

Sensing Characteristics of Tyrosinase Immobilized and Tyrosinase, Laccase Co-immobilized Platinum Electrodes

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Tyrosinase was covalently immobilized on platinum electrode according to the method we developed for laccase (Bull. Korean Chem. Soc. 2002, 23(7), 385) and *p*-chlorophenol, *p*-cresol, and phenol could be detected with sensitivities of 334, 139 and 122 nA/ μ M and the detection limits of 1.0, 2.0, and 2.5 μ M, respectively. The response time ($t_{90\%}$) is 3 seconds for *p*-chlorophenol, and 5 seconds for *p*-cresol and phenol. The optimal pHs of the sensor are in the range of 5.0–6.0. This sensor can tolerate at least 500 times repeated injections of *p*-chlorophenol with retaining 80% of initial activity. In case of tyrosinase and laccase co-immobilized platinum electrode, the sensitivities are 560 nA/ μ M for *p*-phenylenediamine (PPD) and 195 nA/ μ M for *p*-chlorophenol, respectively. The sensitivity of the bi-enzyme sensor for PPD increases 70% compared to that of only laccase immobilized one, but the sensitivity for *p*-chlorophenol decreases 40% compared to that of only tyrosinase immobilized one. The sensitivity increase for the bi-enzyme sensor for PPD can be ascribed to the additional catalytic function of the co-immobilized tyrosinase. The sensitivity decrease for *p*-chlorophenol can be explained by the “blocking effect” of the co-immobilized laccase, which hinders the mass transport through the immobilized layer. If PPD was detected with the electrode that had been used for *p*-chlorophenol, the sensitivity decreased 20% compared to that of the electrode that had been used only for PPD. Similarly, if *p*-chlorophenol was detected with PPD detected electrode, the sensitivity also decreased 20%. The substrate-induced conformation changes of the enzymes in a confined layer may be responsible for the phenomena.

Key Words : Tyrosinase, Laccase, Immobilization, Biosensor, Phenols

Introduction

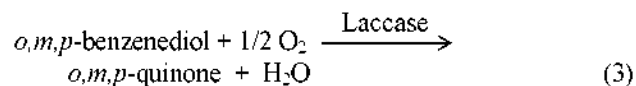
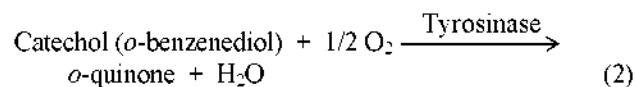
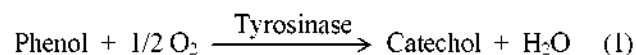
Phenolic compounds are widely distributed throughout the environment.¹ Photometric analyses by DIN (Deutsche Industrie Normen, *i.e.* German Industry Standard) and EPA (Environmental Protection Agency, USA) standard methods are commonly used for the determination of phenols,^{1,2} and these analyses usually require sample pretreatment by filtration and distillation. Recently, tyrosinase or laccase based biosensors have been shown to be useful for this purpose.³ Easy fabrication, fast analysis, and low-cost are the main advantages of the biosensor method.

Tyrosinase (EC 1.14.18.1, monophenol monooxygenase) is the first enzyme to be shown to catalyze the incorporation of molecular oxygen into phenolic compounds.⁴ Tyrosinase active site contains a coupled binuclear copper complex (type 3 copper).⁵ The three dimensional structure of tyrosinase is not yet known, but the recent structural data for a molluscan hemocyanin and a plant catechol oxidase allow a deeper insight into the active site of type 3 copper proteins.^{6–8} Recently, the mechanism of catalytic function of tyrosinase has been proposed and actively investigated.^{9–13}

Laccase (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase) 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.^{9,14–16} Laccase contains one type 1 copper, one type 2 copper, and one binuclear type 3 copper sites. The mechanism of catalytic function of laccase has also been proposed,⁹ and recently the crystal structures of laccases from different sources have been determined by X-ray crystallography.^{17–19}

The oxidation of phenols by tyrosinase or laccase can be presented according to the following reactions:²⁰



The liberated quinone species catalytically oxidized by tyrosinase or laccase can be electrochemically re-reduced and electrochemical sensor can be developed.^{2,21–23} This provides the additional advantages of the enzymatic/electrochemical recycling of the substrate, giving rise to an amplification of the signal (Figure 1). We have reported the detection of di-phenols such as PPD, PAP (*p*-aminophenol),^{24,25} catechol, and catecholamines²⁶ with DeniLite™ laccase covalently immobilized platinum electrode, in which substrate recycling principle was employed and the obtained amplification factors were about 10–30. However, detection of mono-phenols with the same sensor was not successful.

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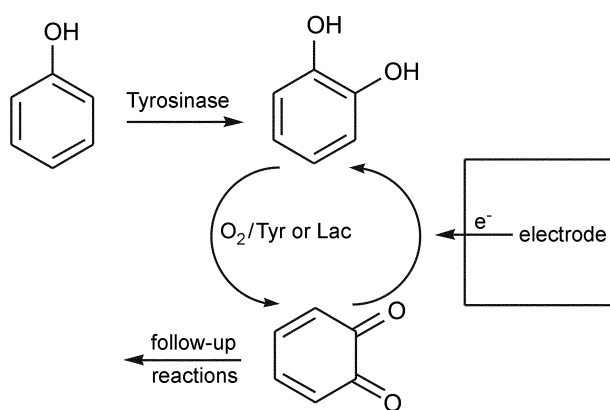


Figure 1. Signal amplification by substrate recycling between the immobilized enzyme and electrode. Tyr: tyrosinase, Lac: laccase.

so in this paper we report detection of mono-phenols with tyrosinase immobilized platinum electrode and detection of both mono- and di- phenols with tyrosinase and laccase co-immobilized platinum electrode. It is more important to measure the total contents of phenolic compounds rather than to determine each of them individually in many cases such as environmental remediation.^{27,28}

Experimental Section

Phenol (99+%), *p*-cresol (99%), *p*-chlorophenol (99+%), *p*-phenylenediamine (PPD, 98%), catechol (99.5+%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS, 98%), 3-aminopropyltriethoxysilane (APTES) (99%), glutaraldehyde (25% aqueous solution) and tyrosinase (from mushroom, 2000 units/mg) 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 until the measurements.

Mellvaine buffer (0.05 M citric acid/0.1 M Na₂HPO₄, pH 2.5–8.0) was used for pH dependence studies. Mellvaine buffer or 0.05 M phosphate buffer solution (PBS, Na₂HPO₄/KH₂PO₄, pH 5.8–8.0) was used for sensor experiment.

Platinum disk working (ϕ 4 mm), platinum 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 cyclic voltammograms (CVs) and measure current - time responses. The electrochemical cell (5 mL in volume) was equipped in a thermostatic water bath and the solution was continuously stirred by magnetic bar during amperometric experiments.

Laccase was isolated and purified from DeniLite™, which is a commercial product for decolorization of indigo dye from Novo Nordisk Co., according to the published method.^{24,29} 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.³⁰ Tyrosinase solution was prepared by dissolving purchased tyrosinase powder in 50 mM PBS, pH 5.5.

Covalent immobilization of tyrosinase was performed according to the method we previously reported for laccase.²⁴ The method consists of oxidation of platinum electrode surface, introduction of amine functional group by silanization with APTES, and immobilization of tyrosinase by glutaraldehyde. In this study, the oxidation of platinum electrode surface was performed by electrochemical oxidation at -1150 mV vs. Ag/AgCl for 20 minutes in 1 N H₂SO₄ solution under argon.³¹ Tyrosinase was covalently immobilized by repetitive applying of 5 μ L of 10 U/ μ L of enzyme to the modified electrode surface several times. The immobilized tyrosinase layer was cross-linked in vapor of glutaraldehyde for 20 minutes at room temperature.

Covalent co-immobilization of tyrosinase and laccase on platinum electrode surface was performed by repetitive applications of homogeneous mixture (2 μ L of 25 U/ μ L of tyrosinase and 1.7 μ L of 6 mg/mL of laccase) several times, and the immobilized bi-enzyme layer was also cross-linked by glutaraldehyde.

Results and Discussion

Tyrosinase immobilized platinum electrode for detection of mono-phenols. Tyrosinase was immobilized by sequential dripping of 5 μ L of 10 U/ μ L enzyme solution on the modified electrode surface. Three times of dripping gave the highest sensitivity in mono-phenols detection. Further addition of the enzyme solution led to a slight decrease in sensitivity. Similar trend has been observed in our previous results by laccase immobilized sensor²⁵ and in Jin *et al.* reported glucose dehydrogenase multilayer electrode.³² Tyrosinases have been reported to be covalently immobilized on various supports, such as reticulated vitreous carbon (RVC) electrode, nylon-66, controlled porous glass or glass beads, magnetite, ion exchange resins, gelatine gels, etc.³³⁻⁴¹ Although APTES-glutaraldehyde method is involved in these examples,³³⁻³⁷ covalent immobilization of tyrosinase on platinum surface with this method, as far as we know, has not been reported. In this study, the detection principle is based on the enzymatic/electrochemical recycling of the produced catechol by tyrosinase^{2,21-23} (Figure 1). According to our previous results,²⁶ if a working potential of -50 mV (vs. Ag/AgCl) is applied on the electrode, the enzymatically oxidized *o*-quinone will electrochemically re-reduced to catechol. Similar working potential was also reported by other authors.^{20,42,43} For the tyrosinase electrode reported here, catechol and three mono-phenols such as *p*-chlorophenol, *p*-cresol and phenol were detected and compared.

Sensing characteristic for catechol. Figure 2 shows catechol sensing signal of the tyrosinase electrode at pH 6.0. The obtained signal is stable, the sensitivity is 323 nA/ μ M and the response time ($t_{90\%}$) is less than 2 seconds. This high sensitivity strongly indicates that the occurrence of chemical

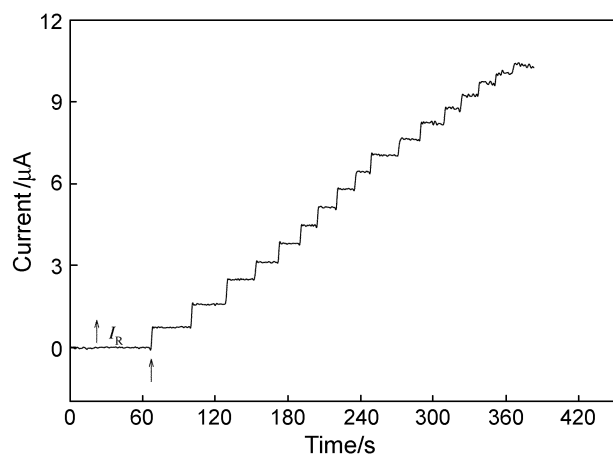


Figure 2. Steady state current response of the tyrosinase immobilized Pt electrode to successive $2.5 \mu\text{M}$ increments for catechol in 0.05 M phosphate buffer, pH 6.0. The applied potential is $-50 \text{ mV vs. Ag/AgCl}$.

amplification due to the cycle of the substrate caused by enzymatic oxidation and following electrochemical regeneration.²⁶ The covalent immobilization can make the redox cycle of the substrate to take place on the surface very closely and minimize the diffusional resistance between electrode surface and enzyme layer to give a fast response.²⁶

Sensing characteristics for *p*-chlorophenol, *p*-cresol and phenol. Figure 3 shows the typical sensor signals obtained with the same electrode for *p*-chlorophenol, *p*-cresol and phenol. For *p*-chlorophenol, the response time ($t_{90\%}$) is 3 seconds (Figure 3a), and the sensitivity is $334 \text{ nA}/\mu\text{M}$ at pH 6.0, which is similar to that for catechol. Figure 3b and 3c show the typical signals for phenol and *p*-cresol. The response times are relatively prolonged (5 seconds for each), which is 2.5 times long as that for catechol. The response time of the sensor is not dependent on the concentration of the substrate as expected for a recycling sensor.¹⁴ The sensitivities of the sensor are $122 \text{ nA}/\mu\text{M}$ for phenol and $139 \text{ nA}/\mu\text{M}$ for *p*-cresol. Although it is essential that to know the detailed information about environment of active site of tyrosinase so as to explain the relative sensitivity for the three substrates, different stability of the enzymatically oxidized *o*-quinone forms originated from the structural differences of the substrates can give a good explanation. From the detection principle, the more stable the produced *o*-quinone form, the higher sensitivity will be obtained. If life-time of the *o*-quinone is short, it will be deactivated before participating in the electrochemical reduction, *i.e.* half of the whole cycling is blocked to some extent. It was reported that the initial product of catechol oxidation by tyrosinase is *o*-benzoquinone, which is unstable in aqueous solution depending on pH, ionic strength, type of anions, the availability of nucleophiles, the catechol concentration. The final products of the oxidation are polymerized dark, humic-like or melanin-like pigments.⁴⁵⁻⁴⁹ In terms of structure of *o*-quinone, β -position of the ketone carbon is active and apt to be attacked by nucleophiles. For *p*-chlorophenol or *p*-cresol, the 4-position is occupied by substitute group of $-\text{Cl}$ or $-\text{CH}_3$,

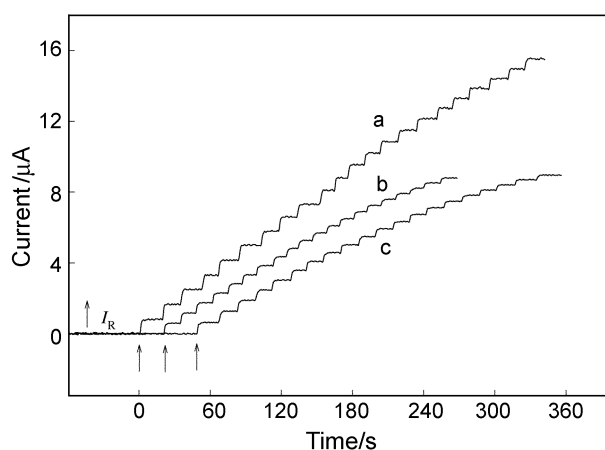


Figure 3. Steady state current responses of the tyrosinase immobilized Pt electrode to successive (a) $2.5 \mu\text{M}$ increments for *p*-chlorophenol at pH 6.0, (b) $5 \mu\text{M}$ increments for phenol at pH 5.5, and (c) $5 \mu\text{M}$ increments for *p*-cresol at pH 5.0 in Mcllvaine buffer. All of the applied potentials are $-50 \text{ mV vs. Ag/AgCl}$ for the three substrates.

so opportunity for nucleophilic addition reaction decreases by 50%, accordingly stabilities of the corresponding *o*-quinones increase compared to native *o*-quinone, *i.e.* sensitivity for *p*-chlorophenol or *p*-cresol should be greater than that for phenol. Furthermore, in case of *p*-chlorophenol, due to the electron withdrawing effect of $-\text{Cl}$, distribution of the delocalized electron on the ring becomes relatively uniform by counteraction with withdrawing effect of oxygen on the other side, accordingly carbon at 5-position is relatively not active for nucleophile attack. On the contrary, for *p*-cresol, the electron donating ability of $-\text{CH}_3$ decreases the stability of the corresponding *o*-quinone form. Therefore, the obtained relative sensitivities in this study, *i.e.* phenol 100%, *p*-cresol 114% and *p*-chlorophenol 274% can be reasonably understood. This sensitivity order of tyrosinase sensor for the three substrates was also reported by other authors.^{2,50,51}

The sensitivities of the sensor for phenol and *p*-cresol are 122 and $139 \text{ nA}/\mu\text{M}$, respectively. These values are comparable to that of Deng *et al.* reported ($43 \text{ nA}/\mu\text{M}$ or $127 \text{ nA}/\mu\text{M}$ for phenol, $\phi 3 \text{ mm}$ glassy carbon electrode),^{21,52} Wang *et al.* reported ($80 \text{ nA}/\mu\text{M}$ for phenol, electrode size unknown),⁵³ and Freire *et al.* reported ($100 \text{ nA}/\mu\text{M}$ for *p*-cresol, 20 carbon fibers, $\phi 8 \text{ mm}$ for each),²⁸ etc. The linear ranges of the sensor are $3\text{--}38$, $6\text{--}90$, and $8\text{--}110 \mu\text{M}$ and the detection limits are 1 , 2 , and $2.5 \mu\text{M}$ for *p*-chlorophenol, *p*-cresol, and phenol, respectively. Detection limits are evaluated as a signal to noise ratio of three ($S/N=3$). The linear range and detection limit are comparable to Kim *et al.* reported⁵⁰ ($\phi 4.7 \text{ mm}$ glassy carbon electrode) in which tyrosinase was immobilized in sol-gel composite film. It was reported that upper limits of linear ranges of a tyrosinase sensor for phenol and *p*-cresol were about $100 \mu\text{M}$,²⁸ and detection limit for phenol was $1 \mu\text{M}$ ($\phi 1.5 \text{ mm}$ graphite epoxy composite electrode, FIA mode,⁵⁴ Clark sensor,⁵⁵ $\phi 3 \text{ mm}$ Ru-dispersed carbon paste electrode⁵⁶).

The $K_{m,app}$ values obtained by Michaelis-Menten type

analysis for *p*-chlorophenol, *p*-cresol, and phenol are 330, 530, and 640 μM , respectively. The $K_{m,app}$ value for the free enzyme in solution was estimated to be 240 μM using catechol as the substrate.⁵⁷ Nistor *et al.* reported that $K_{m,app}$ for immobilized tyrosinase was 230 μM using catechol as the substrate,⁵⁸ and Morales *et al.* and Reviejo *et al.* reported that $K_{m,app}$ for immobilized tyrosinase was about 400 and 440 μM using phenol as the substrate.^{59,60} Compared with these reported data the obtained $K_{m,app}$ values in this study are relatively large, it may be ascribed to the relatively strong immobilization conditions employed in this study (covalent vs. adsorption or graphite composite^{59,60}). Willner *et al.* studied a crosslinked microperoxidase-11 and nitrate reductase (NR) monolayer on a gold electrode and they explained the higher K_m value of the immobilized NR by the partial deactivation of the enzyme in crosslinking process and the increased transport barriers for substrate to the enzyme interface.⁶¹

The reproducibility of the tyrosinase sensor is relatively worse compared to that of laccase immobilized one,^{25,26} with relative standard deviation (R.S.D) of 4.6% in successive detections of *p*-chlorophenol ($n=7$). The sensitivity for successive 10 detections for *p*-chlorophenol (20 times injections for each) retained about 95% of initial value. It can be expected that this sensor can tolerate at least total 500 times repeated injections of *p*-chlorophenol (80% of initial value). This durable long-term stability of the sensor may be originated from more stable enzyme loading due to the covalent immobilization. We have reported that long-term stability of laccase covalently immobilized sensor is as long as two months.^{24,25} It was reported that stability of tyrosinase adsorbed sensor was only few hours.²⁰

pH dependences. Figure 4 shows pH dependences of the sensor for the three substrates. The optimal pHs are 6.0 for *p*-chlorophenol, 5.0 for *p*-cresol, and 5.5 for phenol. Wang *et al.* reported that the high sensitivity was observed in pH range of 4.5–6.5 for phenol and *p*-cresol⁵³ and they ascribed such pH profile to the broad pH-activity profile of tyrosinase. Anh *et al.* reported that the optimal pH was 6.0 for *p*-chlorophenol⁶² and Svitel *et al.* reported that the optimal pH was 5.5–6.0 for phenol,⁴² etc. Ortega *et al.* reported the effect of pH on the responses of solid graphite and carbon paste electrodes modified with tyrosinase. In both configurations the responses for phenol showed an optimum at pH 6.0.^{43,54} Compared with these data, it can be concluded that tyrosinase immobilized platinum electrode reported here shows similar pH response to the previously reported ones.

Tyrosinase, laccase co-immobilized platinum electrode for detection of both mono- and di-phenols. As far as we know, only few papers have been published^{20,22,28} about the co-immobilization of tyrosinase and laccase for detection of phenols, and all of the used electrode materials for the bi-enzyme immobilization were carbons. In this study, co-immobilization of tyrosinase and laccase on platinum electrode was also tested, and the immobilization was done by use of the homogeneous mixture of tyrosinase and laccase. The consideration was based on possible advantage

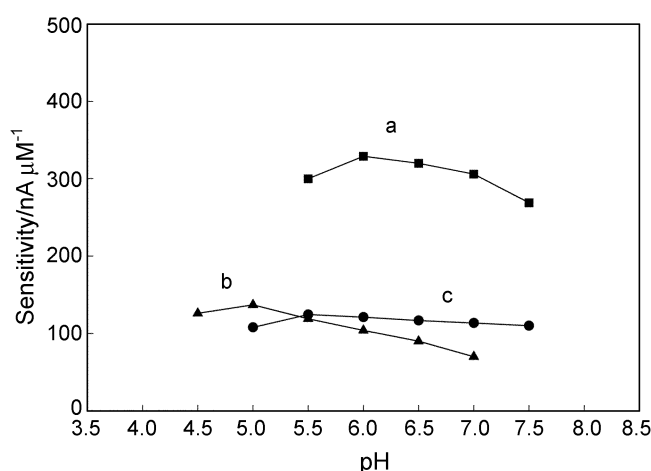


Figure 4. pH dependences of the tyrosinase immobilized Pt electrode in McIlvaine buffer for (a) *p*-chlorophenol (b) *p*-cresol (c) phenol.

of the bi-enzyme immobilized electrode for simultaneous response to mono- and di-phenols. The amounts of enzymes immobilized were same as those of respective enzyme (150 U tyrosinase and 5 μl . of 6 mg/ml. laccase²⁵) so as to compare the sensitivity changes resulted from the additional immobilization of another enzyme. PPD and *p*-chlorophenol were selected as the representatives of mono- and di-phenols because the highest sensitivities were obtained with them among tested di-phenols and mono-phenols. As mentioned in Section 1, the optimal pH for tyrosinase immobilized sensor is 6.0 with *p*-chlorophenol as the substrate and that for laccase immobilized sensor is also 6.0 with PPD as the substrate.²⁵ So the operating pH was fixed at 6.0 for the bi-enzyme co-immobilized sensor.

Sensing characteristics for PPD and *p*-chlorophenol. PPD was detected first with the bi-enzyme electrode. Although sensor signal is not as stable as that of only laccase immobilized sensor,²⁵ response time is fast enough as that of laccase sensor (2 seconds). Sensitivity of the sensor for PPD is 560 nA/ μM (Figure 5a), which is 70% larger than that of only laccase immobilized sensor (330 nA/ μM ²⁵). Obviously, this increased value is due to the additional catalytic capability of tyrosinase for PPD. It was reported that tyrosinase can oxidize *p*-aminophenol which is a structural analogue of PPD, and the relative sensitivity of tyrosinase sensor for this substrate was 25% of that for catechol.² Yaropolov *et al.* reported that the relative responses of laccase electrode and bi-enzyme (tyrosinase and laccase) electrode for catechol were 87 and 590, respectively.²⁰ In that case, the largely increased response after additional immobilization of tyrosinase with laccase originated from the relatively high response of tyrosinase for catechol (relative response of 700). We also measured activity of soluble tyrosinase for PPD by spectrophotometric assay and found that tyrosinase has only 5% activity compared to that of laccase for PPD. Although the measured activity is not large enough to explain the 70% increase in sensitivity, the capability of tyrosinase to oxidize PPD can be considered as one explanation. It seems that

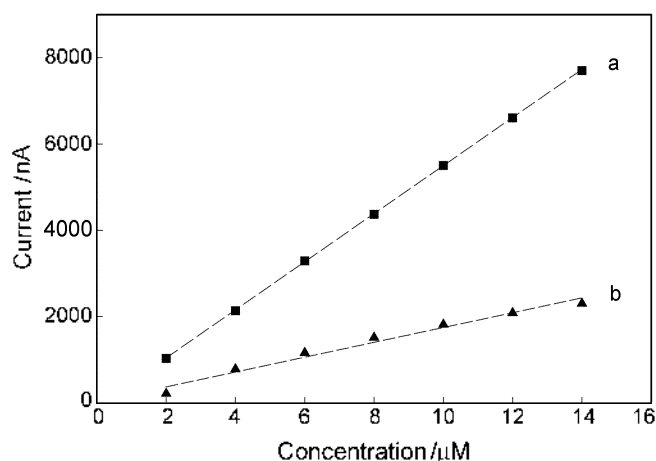


Figure 5. Sensitivities of the tyrosinase and laccase co-immobilized Pt electrode in 0.05 M PBS, pH 6.0 for (a) PPD, (b) *p*-chlorophenol.

cooperative effect of tyrosinase and laccase is enhanced when they are very closely located by the co-immobilization.

p-Chlorophenol was also detected alone with the bi-enzyme electrode. The sensor signal is also not as stable as that of tyrosinase immobilized sensor, and the upper linear range is decreased to 10 μM (vs. 38 μM , Figure 3a). At the same time, the sensitivity of the sensor for *p*-chlorophenol is 195 nA/ μM (Figure 5(b)), which is 40% smaller than that of only tyrosinase immobilized sensor (330 nA/ μM). This result may be mainly due to the "blocking effect" of one enzyme on co-existing another enzyme. We made spectrophotometric assay and concluded that DeniLite™ laccase has almost no activity for *p*-chlorophenol, which is in accordance with the general characteristics of laccases.⁹ The "blocking effect" caused by laccase on the co-existing tyrosinase may increase the resistance for mass transport in the bi-enzyme layer. A similar result was also obtained by Freire *et al.*²⁸ In that case, sensitivity of the bi-enzyme sensor for *p*-cresol decreased about 25% compared to that of only tyrosinase immobilized sensor, and sensitivity of the bi-enzyme sensor for chloroguaiacol decreased about 15% compared to that of only laccase immobilized sensor. In another case, however, the relative responses of the tyrosinase and bi-enzyme sensors for *p*-chlorophenol were 98 and 130, which corresponds to 33% increase due to additional immobilization of laccase with tyrosinase.²⁰ In that case, *C. hirsutus* laccase showed response to *p*-chlorophenol (relative response of 21). It seems that responses of laccases to *p*-chlorophenol differ from source to source.

Sequential detection of PPD and *p*-chlorophenol.

Figure 6 shows the simultaneous detection of di- and monophenols. When PPD was detected prior to *p*-chlorophenol (Figure 6a) the sensitivity of the sensor is almost same as that of PPD detected alone case (560 nA/ μM , Figure 5a), which means that the reproducibility of the bi-enzyme sensor is excellent. The sensitivity for the following detection of *p*-chlorophenol (after detection of PPD, Figure 6b) is 155 nA/ μM , which is about 20% decrease compared to that for

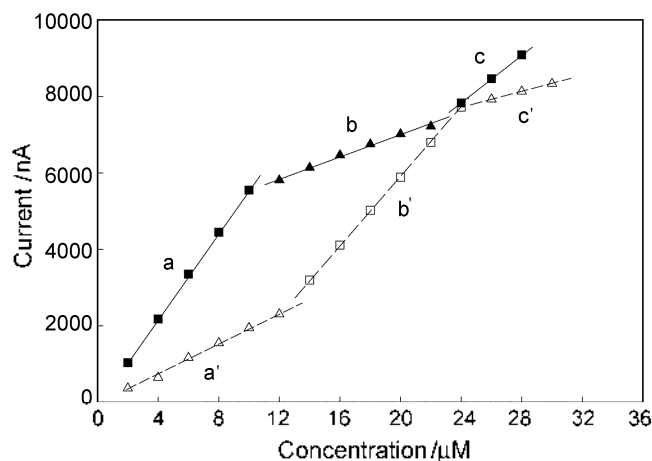


Figure 6. Sensitivities of the tyrosinase and laccase co-immobilized Pt electrode in 0.05 M PBS, pH 6.0 in detection orders of (a) PPD, (b) *p*-chlorophenol, (c) PPD (solid squares and triangles) and (a') *p*-chlorophenol, (b') PPD, (c') *p*-chlorophenol (empty triangles and squares).

p-chlorophenol detected alone (195 nA/ μM , Figure 5b). Also, the sensitivity for the following PPD detection (after PPD and *p*-chlorophenol detection, Figure 6c) is 310 nA/ μM , which is about 45% decrease compared to that for previous PPD detection (560 nA/ μM , Figure 6a). In another case, if the detection was done in sequence of *p*-chlorophenol, PPD and *p*-chlorophenol, the obtained sensitivities are 197, 455 and 105 nA/ μM (Figure 6a', 6b' and 6c'), respectively, which correspond to 0%, 20% and 45% decrease compared to those for these substrates detected alone case. By comparison, these decreased values are same each other in both cases, and this means that sensitivity of the bi-enzyme sensor has no relation with detection sequence.

Although the cross-blocking effect of the bi-enzyme sensor as mentioned above remains to be further studied, a term "substrate memory" of an enzyme may be an explanation. Kermasha *et al.* reported that the presence of chloroform in aqueous solution did not alter the optimal pH for mushroom tyrosinase activity in aqueous solution (pH 6.0).⁶³ They explained this result by the term of "pH memory" of the enzyme. Indeed, it was observed that if a tyrosinase immobilized sensor had been used for successive detection of one substrate, the same sensor would not be sensitive to another substrate at initial stage of the detection. After several uses, sensitivity of the sensor recovered to its original sensitivity to this "another substrate". These findings may suggest that surrounding configuration of the enzyme's active site acquires the corresponding suitable states with a particular substrate which will then remain unchanged to some extent, especially in the immobilized state. Substrate-induced conformation change is also reported for other enzymes.⁶⁴⁻⁶⁶

Conclusions

Tyrosinase can be covalently immobilized on platinum

electrode *via* silanization-glutaraldehyde method, and thus obtained sensor can be used for detection of mono-phenols such as phenol, *p*-cresol and *p*-chlorophenol. The sensor shows the highest sensitivity for *p*-chlorophenol among the selected substrates, which indicates that relatively effective substrate recycling happens due to the most stable *o*-quinone form of the substrate. Tyrosinase and laccase co-immobilized platinum electrode can be used to simultaneous detection of both di- and mono-phenols such as PPD and *p*-chlorophenol. The bi-enzyme sensor shows increased sensitivity for PPD probably due to the cooperative effect of the enzymes. For the sequential detection of PPD and *p*-chlorophenol, sensitivity of the sensor for the following substrate decreases remarkably. This kind of "substrate memory" effect may be resulted from the slow conformational change of the immobilized enzyme upon changing substrates.

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