Characterization and Catalytic Property of Surfactant–Laccase Complex in Organic Media

Shin-ya Okazaki, Masahiro Goto,* Hiroyuki Wariishi,† Hiroo Tanaka,† and Shintaro Furusaki

The oxidation of 4-phenylenediamine catalyzed in anhydrous organic solvents by surfactant–laccase complex was investigated. The complex was prepared by utilizing a novel preparation technique in water-in-oil (W/O) emulsions. The surfactant–laccase complex effectively catalyzed the oxidation reaction in various dry organic solvents, while laccase, lyophilized from an aqueous buffer solution in which its activity was optimized, exhibited no catalytic activity in nonaqueous media. To optimize the preparation and reaction conditions for the surfactant–enzyme complexes, we examined the effects of pH in the water pool of W/O emulsions, the concentration of enzyme and surfactant at the preparation stage, and the nature of organic solvents at the reaction stage on the laccase activity in organic media. Surfactant–laccase complex showed a strong pH-dependent catalytic activity in organic media. Its optimum activity was obtained when the complex was prepared at a pH of about 3. Interestingly, native laccase in an aqueous buffer solution exhibited an optimum activity at the same pH of 3. The optimum preparation conditions of surfactant–laccase complex were [laccase] = 0.8 mg/mL and [surfactant] = 10 mM, and the complex showed the highest catalytic activity in toluene among nine anhydrous organic solvents. The effect of a cosolubilized mediator (1-hydroxybenzotriazole (HBT)) on the reaction was also investigated. The addition of HBT at the preparation stage of the enzyme complex did not accelerate the catalytic reaction because HBT was converted to an inactive benzotriazole (BT) by laccase. However, the addition of HBT at the reaction stage enhanced the catalytic performance by a factor of five compared to that without HBT.

Laccase (polyphenoloxidase, EC 1.10.3.2.) is a blue multicopper-containing enzyme that catalyzes the oxidation of a variety of organic substrates coupled to the reduction of molecular oxygen to water. This enzyme is widely produced in higher plants and fungi. In woody tissues, laccases are implicated in the wound response (1), whereas in fungi, laccases are involved in lignin degradation (2, 3). The enzyme has broad specificities for substrates, especially for phenolic compounds and thus it is potentially useful in the cleanup of environmental pollutants.

The enzymatic degradation of environmental pollutants has been reported to be achieved by peroxidase-type enzymes such as horseradish, lignin, and manganese peroxidases. For example, dibenzo-p-dioxine (4), 2,7-dichlorobenzo-p-dioxine (5), and 2,4-dichlorophenol (6) can be degraded by lignin or manganese peroxidases. Sulfonated azo dyes are also readily degraded by these enzymes (7). This approach of using peroxidases suffers from the need for hydrogen peroxide. Laccases, which utilize molecular oxygen instead of hydrogen peroxide as an electron acceptor, are a potentially attractive catalyst. Moreover, in the presence of a mediator, laccases are able to oxidize nonphenolic compounds that cannot be oxidized by laccase itself (8). The oxidation of nonphenolic lignin model compounds (9, 10) and polycyclic aromatic hydrocarbons (PAHs) (11) utilizing the laccase–mediator system has been reported.

In general, environmental pollutants such as dioxins and PAHs do not dissolve in aqueous media owing to their high hydrophobicity, and hence organic solvents are required to concentrate them. This implies that the use of organic solvents inevitably allows the degradation reaction to proceed at a high concentration of environmental pollutants and in a homogeneous system. However, native enzymes do not exhibit significant catalytic activities in organic media unless they are modified by an appropriate surfactant.

Recently, we have developed a novel method for preparing surfactant–enzyme complexes utilizing water-in-oil (W/O) emulsions. For example, the surfactant–lipase, surfactant–protease, and surfactant–peroxidase complexes that were prepared by this novel method exhibited much higher catalytic activities compared to their native powder enzymes in various anhydrous organic media (12–14). This novel preparation method can be easily applied to many enzymes.

In the present study, we have extended our novel method to the preparation of surfactant–laccase complex, which has hardly been used in anhydrous media, and the optimum preparation and operation conditions to activate the enzyme are discussed. Furthermore, we investigated...
the effect of a mediator on the catalytic efficiency of surfactant–laccase complex in organic media.

Materials and Methods

Reagents. Laccase was isolated from the extracellular culture fluid of Coriolus versicolor IFO 9791, kindly supplied from Merckian Co., Ltd. (Japan). The protein content in a laccase solution was determined by the Bradford method using bovine serum albumin as a standard protein and was estimated to be 6.75 mg/mL. The molecular weight of laccase was evaluated by SDS–PAGE and was found to be approximately 65 kDa. o-Phenylenediamine (o-PDA) was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). 1-Hydroxybenzotriazole (HBT) and benzotriazole (BT) were obtained from Sigma Chemical Co., Ltd. All organic solvents used in this work were analytical grade. They were dried with 3 Å molecular sieves prior to use for more than 24 h. Their water contents, determined by Karl Fischer potentiometric titration using a Mitsubishi moisturemeter (model CA-05), were 80 ± 20 ppm. Sequentially distilled and deionized water was employed throughout the experiments. The nonionic surfactant dioleyl N-o-glucronol-g-glutamate (abbreviated as 2C18AGE) was synthesized in our laboratory according to the procedure described in a previous report (15). A surfactant–laccase complex was prepared as follows. A laccase solution (0.148 mL) was added to an aqueous phase (100 mM potassium phosphate buffer) at the optimum pH; the total volume was 1 mL (enzyme content was adjusted to 1 mg/mL). The surfactant was dissolved in 3 mL of toluene (10 mM). The aqueous and organic phases were mixed with a homogenizer (ULTRA-TURRAX T25, J unke & Kunkel) at 15 000 rpm for 3 min. The resultant stable W/O emulsion was immediately frozen in liquid nitrogen and lyophilized in a freeze-drying apparatus (FD-5N, EYELA) for 24 h. A white-blue solid was obtained, which was employed as the surfactant–laccase complex. In the case of the HBT addition, a stock dimethylformamide (DMF) solution of HBT (1.54 mM) was prepared, and 10 µL of this solution was injected to the phosphate aqueous phase. Thus, in this preparation method, the complex consists of laccase, HBT, and surfactant. The final concentration of HBT was adjusted to 0.154–7.7 mM in the mixture yielding a 10- to 500-fold molar excess over laccase. A lyophilized laccase was also prepared by the lyophilization method, as described in our previous study (15). A surfactant–laccase complex was immediately frozen in liquid nitrogen and lyophilized in a freeze-drying apparatus (FD-5N, EYELA) at −40°C. The lyophilized laccase was used for the next reaction (16).

Measurement of Catalytic Activity in Toluene. Stock toluene solutions of o-PDA (60 mM) and the surfactant–laccase complex (50 µg/mL) were prepared. The o-PDA solution (0.5 mL) and toluene (0.3 mL) were mixed in a 1 mL quartz cuvette for UV measurement. The oxidation reaction was initiated by addition of 0.2 mL of surfactant–laccase complex to the reaction medium at room temperature. The final concentration of o-PDA and laccase were 30 mM and 10 µg/mL (0.154 µM), respectively. The initial oxidation rates of o-PDA were evaluated spectrophotometrically by measuring the absorbance at 470 nm (16). In the case of the HBT addition, a stock DMF solution of HBT (1.54–154 mM) was prepared and 10 µL of this solution was added to the reaction mixture. The initial oxidation rate of o-PDA was evaluated spectrophotometrically by measuring the absorbance at 470 nm (16). In the case of the HBT addition, a stock DMF solution of HBT (1.54–154 mM) was prepared and 10 µL of this solution was added to the reaction mixture. The reaction mixture containing o-PDA and HBT and surfactant–laccase complex was used for the next reaction (16).

Product Analysis. HPLC analysis was carried out using a Shimadzu STR ODS-II column with linear gradient from 10% acetonitrile in water (0.05% phosphoric acid) (isocratic for 5 min) to 100% acetonitrile (20–25 min) at a flow rate of 1.0 mL/min. A UV monitor was utilized at 254 nm. GC–MS was performed at 70 eV on an Autanom 15 system II (J EOL) equipped with a 30-m fused silica column (J&W Science, DB-5). Products were identified by comparing their retention times on GC and HPLC, as well as their mass fragmentation patterns.

Results and Discussion

Comparison of Reaction Systems. Figure 1 shows the time course of o-PDA oxidation catalyzed by either surfactant–laccase complex or lyophilized laccase in anhydrous toluene. The surfactant–laccase complex efficiently catalyzed the oxidation reaction in the dry organic solvent, while the lyophilized laccase exhibited no catalytic activity. In addition, enzyme activity was not observed when enzyme and surfactant were added separately to the reaction medium. Thus, the modification of laccase with surfactant molecules at the preparation stage was found to have a remarkable effect, producing a high enzymatic activity in organic media. Luterek et al. reported the catalytic activity of laccase from Cerrella unicolor immobilized on silanized porous glass beads in organic solvents (17). They used nine water-miscible organic solvents, and when the content of organic solvents increased up to 60%, the laccase activity almost diminished in all solvents, while the surfactant–laccase complex was catalytically active in anhydrous organic media. It is deduced that the surfactant–laccase complex maintains the active conformation of the protein in organic media. Moreover, we considered the reason for the activation of the enzymatic activity in organic media by surfactant complex formation as follows: (i) The surfactant–laccase complex is readily soluble in anhydrous toluene, thus facilitating access of the substrate to the active site, which is advantageous for a homogeneous reaction system. (ii) The surfactant layer surrounding laccase protects the enzyme from denaturation caused by direct interaction of protein surface with organic solvent molecules (18). (iii) The surfactant–laccase complex contains the intact salts that exist in the aqueous buffer phase at the preparation stage. Presumably, salts are useful in maintaining the native structure of an active enzyme in organic media or serve as an immobilization matrix and suppress the deleterious effect of protein–protein interaction (19). Dordick and coworkers reported that adding a large amount of KCl at the preparation stage activates the catalytic activity of subtilisin Carlsberg in organic media. The effect of salts has also been observed in a surfactant–protease complex in our previous study (13).
The oxidized product in the reaction medium was analyzed by direct ionization mass spectrometry (DIMS) and was identified as 2,3-diaminophenazine (M+ 210), which is the same as the reaction product catalyzed by native laccase in an aqueous buffer solution (21) (data not shown). Any other minor products were not observed in organic media. This result indicates that the reaction mechanism is most likely the same in both the aqueous buffer solution and the organic solvent.

**Effect of Aqueous pH on Laccase Activity.** One of the most important parameters for optimizing enzyme preparation in organic media is known to be aqueous pH at the lyophilization stage. Enzymes tend to “memorize” the ionization state in the last aqueous buffer solution and expose this state in organic media (22). Thus, enzymes maintained the state of charge memorized at the preparation stage with the aqueous. The effect of aqueous pH at the preparation stage on the enzymatic activity of the surfactant–laccase complex in organic media is shown in Figure 2. Figure 2 also shows the pH dependency of laccase activity in an aqueous solution. The enzyme and substrate concentrations were kept similar in the aqueous buffer and organic solvent. From the data in Figure 2, the activity of the surfactant–laccase complex was found to be strongly influenced by the aqueous pH at the preparation stage, and the pH profiles are almost similar in both organic solvent and aqueous solution. The optimum pH is found to be approximately 3 in both cases. This result suggests that the active conformation was locked at the preparation stage and the structure was maintained in anhydrous toluene, that is, “pH memory” was expressed. The activity of the surfactant–laccase complex prepared from pH 2 was strongly reduced. At pH 2, the ester groups in the nonionic surfactant are hydrolyzed and the solubility of the resultant complex is lowered compared to that of the complexes prepared from higher pH solutions. These observations demonstrate that the pH adjustment at the preparation was crucial for activating the surfactant–laccase complex. On the basis of this result, the surfactant–laccase complex was prepared at pH 3 in subsequent experiments.

**Effect of Surfactant Concentration on Enzymatic Activity.** The surfactant concentration at the preparation stage would be another important factor for the preparation of enzyme complexes. In this section, the concentration of laccase in the aqueous phase was fixed at 1 mg/mL and the concentration of the surfactant in the organic phase was varied (0.5, 1, 2, 4, 6, 8, and 10 mM). Figure 3 shows the relationship between the initial reaction rate and the concentration of surfactant. At less than 2 mM, the catalytic activity of the surfactant–laccase complex decreased and the solubility of the complexes prepared from the surfactant concentration of 0.5 and 1 mM was very poor. This observation indicates that the critical surfactant concentration (CSC) to obtain a good performance of the complexes is 2 mM, and below this concentration stable W/O emulsions were not formed. As described above, the complexation of enzyme and surfactant plays a crucial role in the expression of the enzymatic activity in organic solvent. The initial oxidation rate did not depend so much on the surfactant concentration above 2 mM. At high concentrations of surfactant, however, as a slight increase in the reaction rate was observed, the concentration of 10 mM was selected to prepare the surfactant–laccase complex in subsequent experiments.

**Effect of Enzyme Concentration at the Preparation Stage.** A weak point in the novel preparation method utilizing W/O emulsions is that the particle size of emulsions (about 6 µm in average) is relatively larger compared to that of enzyme. This means that many enzyme molecules are entrapped in one emulsion droplet (several thousands of enzyme molecules in an emulsion droplet by calculation). There might be a possibility that enzymes entrapped in emulsion droplets have no access to the substrate. The simplest way to resolve this problem is to reduce the enzyme concentration at the preparation stage so as to minimize the enzyme molecules entrapped in emulsion droplets. The concentration of laccase in the aqueous phase was varied (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL), while the concentration of the surfactant in the organic phase was fixed at 10 mM in this experiment. It should be noted that the total laccase concentration at the reaction stage was kept constant (10 µg/mL). The result is shown in Figure 4. In contrast to our expectation, the initial reaction rate was decreased upon decreasing the laccase concentration at the preparation stage. The solubility of the complexes in dry toluene was lowered by using low enzyme content complexes because a large amount of the complex is required to adjust the laccase concentration (10 µg/mL) at the same level. Thus, it was found necessary to keep a critical balance between the laccase content of the complex and its solubility without compromising the optimum enzyme concentration at the preparation stage.

**Effect of Reaction Media.** It is still unclear what characteristics of organic solvents would affect the enzymatic activity, however, the solvent’s hydrophobicity (expressed by the log P value) is known to be one of the useful parameters in predicting the degree of enzymatic activity in anhydrous organic solvents (23). It is established that the catalytic activity of biocatalysts in organic media is relatively lower for hydrophilic solvents having log P < 2 and higher in hydrophobic solvents having log P > 4 (24). To investigate the effect of organic solvents on the enzymatic activity of the surfactant–laccase complex, one of the organic solvents and the aqueous buffer solution.
complex, the oxidation reaction was carried out in various organic solvents covering a wide range of hydrophobicity (log P = -1.1 to 2.5). The results are shown in Figure 5 where the activity in toluene was unit. The complex showed a higher activity in aromatic solvents than in other solvents. Although no strong correlation exists between enzymatic activity and log P value of the solvents, the complex generally exhibited a high catalytic activity in hydrophobic solvents (e.g., toluene and isopropyl ether). Thus, the protective effect of the surfactant layer in the complex largely depended on the property of organic solvents. The effect was rather weak in hydrophilic solvents (e.g., 1,4-dioxane and DMF) and the enzyme complex did not show activity in such hydrophilic solvents. The essential water, which is a monolayer of water surrounding the enzyme molecules that is absolutely required for the catalytic function of enzymes (25), could have been removed from the surfactant–laccase complex, because the water content of the hydrophilic solvents rapidly increased after adding the surfactant–laccase complex (data not shown).

**Effect of Mediator.** The laccase–mediator system is used in the pulp bleaching process to reduce the amount of bleaching chemicals. Both 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and HBT have been shown to be effective in reducing the lignin content of Kraft pulp, and the existence of natural mediators has also been recently reported (26). While little is known about the mechanism of the laccase mediator system, several mechanisms have been suggested (27). The most current theory is based on the diffusion of radical species into the pulp that selectively initiate the oxidation of the residual lignin. It has been reported that not only the model compounds (9, 10) but also PAHs (11) were effectively oxidized by a laccase–mediator system. In our study, the effect of a mediator on the reaction efficiency was investigated in the organic media. As a mediator, HBT was selected because it is the most effective mediator among reported ones and its radical is durable in nonaqueous organic solvents (28). In the present experiment, two methods were employed. Method 1 involved the addition of HBT at the preparation stage, i.e., HBT is complexed with the surfactant and laccase at the same time. This method yielded a surfactant–laccase–HBT ternary complex. Method 2 involved the addition of HBT at the reaction stage. In this case, HBT was not complexed with the surfactant and enzyme. o-PDA solution and a DMF solution of HBT were mixed in the UV cuvette, and the reaction was initiated by the addition of the surfactant–laccase complex (see details in Materials and Methods). The results are shown in Figures 6a and b. Based on the result in Figure 6a (method 1), the initial reaction rate did not increase by the addition of HBT. To explain this result, the aqueous phase containing laccase and HBT was analyzed by GC–MS, which indicated that HBT was converted to benzotriazole (BT, which is inactive as mediator (29)). Thus, active HBT was converted to inactive BT during the preparation of the complexes. In the case of results from method 2 shown in Figure 6b, the initial reaction rate increased with increasing HBT concentration. This indicates that HBT effectively functions as a mediator. The molar ratio of laccase and HBT ([HBT]/[Lac]) in method 2 is significantly higher than in method 1. This is because in method 1, laccase concentration at 1 g/L was higher compared to that in method 2, and the preparation of a HBT stock solution corresponding to over 500 equimolar of laccase is difficult. In other words, method 2 can provide a sufficient amount of HBT for an effective reaction. Moreover, we observed that HBT was oxidized to BT during the preparation step in method 1. When the reaction solution of method 2 was analyzed by GC–MS, however, BT was also detected. These results demonstrated that the conversion of HBT to BT during oxidation of o-PDA by laccase might be the limiting step in the activity of this laccase–mediator system. Other researchers (29, 30) obtained similar results; however,
the mechanism for the formation of BT during oxidation in a laccase—mediator system would require further study.

Conclusion

In this study, it is demonstrated that a surfactant—laccase complex efficiently catalyzes the oxidation reaction of ophenylenediamine in anhydrous toluene. Surfactant—laccase complexes exhibited a high enzymatic activity, while the lyophilized laccase showed no catalytic activity. The activity of the surfactant—laccase complex was strongly dependent on the aqueous phase pH at the preparation step and also the nature of organic solvents used as reaction media. Catalytic activity was observed only after forming a complex with surfactant molecules. The enhanced effect was explained by the existence of the protective surfactant layer surrounding the enzyme. Furthermore, the hydrophobic tails in the nonionic surfactant facilitated the solubility of the surfactant—laccase complex in organic media.

The preparation method for the surfactant—enzyme complexes utilizing W/O emulsions is advantageous by its simplicity and the superior yields of resultant enzyme complexes. It is also versatile in its application because it conforms well to several enzymes without the requirement of additional handling techniques. We hope that the fundamental information in this paper will be useful for the preparation of active biocatalysts that are catalytically active in organic media.

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References and Notes


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