Significance of Uricase in Oxidase-Induced Oxidative Coloring Reaction of *p*-Phenylenediamine

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Uricase (urate oxidase, UOD) is found to induce the oxidative polymerization of *p*-phenylenediamine (PPD) effectively, which is a key reaction of color development in hair-dyeing and fur-dyeing practices. The significance of uricase is described by comparison to glucose oxidase (GOD), which also produces hydrogen peroxide as an oxidizing agent of PPD. In contrast to UOD, GOD inhibits the polymerization reaction. Spectroscopic and electrochemical study has revealed that the inhibition effect of GOD is ascribed to the glucose dehydrogenase activity, in which *p*-benzoquinonediimine (BQI) as the two-electron oxidized form of PPD works as an efficient electron acceptor to be reduced back to PPD, resulting in the inhibition of the subsequent polymerization of BQI. On the other hand, the UOD reaction does not compete with the polymerization of BQI owing to the lack of urate dehydrogenase activity in UOD. In addition, it has been found that UOD catalyzes the oxidation of PPD in the presence of uric acid by PPD oxidase-like and PPD peroxidase-like activities. These properties of UOD are favorable toward the oxidative generation of BQI from PPD and are responsible for the prominent ability in the oxidative coloring of PPD.

Dyeing of hair and/or fur involves synthesis of the colored species by oxidative polymerization of monomeric precursors.[1] Commercial hair dyeing compositions usually consist of three types of reactive species: the dye precursors (dye intermediates or developers), the color couplers (modifiers), and the oxidizing agents. *p*-Phenylenediamine (PPD) and hydrogen peroxide (H_2O_2) are most frequently used as a precursor and an oxidizing agent, respectively, while the color couplers may be *m*-diamines, *m*-aminophenols, phenols, pyrazolones, or *β*-diketones. The oxidizing agents trigger the coloring development by oxidizing PPD into *p*-benzoquinonediimine (BQI),[2] which reacts with the couplers to form several colored species and may also undergo electrophilic attact of PPD to give a trimer where PPD is considered to be a vital catalyst (Scheme 1).[1b]

In usual hair-dyeing practice, the coloring reaction is initiated by mixing H_2O_2 directly with the organic reactants. Such direct use of H_2O_2 at relatively high concentrations (usually about 3%, i.e., 1 M) may damage hair and skin due to the strong oxidizing power of H_2O_2. In order to alleviate the damages, several enzyme reactions were examined to induce *in situ* synthesis of colored species. For example, tyrosinase was utilized to oxidize polyhydroxy aromatic compounds directly to generate melanin dyes.[3,4] However, the methods were not satisfactory in practice. One of the present authors (Y.T.) and co-workers have presented an idea to utilize oxidases as a mild alternative to H_2O_2 in hair-dyeing practice.[5,6] Screening of several oxidases for this purpose revealed that uricase (urate oxidase, UOD), which catalyzes the following reaction,

$$\text{uric acid} + O_2 + 2H_2O \rightarrow \text{allantoin} + CO_2 + H_2O_2 \quad (1)$$

is superior to other oxidases examined including glucose oxidase (GOD), pyruvate oxidase, lactate oxidase, glycerol oxidase, and xanthine oxidase.[6] The UOD-induced hair coloring was as effective as and much milder than the direct oxidation method using H_2O_2.

However, the significance of UOD in the hair-dyeing practice remains unknown. If the UOD reaction worked simply as the H_2O_2 supplying source, other oxidases such as GOD could work as effectively as UOD. This question inspired us to clarify what is happening in the oxidative polymerization of PPD induced by UOD or other oxidases from the viewpoint of enzyme-assisted organic synthesis. In this work, GOD was used as a comparison to UOD. Detailed spectroscopic and electrochemical study has revealed that GOD reduces BQI as an efficient electron acceptor due to its dehydrogenase activity and then inhibits the subsequent polymerization of BQI. On the other hand, UOD lacks such dehydrogenase activity and does not inhibit the polymerization. Furthermore, it has been found that UOD can function as a catalyst of the PPD oxidation as well as the H_2O_2 supplying source in the presence of uric acid to generate BQI.

Materials and Methods

Reagents. Oxidases used in this study are uricase (EC 1.7.3.3, UOD) from *Escherichia coli* cloned with the UOD gene

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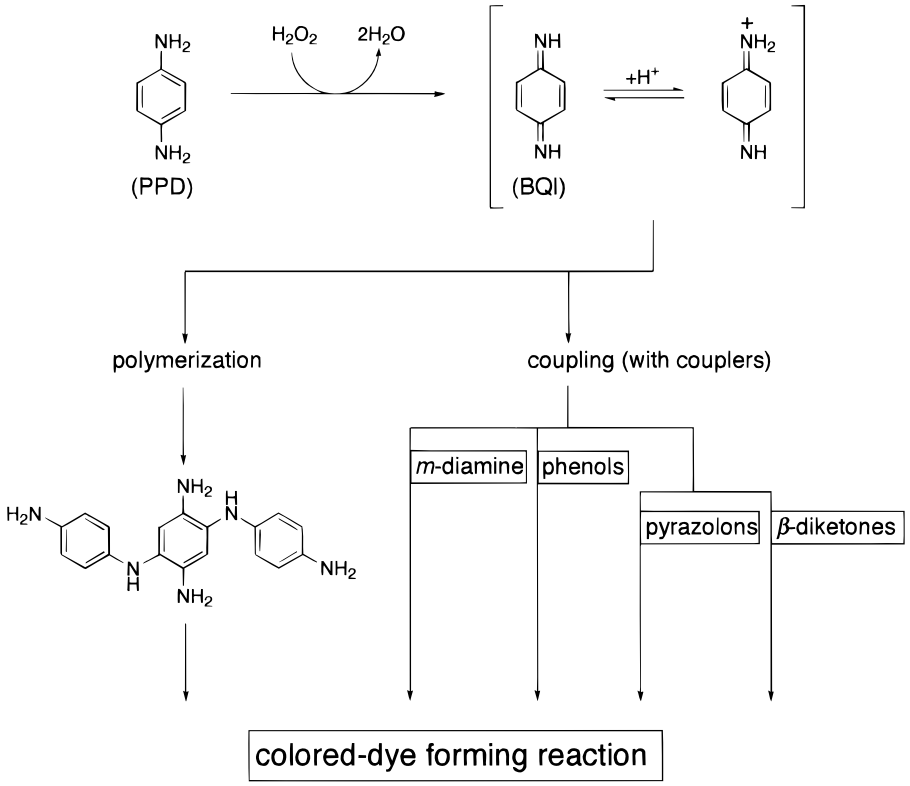
Scheme 1. Mechanism of the Oxidative Polymerization of PPD and the Related Coloring Reaction



of *Cellulomonas flavigena*[7] and glucose oxidase (EC 1.1.3.4, GOD) from *Aspergillus niger*. UOD was donated from Kyowa Hakko Kogyo Co., Ltd., while GOD was purchased from Sigma. The GOD concentrations were determined spectrophotometrically using the molar extinction coefficient of 18 240 at 450 nm.[8] Uric acid, glucose, and *p*-phenylenediamine (PPD) were purchased from Wako and used without further purification. All other chemicals were of reagent grade.

Apparatus. UV−vis spectroscopy and spectrophotometry were performed with a Shimadzu UV-2500 (PC)S spectrophotometer at 30 ± 2 °C. In anaerobic measurements, standard quartz cuvettes with screw caps were used, and nitrogen gas was passed through test solutions in the cuvettes before measurements. Under aerated conditions, the cuvettes were opened to the atmosphere without stirring.

All electrochemical measurements were performed with a Bioanalytical System 100 B/W electrochemical workstation or a Yanagimoto P-1100 potentiostat controlled with an NEC PC-9801 RA microcomputer. Cyclic voltammetry, constant-potential amperometry, and potential step chronoamperometry were carried out with a three-electrode system consisting of a glassy carbon working electrode (Bioanalytical System, Φ = 3.0 mm), a platinum plate auxiliary electrode, and an Ag/AgCl/KCl(sat.) reference electrode using a conventional electrochemical cell at 30 ± 0.2 °C under nitrogen atmosphere. The glassy carbon electrode was polished with 0.05 μm alumina powder (Buehler, No. 3) before each measurement. The alumina powder was removed by washing with water and then by sonication in distilled water. Dioxygen concentration measurements were carried out using an oxygen electrode equipped with an Ag/AgCl/KCl(sat.) reference electrode (two-electrode system, Oriental Electronics) and a 1.0-mL space-free and air-tight electrochemical cell (Oriental Electronics) under constant stirring. The dioxygen concentrations of test solutions were regulated by purging air, nitrogen, oxygen gas, or their mixture just before measurements. All spectroscopic and electrochemical measurements were done using 0.1 M phosphate buffer of pH 7.0, unless otherwise stated.

Oxidase Reaction Rate Measurements and Definition of Enzyme Activity. Steady state enzymatic reaction rates of UOD and GOD were estimated by monitoring the oxygen consumption rate using the oxygen electrode at 30 °C. The Michaelis constant of uric acid in the UOD reaction ($K_{S,UOD}$) was determined as 0.8 mM under oxygen-saturated conditions at pH 7.0, while that of glucose in the GOD reaction ($K_{S,GOD}$) was 86 mM.[9] Because of the difference in the optimum pH between UOD and GOD, one unit (U) of the enzyme activity was defined in this work as the amount of the enzyme that consumes 1 μmol of dissolved dioxygen (O_2) per minute at pH 7.0 and at 30 °C under air-saturated conditions (0.25 mM[10] as a dioxygen concentration ([O_2]) in the presence of the substrate at sufficiently high concentrations (1.3 mM uric acid for UOD and 130 mM glucose for GOD) compared with the corresponding Michaelis constant of the substrate. One unit of UOD and GOD in the present definition were equivalent, respectively, to 2.0 and 1.8 U in the unit scale estimated according to the protocols.[11]

Results and Discussion

Comparison of UOD and GOD in the Oxidative Coloring Reaction of PPD. Panel A of Figure 1 shows spectral change of PPD associated with the UOD reaction at pH 7.0. Upon the addition of 10 U mL^{-1} of UOD into the solution containing PPD (30.8 mM) and uric acid (1.0 mM $\gg K_{S,UOD}$), a broad absorption band increased, giving the maximum absorption around 520 nm and a shoulder around 430 nm. During the reaction, the practically colorless solution of PPD changed to red-purple. Thus,

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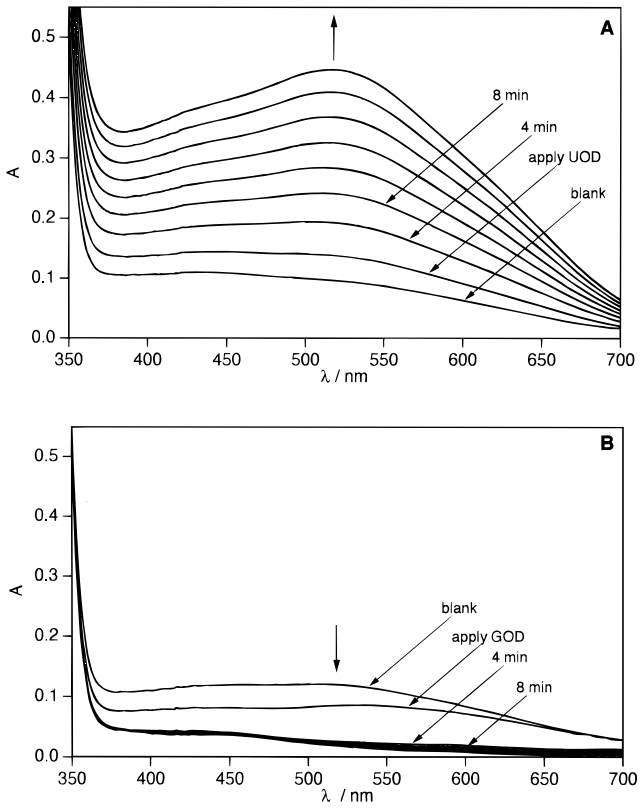

Figure 1. Time-dependent adsorption spectra of PPD (30.8 mM) after addition of (A) 10.0 U mL^{-1} of UOD in the presence of 1.0 mM uric acid and (B) 10.0 U mL^{-1} of GOD in the presence of 130 mM glucose at pH 7.0 and at 30 °C under aerated conditions. The time interval of the spectroscopic measurements after the addition of the enzyme was 4.0 min.

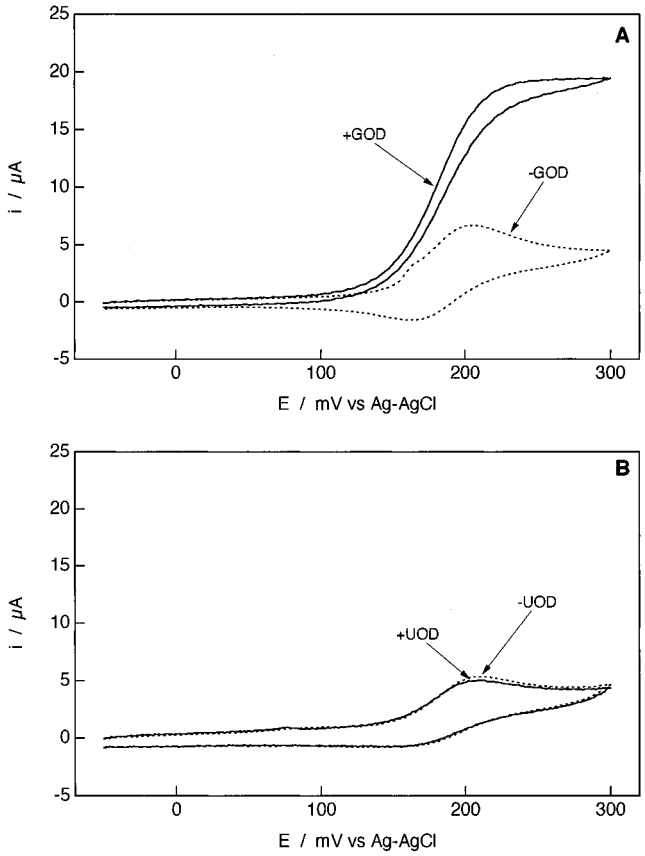

Figure 2. Cyclic voltammograms of 0.308 mM of PPD in the presence of (A) 10 U mL^{-1} GOD and 130 mM glucose and (B) 10 U mL^{-1} UOD and 1.0 mM uric acid. The broken curves in panels A and B are voltammograms in the absence of GOD and UOD, respectively. The voltammograms were recorded at a scan rate of 10 mV s^{-1} with a glassy carbon electrode in pH 7.0 phosphate buffer at 30 °C under deaerated conditions.

the spectroscopic change is reasonably ascribed to the oxidation of PPD with enzymatically generated H_2O_2, yielding BQI and the subsequent polymerization of BQI with PPD. The absorption bands around 430 nm and 520 nm seem to be assigned to BQI and its polymerized product (probably the trimer in Scheme 1), respectively.[12] Such spectroscopic change was not observed when either uric acid or dissolved dioxygen was removed from the reaction solution. Spectral changes similar to those depicted in Figure 1A were reproduced by the direct addition of about 3 mM of H_2O_2 into a PPD solution.[12] These results indicate that the UOD reaction is effective in oxidizing PPD and the subsequent coloring reaction.

Similar experiments were performed using GOD and glucose instead of UOD and uric acid, respectively. In this experiment, the enzymatic activity of GOD was adjusted to be identical with that of UOD given in Figure 1A (10 U mL^{-1}) at 130 mM of glucose. Therefore, the initial rate of the H_2O_2 production by GOD was expected to be identical with that in the case of UOD, though the GOD reaction was expected to generate a larger total amount of H_2O_2 than that in the case of UOD under these experimental conditions, because of the higher concentration of glucose compared with that of uric acid. In spite of the setting of such experimental conditions, the spectroscopic data depicted in panel B of Figure 1 shows marked inhibition effect of GOD on the polymerization reaction of PPD. Any increase in the absorption bands was not observed. Rather, a small broad absorption band around 500 nm disappeared, in which the band would be ascribed to a very small amount of oxidized impurity of PPD. The comparison between panels A and B in Figure 1 clearly reveals that UOD is superior to GOD in the PPD coloring reaction. This inhibiting effect of GOD was observed even *under anaerobic conditions* when H_2O_2 (ca. 3 mM) was directly added into the solution containing glucose and PPD. In the absence of glucose, however, GOD itself did not affect the coloring reaction of PPD induced by the direct addition of H_2O_2.

Difference in Catalytic Function between UOD and GOD. As an explanation for the inhibition effect of GOD described above, we considered that BQI generated by the PPD oxidation with H_2O_2 works as an electron acceptor of GOD. The following cyclic voltammetric experiments support the consideration clearly. Panel A of Figure 2 shows effects of the GOD addition on the cyclic voltammogram of PPD in the presence of glucose under anaerobic conditions. PPD gave a reversible wave with a midpoint potential of 197 mV vs Ag/AgCl/KCl(sat.) in the absence of GOD. The redox wave is ascribed to the two-electron oxidation into BQI and its rereduction. The addition of GOD into the solution resulted in an appearance of a well-defined catalytic oxidation wave and a disappearance of the rereduction wave. This electro-

(12) When PPD was oxidized more strongly with H_2O_2 at concentrations higher than 1 M, an absorption band around 415 nm increased faster than that of 520 nm. Therefore, the former band can be assigned to BQI. This is also supported by considering the longest $\pi-\pi^*$ wavelength of *p*-benzoquinone (425 nm). The shoulder around 430 nm in Figure 1A would be identical with the 415 nm band by considering the overlapping of the 520 nm band.

chemical phenomenon is called mediated bioelectrocatalysis,[13−15] in which GOD functions as a glucose dehydrogenase and BQI serves as an electron transfer mediator; that is, the electrochemically generated BQI is quickly rereduced by the substrate-reduced GOD yielding PPD, which is again oxidized electrochemically. Several redox compounds involving benzoquinones and ferrocenes have been reported to work as mediators in GOD-based bioelectrocatalysis.[16−21] By considering the resemblance in the electronic structure between BQI and *p*-benzoquinone, the function of BQI seems to be reasonable. For some other flavin-containing oxidases such as pyruvate oxidase, xanthine oxidase, sarcosine oxidase, and cholesterol oxidase have been revealed to exhibit corresponding dehydrogenase activity.[22−25]

On the other hand, the addition of UOD did not substantially affect the cyclic voltammogram of PPD in the presence of uric acid (Figure 2, panel B), indicating that BQI does not work as an electron acceptor of UOD. We further examined urate dehydrogenase function of UOD using several possible electron acceptors. Cyclic voltammetric experiments using ferrocene instead of PPD did not show any change in voltammograms after the addition of UOD under conditions similar to those given in Figure 2B, indicating that ferricinium ion does not work as an electron acceptor of UOD. Reactivity of UOD with 2-methyl-1,4-naphthoquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone, and hexacyanoferrate(III) ion were also followed spectroscopically in the presence of an excess amount of uric acid under anaerobic conditions. Any enzyme reaction-related absorbance change of the candidates did not occur upon the addition of UOD. All these results support lack of the dehydrogenase function in UOD.

Dehydrogenase and Oxidase Activities of GOD. It is important to evaluate enzymatic kinetics of the glucose dehydrogenase activity of GOD toward BQI and to compare it with that of the glucose oxidase activity in order to clarify the inhibition effect of GOD. Conventional spectrophotometry might be applied to steady state kinetic measurements of the glucose dehydrogenase activity using BQI as an electron acceptor. However, BQI is not so stable to be isolated.[2] Thus, we employed an electrochemical method, in which the reduced form of electron acceptors can be used preferably to the oxidized form for dehydrogenase activity measurements.[26−28]

Table 1. Kinetic Parameters of Glucose Oxidase and Glucose Dehydrogenase Activities of GOD

electron acceptor	dioxygen	BQI
$k_{cat}K_M^{-1}/10^6\ M^{-1}\ s^{-1}$	1.3	11.5
$k_{cat}/10^3\ s^{-1}$	1.4	4.6[a]
$K_M/10^{-3}\ M^{-1}$	1.1[b]	0.4[a]

[a] Apparent value (see also ref 30). [b] A value of 0.83 mM was reported at 38 °C and pH 5.6 in ref 9.

At higher concentrations (140 mM) of glucose than $K_{S,GOD}$, the bioelectrocatalytic current exhibited a steady state as shown in Figure 2A. The steady state current measured by constant potential amperometry at 0.35 V (i_s) increased with the PPD concentration ([PPD]) at least up to 0.09 mM at the GOD concentration ([GOD]) of 8.5 $\times 10^{-7}$ M in deaerated phosphate buffer of pH 7.0. Under such conditions, i_s is given by[26−28]

$$i_s = nFA\sqrt{Dk_{cat}(\mathrm{BQI})[\mathrm{GOD}]/K_M(\mathrm{BQI})}[\mathrm{PPD}] \quad (2)$$

where k_{cat}(BQI) and K_M(BQI) are the catalytic constant and the Michaelis constant of GOD for BQI: n and D are the number of electrons and the diffusion coefficient of PPD, respectively. F and A are the Faraday constant and the electrode area, respectively. From the slope of the linear i_s vs [PPD] plot, k_{cat}/K_M(BQI) (*i.e.*, the rate constant of the bimolecular reaction between GOD and BQI) was evaluated using an experimental value of $nFA\sqrt{D}$ estimated separately according to the Cottrell equation,[29] and the results are summarized in Table 1.

Concerning electrochemical estimation of K_M(BQI), unfortunately, rigorous analytical methods have not yet been established. In our cyclic voltammetric experiments as shown in Figure 2A, the increased anodic current measured from the diffusion current of PPD in the absence of GOD at 0.3 V exhibited a Michaelis-Menten-type dependence on [PPD] in the [PPD] range from 0.27 to 2.94 mM. Therefore, the increased current vs [PPD] relation was analyzed in terms of the Michaelis-Menten equation to get an apparent value of K_M(BQI),[30] which is summarized in Table 1.

On the other hand, steady state kinetic measurements of the glucose oxidase activity of GOD was performed by means of the oxygen electrode at a glucose concentration of 130 mM and [GOD] = 2.6 $\times 10^{-8}$ M and at pH 7.0.

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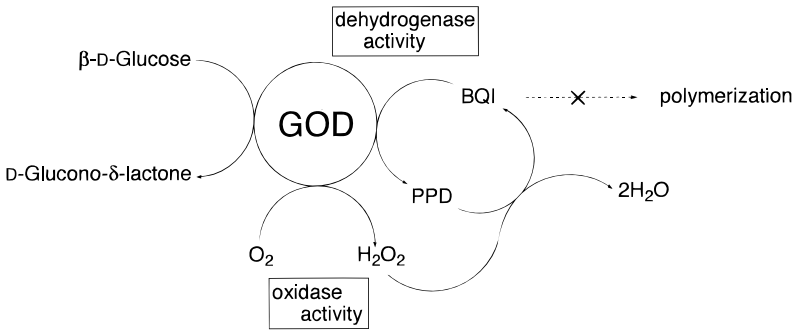
(29) For the Cottrell equation, see, for example: Bard A. J.; Faulkner L. R. In *Electrochemical Methods*; Wiley: New York, 1980; 1980; p 142. In our case, chronoamperometric measurements were done with a potential step from 0 to 0.35 V. The resulting $i\sqrt{t}$ values were practically constant at least within a time (t) range from 1 to 12 s.
(30) Our recent study using digital simulation suggests that dependence of the increased current in mediated bioelectrocatalysis on the mediator concentration follows the Michaelis-Menten-type equation in the presence of an excess amount of substrate. However, the apparent value of K_M as well as k_{cat} is a function of not only the corresponding real value but also time and others, and then the real K_M could not be distinguished from the apparent one. However, the real K_M(BQI) value seems to be smaller than that estimated here, because the latter involves the depletion effect of glucose near the electrode surface.

Scheme 2. Mechanism of the Inhibition of the Oxidative Polymerization of PPD by GOD



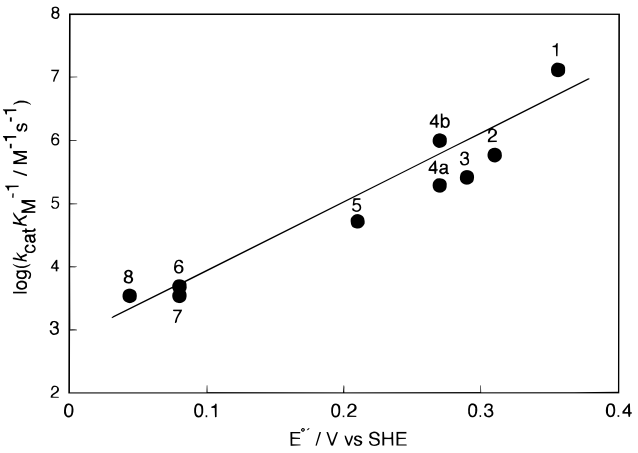


Figure 3. Dependence of the bimolecular rate constant between GOD and quinones (k_{cat}/K_M) on the formal redox potential of the quinones at pH 7.0 ($E^{\circ\prime}_{pH\ 7}$): (1) BQI (this work), (2) 2-bromo-1,4-benzoquinone, (3) tetrabromo-1,4-benzoquinone, (4) 1,4-benzoquinone, (5) 2-methyl-1,4-benzoquinone, (6) 2,3-dichloro-1,4-naphthoquinone, (7) 2-methyl-5-methoxy-1,4-benzoquinone, (8) 1,4-naphthoquinone. The k_{cat}/K_M values (at 25 °C) for compounds **2**−**8** were taken from ref 16, except for **4b**, which were taken from ref 18. The redox potentials are referred to the standard hydrogen electrode (SHE).

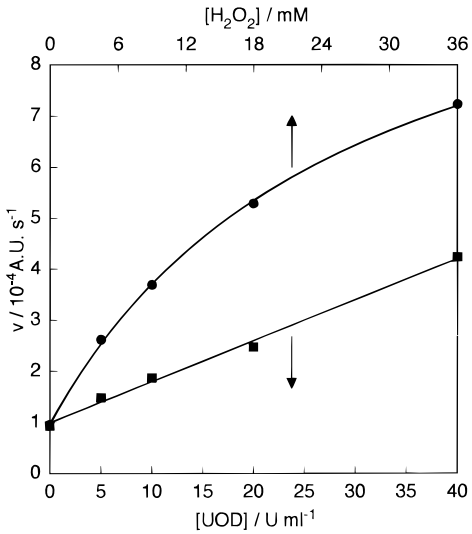


Figure 4. Oxidative polymerization rate of PPD (v) as a function of (●) the H_2O_2 concentration in the absence of UOD and uric acid and (■) the UOD concentration in the presence of 1.0 mM uric acid without addition of H_2O_2, at pH 7.0 and at 30 °C under aerated conditions. The v value was defined as an increase in the absorbance at 520 nm in a given period (see Figure 1A), because the absorbance coefficient of the polymerized product was unknown. The reaction at $[H_2O_2] = 0$ (or [UOD] = 0) is related to the auto-oxidation by dissolved dioxygen.

The dioxygen concentration dependence of the reaction rates followed the Michaelis-Menten equation. Table 1 summarizes the kinetic parameters thus evaluated.

Surprisingly, the k_{cat}/K_M(BQI) value is ca. 9 times larger than that of dioxygen ($k_{cat}/K_M(O_2)$) under the present conditions. This implies that BQI is reduced much faster than dioxygen when BQI and dioxygen coexist at identical concentrations less than the corresponding K_M values. For quinone-mediated and GOD-catalyzed systems, the bimolecular reaction rate constants between quinones and GOD have been found to satisfy a linear free energy relation.[16−18] Interestingly, the present value of k_{cat}/K_M(BQI) falls on the relation as shown in Figure 3. Thus, the large value of k_{cat}/K_M(BQI) seems to originate from the high redox potential of the BQI/PPD redox couple. On the other hand, although some ambiguity exists in the estimation of K_M(BQI),[30] the value is smaller than that of dioxygen ($K_M(O_2)$), indicating stronger affinity of BQI to GOD compared with O_2. This strong glucose dehydrogenase activity of GOD toward BQI is responsible for the inhibition effect of GOD in the PPD polymerization. GOD produces H_2O_2 with consumption of dissolved dioxygen, of which the concentration is initially smaller than $K_M(O_2)$ under aerated conditions. The generated H_2O_2 oxidizes PPD to yield BQI. With a decrease in the O_2 concentration and then with an increase in the BQI concentration, GOD becomes liable to switch the function from glucose oxidase to glucose dehydrogenase. Thus, BQI is reduced back to PPD, resulting in the inhibition of the subsequent polymerization (or coloring) of BQI (Scheme 2). Disappearance of the small peak around 500 nm (probably due to oxidized impurity of PPD) upon the GOD addition (Figure 1B) would be also attributable to the dehydrogenase reaction of GOD. In other words, the polymerization and the glucose dehydrogenase reaction compete with each other toward BQI.

Comparison of UOD-Induced Oxidation and Direct H_2O_2 Oxidation. We further investigated the effectiveness of the UOD-induced oxidative polymerization of PPD as compared with the conventional oxidative polymerization by the direct addition of H_2O_2. Figure 4 shows the dependence of [UOD] and $[H_2O_2]$ on the oxidative polymerization rate (v). In the direct oxidation with H_2O_2, the reaction rate increased parabolically with $[H_2O_2]$. On the other hand, when the UOD reaction was utilized for the oxidative polymerization, the rate increased linearly with [UOD]. The UOD-induced oxidation using 40 U mL^{-1} of UOD is as effective as the direct oxidation with ca. 10 mM of H_2O_2 under the present conditions. This is very interesting from the stoichio-

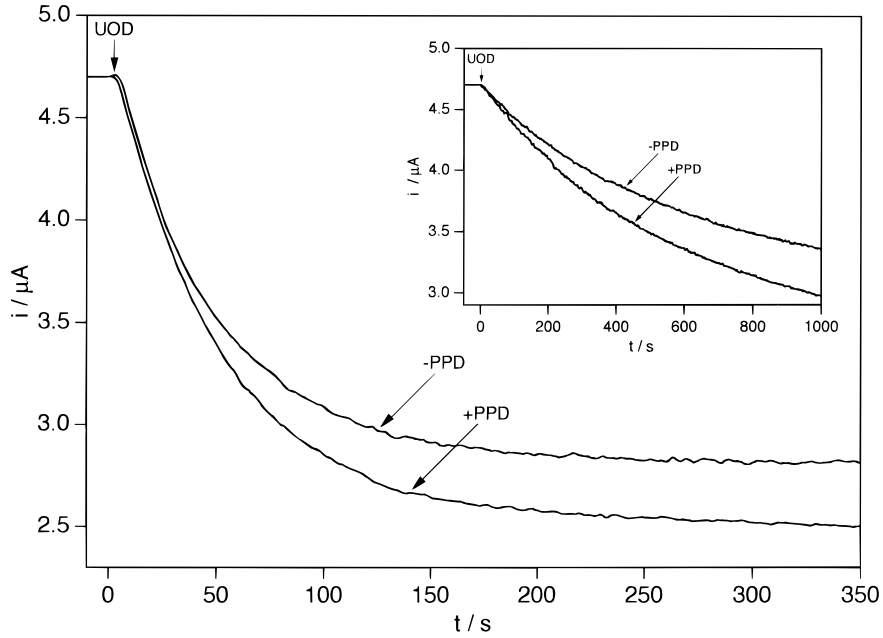


Figure 5. Effect of PPD (30.8 mM) on the time-dependent dioxygen concentration change caused by 0.3 U mL^{-1} of UOD in the presence of 0.1 mM uric acid at pH 7.0 and 30 °C in a space-free and air-tight electrochemical cell. The oxygen concentrations were expressed by the output current measured using an oxygen electrode. The cell was aerated before the experiments. The inset shows the result under identical conditions except for the UOD concentration, which was 0.03 U mL^{-1}.

metric point of view. During the experiments, the spectroscopic cuvettes were opened to the atmosphere without stirring. However, the dioxygen depletion occurred in the cuvette due to the larger amount of uric acid (1 mM) compared to the dissolved dioxygen (0.25 mM). This is evidenced by the experimental result that the reaction rate increased up to at least about two times by periodic stirring to supply dioxygen into the cuvette during the kinetic measurements. Therefore, the total amount of H_2O_2 produced by the UOD reaction would be ca. 0.25 mM, or at most 1 mM even when dioxygen could be supplied sufficiently. The direct oxidation with H_2O_2 at such low concentrations cannot oxidize PPD so effectively as the UOD-induced oxidation, as judged from Figure 4. Therefore, some other functions to enhance the PPD oxidation would be expected in UOD.

Enhanced Oxidation of PPD by UOD. In order to understand the enhanced oxidation of PPD by UOD, we have selected a working hypothesis that UOD exhibits PPD oxidase-like and/or PPD peroxidase-like function(s) to oxidizePPD catalytically. Figure 5 shows a time-course of the dioxygen consumption during the UOD reaction in the absence and presence of PPD (30.8 mM) at 0.1 mM of uric acid and 0.25 mM of O_2 (air saturation). As shown in Figure 5, the total dioxygen consumption as well as the initial dioxygen consumption rate (see the inset) were increased by the addition of PPD. Such PPD-dependent acceleration of the dioxygen consumption did not occur in the absence of uric acid. This means that UOD exhibits PPD oxidase-like activity in the presence of uric acid, that is, that UOD catalyzes the oxidation of PPD by dioxygen to produce BQI in the presence of uric acid. Because the PPD oxidase-like reaction proceeds parallel to the inherent urate oxidase reaction, detailed enzymatic kinetics of the PPD oxidase-like reaction could not be evaluated. Uchiyama et al. have reported a similar property for other UOD (origin: not specified) using dithiothreitol (DDT) as a reductant and explained the phenomena by the proposed mechanism that the UOD−urate−O_2 ternary complex is reduced with DDT yielding UOD, uric acid, hydroxy radical, and the oxidized form of DDT.[31]

We have further found that UOD accelerates the PPD oxidation with H_2O_2 under deaerated conditions in the presence of uric acid, as shown in panel A of Figure 6. Uric acid is essential to the acceleration of the PPD oxidation. This means that UOD exhibits PPD peroxidase-like activity in the presence of uric acid, that is, that UOD catalyzes the oxidation of PPD by H_2O_2 to produce BQI in the presence of uric acid. The dependence of the PPD peroxidase-like reaction rate on [H_2O_2] depicted in panel B of Figure 6 follows the Michaelis-Menten-type kinetics with a Michaelis constant around 2−4 mM. Such peroxidase-like activity of UOD was also effective for the oxidation of 4-methylcatechol: under anaerobic conditions in the presence of uric acid, 4-methylcatechol solution exhibited a new adsorption band with the maximum at 390 nm, which is almost the same as that of the 4-methyl-1,2-benzoquinone in aqueous solution (401 nm)[32] or that generated from 4-methylcatechol using horseradish peroxidase in ethyl acetate saturated with aqueous phosphate buffer (382 nm).[33] These results suggest that the UOD−urate complex is responsible for the PPD peroxidase-like activity and probably for the PPD oxidase-like activity also. The expression of such alternative function of the enzyme−substrate complex is not unique for UOD. For example, nucleoside oxidase from *Pseudomonas maltophilia* LB-86 is reported to show laccase-like activity only in the presence of the substrate (nucleoside) together with the inherent nucleoside oxidase activity.[34,35]

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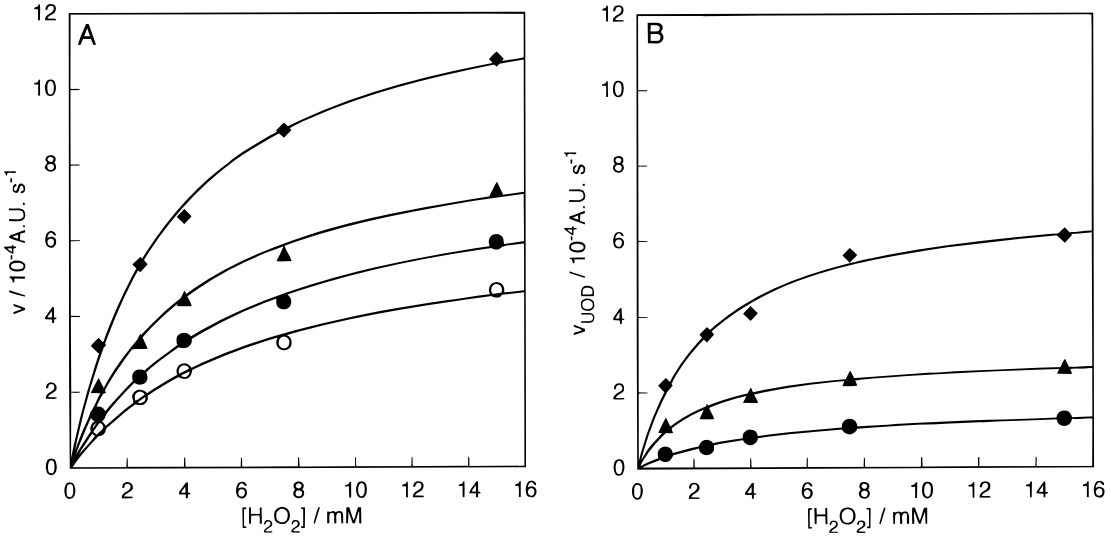
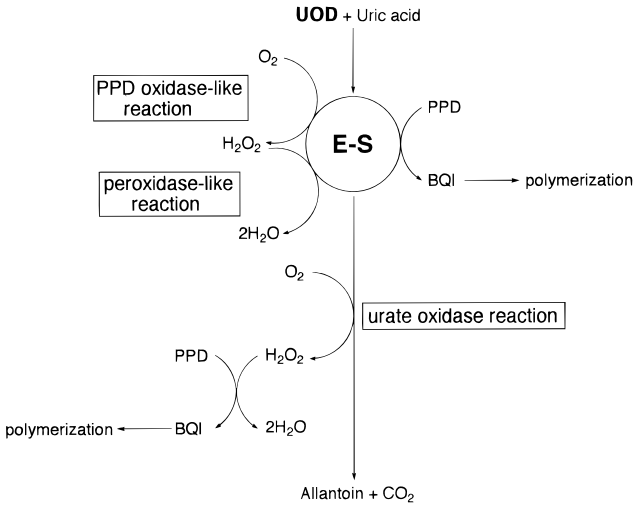


Figure 6. Panel A: Effect of the UOD concentration on the H_2O_2 concentration-dependent oxidative polymerization rate of PPD (v) in the presence of 1.0 mM uric acid at pH 7.0 and at 30 °C under deaerated conditions. The UOD concentration/U mL^{-1}: (○) 0, (●) 23.3, (▲) 45.8, (◆) 88.7. The v values were evaluated as described in the caption of Figure 4. Panel B: UOD-accelerated oxidative polymerization rate of PPD (v_{UOD}) as a function of the H_2O_2 concentration. The symbols represent the UOD concentration given in panel A. The v_{UOD} values were evaluated by subtracting v in the absence of UOD (○ in panel A) from the observed v in the presence of UOD (●, ▲, ◆ in panel A). The Michaelis constant of H_2O_2 were evaluated as (●) 2.6 mM, (▲) 2.1 mM, and (◆) 4.2 mM on the basis of the Michaelis-Menten equation.

Scheme 3. Proposed Mechanism of UOD-Induced Oxidative Polymerization of PPD



Conclusion

The oxidation of PPD induced by UOD can be illustrated as shown in Scheme 3. Under aerobic conditions, UOD oxidizes PPD as well as uric acid, yielding BQI and H_2O_2 (as well as allantoin). The generated H_2O_2 also oxidizes PPD to yield BQI. The PPD oxidation with H_2O_2 is catalyzed by the UOD−urate complex. Because of the lack of urate dehydrogenase activity in UOD, BQI can undergo the subsequent polymerization effectively. The latter property as well as the expression of the PPD oxidase-like and PPD peroxidase-like activity is essential for the novel UOD-induced oxidative polymerization of PPD. The detailed mechanism of the expression of the PPD oxidase-like activity and the PPD peroxidase-like activity remains to be solved, and the study is in progress.

Recently, high cell density cultivation and high recombinant UOD production of *E. coli* cloned with cDNA of UOD has been established for the large-scale commercial production of UOD.[7,36] Therefore, the UOD is available in high purity and inexpensively. In addition, this enzyme is very stable.[37] Therefore, the UOD-induced PPD oxidation method can be subjected to practical hair-dyeing purposes. In this method, uric acid with low solubility can be formulated as suspension and be continuously supplied into the reaction phase with a decrease in the concentration. As a result, H_2O_2 will be continuously generated at relatively low concentrations. This situation is also very convenient in practice in order to minimize the toxic effect of H_2O_2. In this UOD-induced method, the reaction is triggered simply by exposure to air. Then, all organic reactants and UOD can be formulated together. This is another advantage to develop one-pack-type products for hair-coloring.

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(37) Under storage at 40 °C, over 90% of the UOD activity was retained after 6 months.