Highly Efficient Biodecolorization/Degradation of Congo Red and Alizarin Yellow R by Chloroperoxidase from Caldaromyces fumago: Catalytic Mechanism and Degradation Pathway

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ABSTRACT: Chloroperoxidase (CPO) from Caldaromyces fumago could mediate a very efficient oxidative decolorization/degradation of aqueous azo dye. The decolorization efficiencies for Congo Red and Alizarin Yellow R reached 88.25% in 3 min and 92.38% in 5 min, respectively, under mild conditions at enzyme concentrations of 0.025 and 0.03 μmol L⁻¹, respectively. Compared with the typical mechanism involved in oxidative decolorization of azo dyes catalyzed by other peroxidases, a different enzymatic catalytic cycle was found in this work. The oxidative decolorization was carried out in bulk solution, which would get rid of the restriction of substrate required by the channel access to the substrate pocket in the enzymatic active site. Nine degradation products of Congo Red were detected by HPLC–MS analysis, and two types of degradation pathways were proposed accordingly. The strong toleration of the typical salt species in industrial effluents by this system ensured that it has potential applications in treatment of industrial wastewater.

1. INTRODUCTION

The textile industry generates large amounts of liquid effluent pollutants every year, in which azo dyes account for the majority (more than 3000 different varieties) of all textile dyestuffs produced.¹ Textile industry wastewater containing azo dyes and their metabolites in aqueous ecosystems is not only aesthetically unpleasant but also leads to a reduction of sunlight penetration, which in turn decreases the dissolved oxygen concentration and water quality. It even has acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide.² Therefore, treatment of industrial effluents containing azo dyes prior to their final discharge into the environment is necessary, and hence, there is an urgent need for technically feasible and cost-effective methods.

Various physical/chemical methods, such as adsorption, chemical precipitation, photolysis, chemical oxidation and reduction, and electrochemical treatment, have been used for the removal of dyes from wastewater. However, these methods mostly suffer from serious limitations of being economically unfeasible (as they require more energy and chemicals), being unable to completely remove the recalcitrant azo dyes, generating a significant amount of sludge that may cause secondary pollution problems, and involving complicated procedures.³⁻⁵ In contrast, microbial or enzymatic treatment for decolorization/degradation of azo dyes is an eco-friendly, cost-effective alternative to conventional physicochemical methods.⁶⁻⁸

Azo compounds are susceptible to biological degradation under both aerobic and anaerobic conditions.⁹ Microbial treatment is effective in decolorization of azo dyes, but too much time is needed (from 20 h up to even 26 days).¹⁰ Moreover, general microbial degradation of azo dyes involves a reductive cleavage of azo bonds (−N=N−) under anaerobic conditions, which would result in the formation of potentially hazardous aromatic amines.¹¹,¹²

The enzymatic approach is much more effective that the microbial method with regard to decolorization/degradation of azo dyes, especially of the streams that have the highest concentrations of target contaminants and the lowest concentration of other contaminants. The enzymes involved in decolorization/degradation of azo dyes are mainly oxidoreductive enzymes. Azoreductase catalyzes reductive cleavage of azo bonds, forming undesired colorless aromatic amine products. Conversely, peroxidases, such as lignin peroxidase, laccase, and Mn peroxidase, carry out the oxidative destruction of chromophore with the release of N₂.

Chloroperoxidase (CPO) is a glycohemoprotein isolated from the mold Caldaromyces fumago. It is now considered to be the most versatile heme-containing enzyme, exhibiting peroxidase, catalase, and cytochrome P450-like activities in addition to catalyzing the chlorination of activated C–H bonds. Recently, our group focused on the biodegradation of environmental contaminants by this enzyme.¹³ In the present work, this very efficient CPO-mediated oxidative method was applied to the decolorization/degradation of Congo Red and Alizarin Yellow R. Moreover, it was found that the chlorination activity of CPO was involved in the enzymatic catalysis cycle, and an interesting route is illustrated. A degradation pathway has been proposed on the basis of the characterization of degradation intermediates and final products of the azo dyes by HPLC–MS analysis.

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2. MATERIALS AND METHODS

2.1. Enzyme Preparation. *C. fumago* was cultured according to the method established by Morris and Hager.\textsuperscript{14} CPO was isolated from the growth medium of the fungus with minor modifications, using acetone rather than ethanol in the solvent fractionation step. Next, CPO was purified by DEAE-Sephadex A-50 ion-exchange column chromatography. The enzyme solution was concentrated to 11.6 mg of CPO/mL with $R_z = 1.00$ ($R_z$ = purity standard = $A_{398}/A_{280} = 1.40$ for the pure enzyme), after which it had an activity of $6.13 \times 10^5$ units/L based on the standard monochlorodimedon (MCD) assay.\textsuperscript{15} Both dyes were reacted in the following experiments using this enzyme solution through appropriate dilution to the fixed concentration.

*C. fumago* is the best fungus for obtaining CPO, as it can secrete more and better CPO than other fungi.\textsuperscript{16,17}

2.2. Reagents and Buffer. Congo Red and Alizarin Yellow R (Figure 1) were obtained from Sigma-Aldrich. All of the other chemical regents, such as dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and hydrogen peroxide (30% in aqueous solution), were obtained from Xi’an Chemical Co. Ltd. These chemicals were of analytical grade unless otherwise indicated.

A 0.1 mol L$^{-1}$ potassium phosphate buffer was prepared by mixing and diluting appropriate volumes of 1 mol L$^{-1}$ KH$_2$PO$_4$ and K$_2$HPO$_4$ stock solutions, and its pH was then adjusted to various fixed values using 1 mol L$^{-1}$ HCl.

2.3. Decolorization/Degradation Assays. Decolorization assays were performed at 20–60 °C in 0.1 mol L$^{-1}$ phosphate buffer (containing Cl$^-$) in a centrifuge tube with total volume of 1.0 mL containing CPO (0–0.6 μmol L$^{-1}$) and azo dye (0.05–0.50 mmol L$^{-1}$) at pH 2–4. The reaction was started by the addition of H$_2$O$_2$ (0–5.0 mmol L$^{-1}$). The effects of the operating parameters (pH, amount of enzyme, H$_2$O$_2$ concentration, initial dye concentration, reaction time, and temperature) on the decolorization efficiency were investigated by changing one factor at a time while the other parameters were kept constant.

The decolorization efficiency was determined by monitoring the absorption at the wavelength of maximum absorption ($\lambda_{max} = 567$ nm for Congo Red and 373 nm for Alizarin Yellow R) using a UV–vis spectrophotometer (Shimadzu UV-1700) and employing the following equation:

$$\text{decolorization efficiency} = \frac{A_0 - A_t}{A_0} \times 100\%$$

2.4. Determination of Products. Samples were collected after 30 min of reaction. The crude solutions were treated by extraction using ethyl acetate and dichloromethane at an exact ratio of 1:1 (three times). The combined organic extracts were purified by rotary evaporation, and the sample was dissolved in 750 μL of methanol for HPLC–MS analysis.

An Esquire LC–ion trap mass spectrometer (Bruker Daltonics, Germany) equipped with an orthogonal-geometry electrospray ionization (ESI) source was employed to determine the main products. Nitrogen was used as the drying (8 L min$^{-1}$) and nebulizing (0.8 kPa) gas at 180 °C. Scanning was performed from $m/z$ 100 to 1000 in the standard resolution mode.

3. RESULTS AND DISCUSSION

3.1. Enzymatic Catalytic Cycle and Oxidative Decolorization Reaction. It is well-known that CPO is oxidized by H$_2$O$_2$ through heterolytic cleavage of H$_2$O$_2$ to generate a

![Figure 1. Chemical structures of azo dyes treated in this work.](image1.png)

![Figure 2. Schematic illustration of the enzymatic catalytic cycle.](image2.png)
Compound I intermediate.\textsuperscript{18,19} Several reported results have suggested that Compound I could best be described as an oxyferryl \( \pi \) radical cation that in turn promotes the oxidation of the substrate. In regard to the chlorination reaction catalyzed by CPO, there is a competing oxidation by Compound I between the organic substrate (here an azo dye) and chloride ion. It was found that the oxidation of Cl\textsuperscript{−} is much faster than the overall dye oxidation process. Chloride ion combine with the oxygen of Compound I through a nucleophilic attack process, producing the enzyme iron(III) hypohalite complex (EOCl) with concomitant one-electron reduction of both the iron and the porphyrin.\textsuperscript{20−22} Once formed, EOCl can react rapidly with the chlorine-acceptor substrate. In the absence of chlorine-acceptor substrate, EOCl reacts with chloride ion or water to

Figure 3. Dependence of the efficiency of CPO-catalyzed oxidative decolorization on (A) pH, (B) temperature (\( T \) in \(^\circ\text{C} \)), (C) \( \text{H}_2\text{O}_2 \) concentration (mmol L\textsuperscript{−1}), (D) CPO consumption (\( \mu \text{mol L}^{-1} \)), (E) dye concentration (mmol L\textsuperscript{−1}), and (F) the presence of NaCl, NaNO\textsubscript{3}, or Na\textsubscript{2}SO\textsubscript{4}. 
produce molecular chlorine and free hypohalite ion (Figure 2). This behavior was evidenced by two phenomena observed in this work: (1) when CPO was incubated with chloride ion (in phosphate buffer adjusted to acidic pH using 1 mol L⁻¹ HCl) and hydrogen peroxide in the absence of chlorine-acceptor substrate (azo dyes are not suitable chlorine-acceptor substrates for CPO), the formation of a transient typical odor of chlorine water or bleach could be detected; (2) when the buffer was adjusted to acidic pH using H₂SO₄ or HNO₃ instead of HCl, no decolorization of Congo Red/Alizarin Yellow R was observed. These results are consistent with the previously reported observation that hypohalite ion produced by CPO is an exceptionally good oxidant compared with hydrogen peroxide.²³⁻²⁵

Furthermore, when HClO was applied to the dye solution directly instead of being produced by this CPO-mediated system, the decolorization efficiency was much lower than that of the enzymatic method. This was possibly due to the fact that the Cl₂ or HClO freshly generated by the CPO–H₂O₂ system through the Compound I hypohalite complex EOCI has very high oxidative activity. On the other hand, this result indicated that although these oxidative species (Cl₂/HClO) were formed in the active site of CPO, the oxidation of the azo dye was in fact carried out in the bulk solution (outside the enzyme active site) because the azo dye molecules were too large to access the active site through a substrate tunnel in CPO, which allows only substrates with straight chains containing fewer than nine carbons to go through. Therefore, this method should be very beneficial for oxidative decolorization of azo dyes because there is no restriction on the size of the dye molecule, which should ensure that it will have potential industrial applications.  

3.2. Factors Affecting CPO-Mediated Decolorization.

Since various physicochemical parameters directly influenced the decolorization performance, optimization of these factors was very essential in order to make the process more efficient, faster, and practically applicable.

3.2.1. Effect of Buffer pH. The pH of the medium had a major effect on the efficiency of dye decolorization. The dependence of the decolorization efficiency on pH was found to be bell-shaped. The decolorization efficiency was highest at the optimum pH, and decreased gradually at more acidic or alkaline pH. The optimum pH values for both dyes were acidic: 2.5–3.5 for Congo Red and 3.0–3.5 for Alizarin Yellow R (Figure 3A). The acidic pH required in this work is due to the environment around the CPO heme. The crystal structure of CPO revealed the heme pocket in CPO to be polar, in which the acid–base catalytic group is Glu183. Glu183 in CPO is the only polar or charged group to serve a direct catalytic role. This could explain some of the solution properties of CPO concerning the pH. While ligands bind to the heme iron as anions, CPO accepts weakly acidic ligands (pKₐ > 3) into the active site in the neutral protonated form. Strong acids (pKₐ < 0) are in anionic form at low pH. Because the negatively charged carboxylate on Glu183 would be expected to repel the entry of anionic ligands as a result of unfavorable electrostatic interactions, strong acids (here HCl) could enter the active site in their anionic form only at low pH, where Glu183 is protonated.²⁶

3.2.2. Effect of Temperature. The azo compound decolorization efficiency of CPO-mediated oxidation, like other enzymatic reactions, was dependent on the temperature. In this work, the decolorization reaction was carried out at 20, 30, 40, 50, and 60 °C. According to the results illustrated in Figure 3B, the decolorization efficiencies for Congo Red and Alizarin Yellow R remained above 80% at 20–50 °C and were not smaller than 77% even at 60 °C. In fact, though CPO is not as stable at 60 °C as it is at 4 °C (the storage temperature, at which CPO can stand stably for around 2–3 years), it lost its activity completely in 100 min at 60 °C, as illustrated in Figure S1 in the Supporting Information. Therefore, the above results indicate that the decolorization reactions reported here are so rapid and efficient that they are almost independent of the thermal stability of CPO. For example, at 60 °C about 78% of the activity remained after 5 min (the time needed for decolorization in this work), which can ensure the accomplishment of the decolorization reaction. This property would also ensure the potential practical application of this versatile enzyme from marine fungus.

3.2.3. Effect of H₂O₂ Concentration. It is well-known that high concentrations of H₂O₂ are not favorable for heme
proteins such as cytochrome P450, horseradish peroxidase, and CPO because of inactivation, which is mainly caused by internal oxidative destruction of the porphyrin moiety.\textsuperscript{27}−\textsuperscript{29} On the other hand, CPO can also act as a catalase that catalyzes the decomposition of hydrogen peroxide. Therefore, less $\text{H}_2\text{O}_2$ could not generate enough Compound I. According to the results shown in Figure 3C, an $\text{H}_2\text{O}_2$ concentration of 0.5 mmol L\textsuperscript{−1} was found to be optimal for both Congo Red and Alizarin Yellow R decolorization.

3.2.4. Effect of the Amount of CPO. The amount of enzyme needed for decolorization was investigated in the range 0−0.6 $\mu$mol L\textsuperscript{−1} (Figure 3D). The decolorization efficiency at first increased linearly with increasing amount of CPO, but thereafter the increase slowed and the efficiency reached a plateau even when the CPO concentration was doubled. To ensure both the completion of decolorization reaction and less consumption of enzyme, CPO concentrations of 0.025 and 0.03 $\mu$mol L\textsuperscript{−1} for Congo Red and Alizarin Yellow R, respectively, were used in all experiments, indicating that a very small amount of enzyme (<ppm level) is required. This is also an outstanding feature for its potential practical applications.

3.2.5. Effect of Dye Concentration. For application purposes, a higher initial concentration of dye would be favorable. Figure 3E shows that when the concentrations of Congo Red and Alizarin Yellow R were as high as 0.2 and 0.4 mol L\textsuperscript{−1} respectively, the decolorization efficiency still remained higher than 80%. This result was satisfactory compared with other reports of enzymatic decolorization.\textsuperscript{30,31}

Figure 5. ESI ion trap mass spectra (positive-ion mode) of the fragmentations in the Congo Red degradation process with (A1, A2) dichloromethane and (B1, B2) ethyl acetate as the extraction solvent.
Under all of the above optimum reaction conditions, the maximum decolorization efficiencies reached 88.25% in 3 min for Congo Red and 92.38% in 5 min for Alizarin Yellow R (Figure 4), indicating that the CPO-mediated system is very efficient for decolorization of aqueous azo dyes. The recorded UV−vis spectra showed the disappearance of the sharp peaks at $\lambda_{\text{max}}$ demonstrating that the main chromophores in the dye molecules were destroyed by this enzymatic oxidative process. A control experiment in the absence of CPO showed no indication of dye degradation under the same conditions.

3.3. HPLC−MS Analysis of the Degradation Products.
A detailed characterization of the intermediates and final stable products produced during biodegradation was helpful in disclosing the possible pathways of dye decolorization. In this work, HPLC−MS (ESI) was employed to identify the molecular weights and structural information of the dye metabolites. Each sample was collected after the decolorization efficiency reached the maximum (after 30 min of reaction time) and was extracted with two solvents (dichloromethane and ethyl acetate) at a 1:1 ratio of organic solvent to sample solution. The sample was then dissolved in 750 $\mu$L of methanol for HPLC−MS analysis. MS analysis in positive-ion mode was used in the determination process. Nine products were identified (taking as an example Congo Red, which has a more complicated diazo structure), as shown in Figure 5. The fragmentation products detected in the Congo Red degradation process are summarized in Table S1 in the Supporting Information.

Two types of cleavage during the degradation process were observed. The first type was asymmetrical cleavage beside the two azo bonds [(2) in Figure 6]. Congo Red was first decomposed into 4-amino-3-naphthol-1-sulfonate (M11) and 6-[4-(4-azophenyl)phenylazo]-4-aminonaphthalene-1-sulfonate sodium salt (M2). [M2 + Na+] corresponding to the peak at $m/z$ 475.31 in Figure 5B2. Next, asymmetrical cleavage in M2 occurred in two ways. One involved cleavage beside the azo bond and yielded two main products: 1-azobiphenolate (M21) and 3-azo-4-aminonaphthalene-1-sulfonate (M13). [M21 + H] corresponds to the peak at $m/z$ 196.95 in Figure 5A1. M13 gave off N$_2$ and turned finally into M11. The other cleavage was between the two benzene rings and produced p-hydroxyphenyl diazene (M22) and 6-phenylazo-4-aminonaphthalene-1-sulfonate (M1). M22 was not detected. It possibly gave off N$_2$ and turned into a stable species. Meanwhile, M1 would lose the azo bond and sulfonate group, turning into p-nitrophenol (M17) finally.

The other type of cleavage was symmetrical cleavage between the two benzene rings in Congo Red [(1) in Figure 6].
degradation products of Congo Red by this pathway were two molecules of M4. [M4 + Na] corresponds to the peak at m/z 349.17 in Figure SA2. M4 could further degrade in two ways into a series species. One way would generate M11 and phenyl diazene (M12). [M11 + Na] corresponds to the peak at m/z 261.13 in Figure SA2, and [M11 + H] gives rise to the peak at m/z 239.08 in Figure SB1. [M11 + H + Na] corresponds to the peak at m/z 129.03 in Figure SB1. M11 possibly decomposed to an unstable intermediate, 1-nitroso-6-sulfonatophthalate (M13), with [M13 + Na] at m/z 294.93 in Figure SA1. M13 would then lose the sulfonate group to give 1-nitrosophthalate (M16), with [M16 + Na] at m/z 140.95 in Figure SA2 and [M16 + K] at m/z =177.03 in Figure SB2. The other degradation pathway of M1 possibly produced M13 and phenol (M14) (not detected). [M13 + 2Na] was observed at m/z 294.93 in Figure SA1, and it would give off N2 and turn first into M11, and then into stable M17 finally.

Compared with the work reported by others,32-34 this work has two advantages: (1) The cleavage of the azo bond is asymmetrical, in which N2 is given off, and no toxic aniline compounds were found in the final stable products. (2) The detected products were smaller fragments than that reported in the other studies. These small fragments are more susceptible to biodegradation and complete removal than the dye molecules themselves. Therefore, this method can be taken as a very efficient "pre-stage" treatment of the whole degradation process.

3.4. Application of CPO-Mediated Degradation in High-Salinity Wastewater Treatment. In textile dyeing, various salts are utilized to separate organic contaminants (brine rinse), help precipitation of dyestuffs (salting out), maintain a fixed pH, and so on. Therefore, large amounts of salts are copresent with the azo dyes in many textile effluents.35 However, high salt concentrations would inhibit the catalytic activity of enzymes, affecting the decolorization of the azo dyes. Dilution of effluents might alleviate salt stresses, but this would result in larger volumes of wastewaters and thus an increased treatment burden.

To investigate the efficiency of the CPO-mediated decolorization of azo dyes in the presence of typical salt species in industrial effluents (NaCl, NaNO3, and Na2SO4), an imitative high-salinity wastewater was prepared. The results in Figure 3F indicate that CPO tolerated Na2SO4 was much better than NaCl and NaNO3. The decolorization efficiency remained higher than 80% for Alizarin Yellow R and 65% for Congo Red even when the concentration of Na2SO4 was more than 1 mol L−1. In the case of NaCl, though the decoloration was a bit weaker, the decolorization efficiency still remained at 50% for Alizarin Yellow R and 70% for Congo Red in the presence of 0.5 mol L−1 NaCl. The tolerance of NaNO3 was a little disappointing. The decolorization efficiency dropped sharply below 10% both for Alizarin Yellow R and Congo Red when the NaNO3 concentration was 0.25 mol L−1. However, the investigated salt concentrations all were much higher than those in textile effluents generally, which demonstrates that this enzymatic decolorization of azo dyes could be applied to industrial dye wastewater treatment.

4. CONCLUSION

Chloroperoxidase isolated from the fungus Caldariomyces fumago can mediate a very efficient oxidative decolorization/degredation of aqueous azo dyes (Alizarin Yellow R and Congo Red) under mild conditions with a very small dosage of enzyme.

The substrate of the CPO + H2O2 reaction is Cl− instead of the dye molecule. The enzymatic reaction continually produces HClO and Cl2 with high oxidative activity, which are the oxidants for azo dye decolorization in the bulk solution. This would get rid of the restriction on the size of substrate imposed by the channel that provides access to enzymatic active site.

Nine main products of Congo Red degradation were detected by HPLC−MS analysis. Accordingly, two types of cleavage are proposed: (i) a symmetrical splitting between the two benzene rings and (ii) an asymmetrical cleavage on the either side of the azo linkage. N2 is produced in these pathways, and no toxic aniline compounds were found in the final stable products.

The strong tolerance of typical salt species in industrial effluents (NaCl, NaNO3, and Na2SO4) by this CPO-mediated decolorization of azo dyes ensures that it has potential applications in the treatment of industrial wastewater.

■ ASSOCIATED CONTENT

5 Supporting Information

Remaining activity of CPO as a function of incubation time at 60 °C (Figure S1) and fragmentation products detected in the Congo Red degradation process (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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