N-hydroxyphthalimide and its derivatives for laccase-catalyzed oxidative degradation of Indigo Carmine

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A comparative study of N-hydroxyphthalimide and its derivatives as redox mediators of Indigo Carmine oxidative degradation catalyzed by laccase Trametes versicolor was conducted. The experimental results show that the efficiency of mediators is increased in the order: N, N-dihydroxypyromellitimide < 4-Cl-N-hydroxyphthalimide < N-hydroxyphthalimide < 4-CH_3O-N-hydroxyphthalimide.

Decolorization, Indigo Carmine, *N*-hydroxyphthalimide, laccase *Trametes versicolor*, mediator, oxidation, radical

Introduction

Wastewater treatment from anthropogenic pollution using biocatalysts is a promising "green" technology to ensure global water security in the future. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) have a huge potential for the complex degradation of ecopollutants in wastewater and oxidize a wide range of substrates using molecular oxygen as the final electron acceptor [1]. We chose *Trametes versicolor (T. versicolor*) laccase for research, which is an efficient biocatalyst with a high redox potential (E°) (T1 Cu E° ~ 800 V) [2] and broad substrate specificity. In recent years, a lot of information has appeared on the commercial use of laccase for various analytical, industrial and environmental purposes. *T. versicolor* laccase is one of the most common tools in modern biotechnology for wastewater treatment, biofuel production, delignification and bleaching of cellulose, the transformation of lignin degradation products [3].

Mediators are used for increasing the efficiency of the catalytic action of the enzyme in the oxidation of organic compounds with a high redox potential. They act as intermediate substrates for laccase, significantly increasing the rate of the oxidation process, and expanding the range of compounds that can be oxidized by the enzyme [4]. The most effective mediators of laccase-catalyzed oxidation are compounds with a NOH group, such as hydroxylamines [5], oximes [6], which are easily oxidized *in situ* by laccases to reactive >N-O$^{\bullet}$ radicals. Searching for new effective mediators is a difficult task, since certain requirements are imposed on them.

N-hydroxyphthalimide (NHPI) and its derivatives are well-known organic catalysts for aerobic oxidation of a wide range of organic molecules [7]. They selectively functionalize C(sp^3)-H bonds *via* a radical chain process involving the active intermediate phthalimide-*N*-oxyl radical (PINO), which is easily formed during photo-, enzyme-, electro-oxidation of NHPI. It is known that the structure of NHPI has a significant effect on the efficiency of catalysis [8,9]. Previously, unsubstituted NHPI was used by us in the oxidation of the synthetic dye Indigo Carmine (IC) [5]. 4-Cl-NHPI and 4-CH_3O-NHPI with electron-withdrawing and electron-donating substituents in the benzene ring, as well as *N*, *N*-dihydroxypyromellitimide (NDHPI) with two NOH-functional groups were tested as mediators in a model oxidation reaction of IC. This dye is widely used in the textile industry, in biomedical research, as a food additive, indicator. IC has a carcinogenic, mutagenic effect, the presence in water makes it difficult for sunlight to pass through water, reduces the solubility of oxygen in water bodies, which leads to a violation of the photosynthetic activity of aquatic organisms.

Experimental

The mediator concentration effect on the IC decolorization by laccase *T. versicolor* was studied by the used of 20 μM dye at the presence of mediator such as NHPI, 4-Cl-NHPI, 4-CH_3O-NHPI or NNDHPI in the range of concentrations 60 ÷ 840 μM by step 30 μM with 1.0 U mg^{-1} laccase in the 0.1 M citrate–phosphate buffer at pH 4.5 and 35 °C in 3.0-mL quartz cuvettes. The reaction mixture included 0.1 mL of mediator in CH_3CN, 0.6 mL of laccase in the citrate–phosphate buffer, 0.1 mL of IC and 2.2 mL of the citrate–phosphate buffer. The IC decolorization was monitored at $\lambda_{max} = 614$ nm using UV-Vis spectrophotometer Specord 50 (Analytic Jena AG, Germany). All reactions were performed in triplicate until complete decolorization of the solution. Control samples were run in parallel without laccase. Doubly distilled water was used to prepare solutions.

Results and discussion

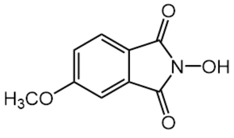
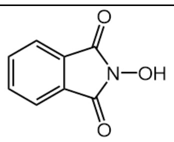
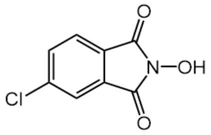
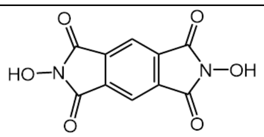
Screening of mediators was performed with various combinations of enzyme-mediator-substrate. To optimize the processes, the influence of temperature, pH, concentration of the components of the catalytic system, and incubation time were studied. Under experimental conditions, IC is not directly oxidized by laccase, i.e., the dye is not a direct substrate of laccase, but in the presence of mediators, the decomposition process proceeds almost quantitatively until complete discoloration. The kinetics of IC oxidation in the presence of laccase and NHPIs of various structures was studied in detail, which is described by the classical Michaelis-Menten equation. The Lineweaver-Burk plot (or double reciprocal plot) was used to determine the kinetic parameters: effective Michaelis constants K_M and maximum rate V_{max}, the values of which are given in Table 1. It can be argued that all studied mediators are laccase substrates, but their effectiveness depends on the structure. IC oxidizes at the fastest rate in the presence of 4-CH_3O-NHPI ($V_{max} = 9.2 \cdot 10^{-9}$ M s^{-1}). Based on the data in Table 1 there is an antibatic dependence of the maximum rate of IC oxidation on the redox potential of mediators and the decay rate constant of the corresponding aminoxyl radicals, which are the active form of mediators.

The data presented in the Table 1 can be explained considering the known mechanism of laccase-mediator oxidation of substrates. NHPIs under the action of laccase *in situ* form active PINO radicals, which further oxidize organic compounds in a non-enzymatic process (Fig. 1).

As a result of two successive catalytic cycles occurs of mediated proton-conjugated electron transfer by the mediator from the substrate to oxygen. The reduction of the mediator radicals to the molecular form proceeds by the reaction of a hydrogen atom abstraction (HAT) from the substrate molecule. The rate of electron transfer by the mediator from the substrate to copper in the active center of laccase is usually considered as a stage that determines the overall rate of the process and depends on the difference in the electrochemical potential of type T1 copper in the active center of laccase (for *T. versicolor* T1 Cu E° ~ 800 mV [2]) and the redox potential of the mediator.

Table 1

Kinetic parameters of the IC oxidation process in the presence of NHPIs, redox potentials of mediators, and decay constants of the corresponding radicals

Mediator	$K_M \cdot 10^4$, M	$V_{max} \cdot 10^9$, M s^{-1}	$E_{1/2}$, (V vs NHE) [10]	k_d 10^4 (s^{-1}) [11]
 4-CH_3O-NHPI	5.6±0.2	9.2±0.4	0.91	2.7
 NHPI	2.8±0.2	2.1±0.2	0.94	5.0
 4-Cl-NHPI	1.35±0.08	1.4±0.2	0.95	12.0
 NDHPI	9.2±0.5	0.73±0.02	–	33.0

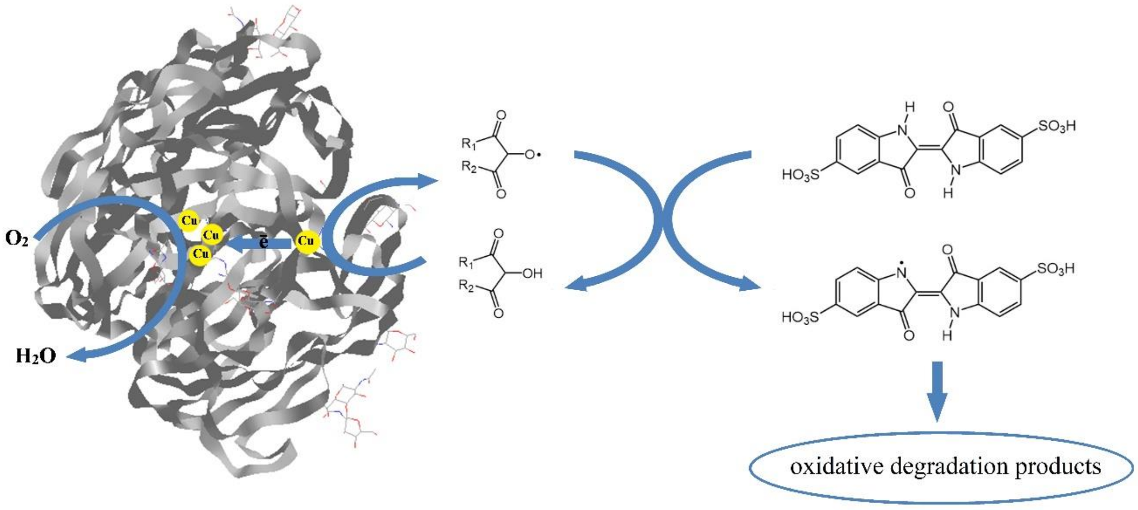


Fig.1 Mechanism of laccase-mediated oxidation of IC.

The 4-CH_3O-NHPI mediator has the lowest redox potential (0.91 mV); therefore, it is easily oxidized by the laccase to the corresponding aminoxyl radical. The second reason for the high activity of the mediator is the stability of 4-CH_3O-PINO radicals (k_d = $2.7 \cdot 10^{-4}$ s^{-1}), which contributes to an increase in their quasi-stationary concentration in the system through the turnover of the 4-CH_3O-NHPI/4-CH_3O-PINO redox-pair and an increase in the number of catalytic cycles. At the same time, 4-CH_3O-PINO exhibits low reactivity in HAT reactions compared to unsubstituted PINO and 4-Cl-PINO [9], as well as NDHPI, but this fact is not decisive for the action of 4-CH_3O-NHPI as a mediator. (see Table 1). This observation, as well as the zero order with respect to the substrate, indicate that it is the enzymatic cycle that is the rate-limiting step in the oxidation process (Fig. 1, A), and not the chemical reaction (Fig. 1, B). In addition, the high K_m value and low mediator activity of NDHPI are due to the instability of the corresponding radical ($k_d = 33 \cdot 10^{-4}$ s^{-1}) and steric hindrances in the enzyme pocket during interaction of mediator with the laccase active site.

Conclusions

The introduction of an electron-donating substituent into the aromatic ring of NHPI has a beneficial effect on the process of IC laccase-catalyzed decolorization. This result can be explained by a complex of various factors, but the main contribution to the overall rate of the process is provided by the redox potential of the mediator and the stability of its radical.

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