0.2-35 μ M ($r^2 = 0.998$).

We already reported the similar stable and fast response of the laccase immobilized Pt electrode used as a biosensor^{26,27} and attributed these characteristics to the covalent immobilization. Undoubtedly, covalent immobilization can provide the most stable enzyme loading. Furthermore, covalent immobilization could make the redox cycle of the substrate to take place on the surface very closely and minimize the diffusional resistance of the substrate. Comparatively, response times of some reported sensors for HQ are 5s, 90s and 7.5 min for laccase,¹⁹ GDH²¹ and GOD²² immobilized sensor, respectively.

It was reported that sensitivity of *T. versicolor* laccase immobilized sensor prepared by using reticulated vitreous carbon electrode (ϕ 5 mm) impregnated with epoxy resin (Epoxy-RVC) for HQ was about 120 nA/ μ M at pH 5.5,¹⁹ and that of *T. versicolor* or *C. hirsutus* laccase adsorbed graphite electrode (ϕ 3.05 mm) for the substrate was 183 or 145 nA/ μ M at pH 5.0.²⁰ The sensitivity of the sensor reported here combined with these reported sensitivities can be sequentially converted to relative ones of 100%, 27%, 112% and 89%. Taken into account of the fact that FIA (flow injection analysis) mode were employed in case of graphite electrode (ϕ 3.05 mm) the sensitivity reported here may be said highly improved. The detection principle in this study is substrate recycling, i.e. enzymatic oxidation of substrate followed by electrochemical regeneration. Obviously, the cycling of the substrate between laccase and electrode is quite efficient. Although exact reason for the improved sensitivity of the sensor is not fully understood several effects may be included such as the high affinity of the

enzyme for the substrates, the increased catalytic rate of the enzyme, and the better mobility of the substrate in the enzyme layer.²⁷ Besides laccases, other enzymes were also immobilized for HQ detection, and the converted relative sensitivities are as follows: 100%, 125%, 70% and 4000% for this study, CDH (FIA mode),²³ GOD²² and GDH²¹ immobilized sensor, respectively. Therefore, except for the sensitivity of GDH modified carbon paste electrode, an unusual highly amplified one, it can be concluded that sensitivity of the laccase immobilized sensor reported here is totally comparable with those of reported other enzymes immobilized ones.

The relative standard deviation (R.S.D) of the current response for successive measurements for HQ is 3.1% ($n = 7$). The obtained amplification factor (A.F.) for HQ is 19, which is perfectly comparable to Wasa et al reported value of 18¹⁹ or Lindgren et al reported value of 19.²³ The obtained linear response range is 0.2–35 μM ($r^2 = 0.998$), which is superior to that of *T. versicolor* laccase adsorbed graphite electrode, i.e. 0.5–8 μM ²⁰ or that of GOD immobilized sensor, i.e. upper limit of 4 μM .²² The relatively low sensitivity in high substrate concentrations can be ascribed to saturation of the enzyme active site by the substrate or formation of polymer film on the enzyme layer upon oxidation. It is known that oxidation of HQ by laccase can lead to polymerization.³⁰ The detection limit can be estimated to be 50 nM ($S/N = 3$) with the background noise of about 5 nA. The detection limit of *T. versicolor* laccase immobilized sensor for HQ is 440 nM²⁰ or 600 nM,³¹ and that of CDH immobilized sensor or GOD immobilized sensor is 3.5 nM²³ or 1 nM.²² In spite of the similar sensitivities among the sensor reported here, CDH sensor and GOD sensor the obtained detection limit in this study is relatively high, which may be due to the relatively high background noise possibly caused by the strong modification condition. It was reported that CDH was immobilized by adsorption²³ and GOD was done by entrapment behind polymer membrane.²² The long-term stability of the sensor is 60 days for HQ with retaining 80% of initial activity. Wasa et al reported that *T. versicolor* laccase immobilized Epoxy-RVC sensor retained about 65% of initial activities after repetitive use for 2 months.¹⁹ Wollenberger et al reported that response of GDH modified carbon paste electrode dropped by 50% after 10 days²¹ and Lindgren et al reported that half the response remained after storage in 4 °C for 1 week for CDH sensor.²³ The very fast response and the durable long-term stability are the principal advantages of the laccase covalently immobilized sensor.

pH profile of the laccase immobilized sensor for HQ shows bell-shaped profile with the optimal pH of 6.0 (Figure 3). From the principle of substrate recycling employed in this study, sensitivity of the sensor is mainly determined by two factors, i.e. enzyme activity and reversibility of the substrate. To check the reversibility, CVs of HQ were run on bare Pt electrode from pH 4 to 7. The reversibility of the substrate did not show remarkable change upon changing pH in this range (data not shown). Therefore, for HQ, only

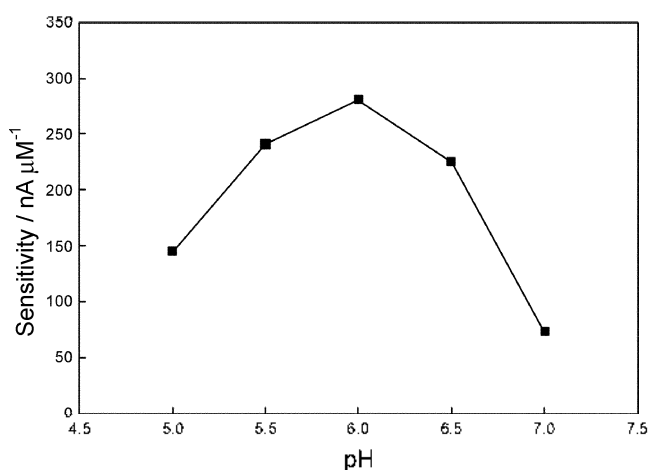


Figure 3. pH dependence of the laccase immobilized Pt electrode in McIlvaine buffer for hydroquinone.

enzyme activity will control the pH profile. We have reported that the optimal pH of the sensor for PPD is 6.0 and explained this pH profile by ΔE^0 effect and OH^- inhibition.²⁶ Both PPD and HQ are the typical substrates for laccases.^{29–31} Therefore, obtained optimal pH 6.0 for HQ can be said reasonable. It was reported that *T. versicolor* laccase immobilized Epoxy-RVC electrode showed optimal pH at 5.0–5.5¹⁹ and the same enzyme adsorbed graphite electrode showed optimal pH at 5.0.²⁰ It is worthy to note that in these cases the optimal pHs for the sensors are also the optimal pH for *T. versicolor* laccase. Obviously, these differences in optimal pH for the different laccases immobilized sensors are mainly resulted from the different sources from which laccases are purified.

HGA detection. Figure 4 shows the typical steady state current response of the laccase immobilized sensor to successive 5.0 μM increments in HGA concentrations at pH 5.0. The current response is also very stable and fast with around 2 seconds response time ($t_{90\%}$). The inset of Figure 4 shows the sensitivity of the sensor for HGA is 53 nA/ μM .

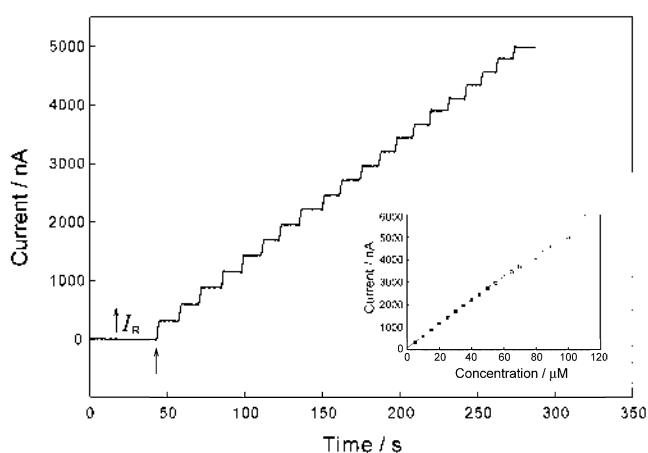


Figure 4. Steady state current response of the laccase immobilized Pt electrode to successive 5.0 μM increments for homogentisic acid in McIlvaine buffer at pH 5.0. The applied potential is -130 mV vs. Ag/AgCl . Inset: Calibration curve.

the linear response range is 1.0–50 μM ($r^2 = 0.999$).

Although the determination and quantification of HGA is important in clinical field as mentioned in introduction section, as far as we know, reports on the detection with electrochemical biosensor are rare in literature. Therefore, we can not fully compare our result with those of others, however, at the same time it may provide a possibility of the sensor to be used in real sample determination. Due to the basic structure of HQ for HGA, we also tested the detection with laccase immobilized biosensor. As early as 1984, Wasa *et al.* reported that sensitivity of *T. versicolor* laccase immobilized epoxy-RVC electrode (ϕ 5 mm) for HGA was about 26 $\text{nA}/\mu\text{M}$.¹⁹ Compared with this value the sensitivity of the sensor reported here is about 3 times higher. Another reported detection in which enzyme was used is made by spectrophotometric detection by use of homogentisate dioxygenase.¹² Therefore, we can not compare the sensitivity reported here with that of the spectrophotometric method. The sensitivity of 53 $\text{nA}/\mu\text{M}$ of the sensor for HGA is only about 20% of that for HQ, which means that substrate recycling for HGA is prohibited to some extent, which is mainly resulted from the relatively worse reversibility of HGA and limited accessibility of the substrate to the active site of the enzyme, which will be discussed in following section on pH effect.

The R.S.D value of the current response for successive measurements for HGA is 3.7% ($n = 7$). The obtained A.F value for HGA is 9, which is much lower than Wasa *et al* reported value of 18.¹⁹ The detection limit of the sensor reported here can be estimated to be 0.3 μM ($S/N = 3$), and the linear response range is 1–50 μM ($r^2 = 0.999$), which is comparable to that of *T. versicolor* laccase immobilized epoxy-RVC electrode, i.e. 0.5–80 μM .¹⁹ It was reported that HGA content in urine of a patient is in the range of 30–40 mM ^{12,17} and that in plasma is in the range of 33–38 μM .¹⁷ Therefore, determination of HGA with the sensor reported here is possible in terms of detection limit and linear response range. Furthermore, the durable long-term stability of the sensor as long as 40–50 days (retaining 80% of initial activity) is another advantage. The reported linear response range of the spectrophotometric enzymatic method by use of homogentisate dioxygenase is 12.5–200 μM .¹²

pH profile of the laccase immobilized sensor for HGA shows optimal pH of 4.5–5.0 (Figure 5). To check the reversibility, CVs of HGA were also run on bare Pt electrode from pH 4 to 7 (data not shown). The reversibility of HGA is worse than that of HQ, which is responsible for the relative sensitivities for HQ and HGA, because the recorded current in amperometric experiment is a reduction current, which is resulted from electrochemical reduction of the enzymatically produced oxidized form. Reversibility of HGA also does not show remarkable change upon changing pH in this range. Therefore, in order to explain the acidic shift of the optimal pH compared to that for HQ (pH 6.0) the carboxylic acid side chain of HGA should be taken into account. It was reported that $\text{p}K_a$ value for phenylacetic acid is about 4.31.³⁵ HGA will basically exist in neutral form at pH \sim 4.3 (or

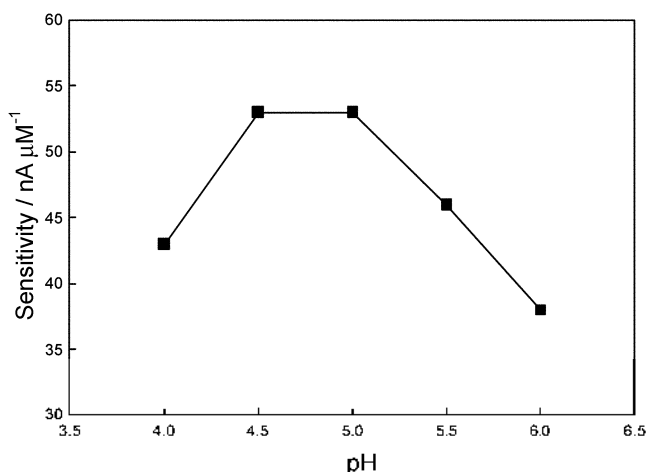


Figure 5. pH dependence of the laccase immobilized Pt electrode in McIlvaine buffer for homogentisic acid.

smaller than 4.3), which is advantageous for access of the substrate to the active site of laccase because active site of the enzyme was reported to be composed of hydrophobic patch.^{29,36} On the other hand, the optimal pH for Denilite™ laccase function is 6.0.²⁶ Therefore, optimal pH can be reasonably expected to be between 4.3–6.0, and degree of the pH shift towards acidic range will be decided by balance of the two opposite effects as the case of catechol and catecholamines detections.²⁷

To test the accuracy of the biosensor, several assays were made for standard substrates samples. Substrates concentrations were determined by standard curve in the linear ranges of the sensor. The current responses were normalized by the response for PPD to compensate the variations resulted from the different fabrications. Regression equations for the two substrates are as follows: $I = 280.56 C - 453.71$ (for HQ), and $I = 53.36 C + 76.20$ (for HGA), where I is current response and C is concentration, and units of I and C are nA and μM . As shown in Table 1, reliable results can be obtained. In tested concentration ranges for the two substrates, both R.S.D values increase with increase of the concentrations with R.S.D 4.2% for HQ and 3.5% for HGA at 10 μM . In spite of the relatively high R.S.D values the biosensor method described here do not require expensive equipment. It is possible to develop a reliable biosensor with rapidity, durability, simplicity and convenience for application

Table 1. Recovery tests of the biosensor for hydroquinone and homogentisic acid

	Added (μM)	Measured ^a (μM)	Recovery (%)	R.S.D (%)
Hydroquinone	2.5	2.4	96	2.8
	5.0	4.7	94	4.2
	10.0	9.4	94	4.2
Homogentisic acid	2.5	2.6	104	2.8
	5.0	5.2	104	2.8
	10.0	10.5	105	3.5

^aAn average of three detections

in real samples with good accuracy. Related work is under way.

Conclusions

The DeniLite™ laccase covalently immobilized sensor shows fast response and durable long-term stability for hydroquinone and homogentisic acid. Detection limit for hydroquinone is in nano-molar level. The relatively higher sensitivity for hydroquinone is resulted from the better reversibility and easier access of the substrate to the active site of laccase. In spite of the relatively lower sensitivity for homogentisic acid compared to that for hydroquinone the sensor may still be used for determination of homogentisic acid in urine or in plasma of a patient in terms of detection limit and linear response range of the sensor.

Acknowledgments. The authors acknowledge the financial support from the Korea Research Foundation (KRF-2001-042-G00015).

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