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ARTICLE

Peroxidase Inactivation by Covalent Modification with Phenoxyl Radical during Phenol Oxidation

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Abstract The mechanism-based inactivation of peroxidases limits the usefulness of these versatile enzymes. In this study, we propose a dominant inactivation mechanism for peroxidase during phenol oxidation. Two peroxidases, *Coprinus cinereus* peroxidase (CiP) and horseradish peroxidase isozyme C (HRPC) showed much higher inactivation rates after simultaneous addition of phenol and hydrogen peroxide, whereas addition of hydrogen peroxide alone or polymeric products had relatively little impact on peroxidase activity. During the oxidation of a phenol substrate, the molecular weights of polypeptides originating from inactivated peroxidases were slightly increased, and a large fraction of CiP and HRPC hemes remained intact even after phenoxyl radical coupling. Our study strongly supports the hypothesis that the inactivation of a peroxidase during the oxidation of phenolic compounds occurs by a covalent modification of the peroxidase polypeptide chain with a phenoxyl radical. These findings will elucidate the method of inactivation for peroxidase and other heme proteins.

Keywords *Coprinus cinereus* peroxidase · covalent bonding · horseradish peroxidase isozyme C · inactivation · phenoxyl radical · peroxidase

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Introduction

Peroxidases are heme enzymes that contain a ferric protoporphyrin IX prosthetic group and use hydrogen peroxide and other peroxides to catalyze the oxidation of a large number of aromatic compounds (Dunford, 1999). The catalytic cycle of peroxidase occurs in three distinct steps. Peroxidase is oxidized by hydrogen peroxide and is converted to Compound I. Compound I oxidizes aromatic substrate into a free radical, whereas Compound I is reduced to Compound II. Compound II is further reduced by another aromatic substrate, returning to its native form of peroxidase and producing another free radical (Yu et al., 1994).

Peroxidases have attracted industrial attention because of their usefulness as catalysts in the pulp, paper, textile and laundry industries and for their use as biosensors and in other applications (Vyas and Molitoris, 1995; Harazono et al., 1996). The ability of peroxidases to catalyze free radical formation from a variety of aromatic compounds, followed by spontaneous polymerization, can be used in the bio-remediation of water and soil containing phenolic compounds (Klibanov et al., 1983; Bollag, 1992) and in the synthesis of various aromatic polymers (Won et al., 2004; Hollmann and Arends, 2012). Despite their potential applications, the commercial uses of peroxidases are limited by their rapid inactivation during reactions (Aitken, 1993).

Three mechanisms of horseradish peroxidase (HRP) inactivation have been proposed. The first is associated with excess peroxide. Active intermediate peroxidase compounds react with excess peroxide, which results in the formation of inactive species (Nakajima and Yamazaki, 1987; Arnao et al., 1990). The second involves absorption by polymeric products. Peroxidase is absorbed on a polymeric product, and its active sites are occluded (Nakamoto and Machida, 1992). In the third mechanism, free phenoxyl radicals generated by the oxidation reaction attack peroxidase, leading to its inactivation (Klibanov et al., 1983). The severity of inactivation from each mechanism depends on the reaction conditions.

It is necessary to understand the factors involved in the inactivation process and to describe the molecular mechanism underlying peroxidase inactivation. The overall objective of the present study was to evaluate the dominant mechanism of peroxidase inactivation during the oxidation of phenol. Using two types of peroxidases, CiP (*Coprinus cinereus* peroxidase) and horseradish peroxidase isozyme C (HRPC), we investigated the effects of hydrogen peroxide and polymeric products on peroxidase activity. Modifications that were made to the peroxidases and the proportion of intact hemes were measured using electrophoresis and high-performance liquid chromatography (HPLC). This work provides a basis for understanding the molecular mechanism of peroxidase inactivation and for the rational protein engineering of peroxidases to improve their stability in the phenol oxidation process.

Materials and Methods

Chemicals and reagents. Phenol (99%), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (98%, in diammonium salt form), formic acid (96%), hemin (bovine, 90%) and Pronase (from *Streptomyces griseus*) were purchased from Sigma-Aldrich (USA). Hydrogen peroxide (H_2O_2, 30%, v/v) was obtained from Junsei Chemical Co., Ltd. (Japan). HPLC-grade water, acetonitrile, and formic acid were supplied by Honeywell Burdick & Jackson (USA). A Bradford protein assay kit was purchased from Bio-Rad (USA).

Enzymes. HRPC (P8415) was purchased from Sigma-Aldrich and used without any further purification. The heterologous expression and purification of *Coprinus cinereus* peroxidase (CiP) was performed as previously described (Kim et al., 2010). Briefly, the synthetic CiP gene was expressed in the supernatants of *Pichia pastoris* cell cultures using a pPICZαA vector-based expression system (Invitrogen, USA). CiP was purified with a size exclusion column (Superose^{TM} 6 10/300 GL, GE Healthcare, USA) using an fast protein liquid chromatography system (GE Healthcare).

Peroxidase stability. To investigate the effect of substrates and reaction products on peroxidase activity, CiP and HRPC were treated with hydrogen peroxide, polymer products and phenol. CiP and HRPC were diluted in 100 mM phosphate buffer, pH 7.0, to yield a concentration of 1 U/mL, and enzyme solutions were then treated by the addition of the following solutions: 0.5 mM hydrogen peroxide, phenol polymer or 0.5 mM hydrogen peroxide plus 0.5 mM phenol. Each reaction mixture was incubated and stirred for 20 min at room temperature. Samples were taken every 5 min after initiating the reaction. Peroxidase activity was assayed by mixing enzyme samples with 2 mL of ABTS-H_2O_2 (0.18 mM ABTS and 2.2 mM H_2O_2, pH 5.0), and changes in absorbance at 420 nm (molar extinction coefficient of ABTS, 34,700 M^{-1} cm^{-1}) were determined using a UV-Vis spectrometer (Shimadzu, Japan) at room temperature. One unit of activity (U) is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol ABTS for 1 minute at 25°C.

The phenol polymer used to estimate peroxidase stability was obtained using CiP and HRPC catalysis. The reaction solution was a mixture of 100 mM potassium phosphate buffer at pH 7.0, 10 mM phenol, and 20 U/mL of CiP or HRPC. Each reaction was initiated by adding 10 mM hydrogen peroxide to the reaction solution. After the reaction was complete, the precipitates were collected by centrifugation for 15 min at 13,000 rpm. The collected materials were washed twice with deionized distilled water to remove residual peroxidases and then dried in an oven at 100°C for 1 day. The precipitate was added to the reaction mixture to give a final concentration of 0.1 mg/mL.

Peroxidase-catalyzed reactions. The phenol oxidation reaction was initiated by adding 0.5 mM hydrogen peroxide to reaction mixture containing 100 mM phosphate buffer (pH 7.0) and 10 U/mL of CiP or HRPC. The control reaction was performed without hydrogen peroxide. After stirring vigorously at room temperature for 1 h, the precipitates were eliminated by centrifugation at 13,000 rpm for 20 min. The reaction supernatants were desalted through a HiTrap desalting column (GE Healthcare) to remove the remaining hydrogen peroxide and phenol. Protein samples were concentrated with an Amicon concentrator (Millipore, USA) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC.

SDS-PAGE. Concentrated samples from CiP- and HRPC-mediated oxidation reactions were subjected to SDS-PAGE using a 4% stacking gel and a 15% running gel. A total of 10 μg of each protein was loaded, and protein bands were visualized by Coomassie Brilliant Blue staining.

HPLC analysis. CiP and HRPC samples that were separated from the phenol oxidation reactions were concentrated to approximately 1 mg/mL. Pronase (0.25 mg/mL) was added to the protein samples, which were incubated overnight at 37°C. Hemes were analyzed by HPLC using an OptimaPak C18, 4.6×250 mm column (RS tech Co., Korea). The heme was eluted with a linear gradient of 20–80% acetonitrile in water (0.1% formic acid) for over 50 min. The flow rate was 1 mL/min, and the elution was monitored at 400 nm. Under the HPLC gradient, the heme group was detected at a retention time of 44.8 min. The heme content was calculated according to the relative peak areas, and 100 μM hemin was used as a control.

Results and Discussion

Inactivation factors for peroxidase during the phenol oxidation reaction. There are three proposed pathways for peroxidase inactivation during phenol oxidation. The first is a reaction with excess hydrogen peroxide, the second is the absorption by polymeric products, and the third is a reaction with free phenoxyl radicals produced by the oxidation reactions (Klibanov et al., 1983; Nakajima and Yamazaki, 1987; Arnao et al., 1990; Nakamoto and Machida, 1992). To determine which

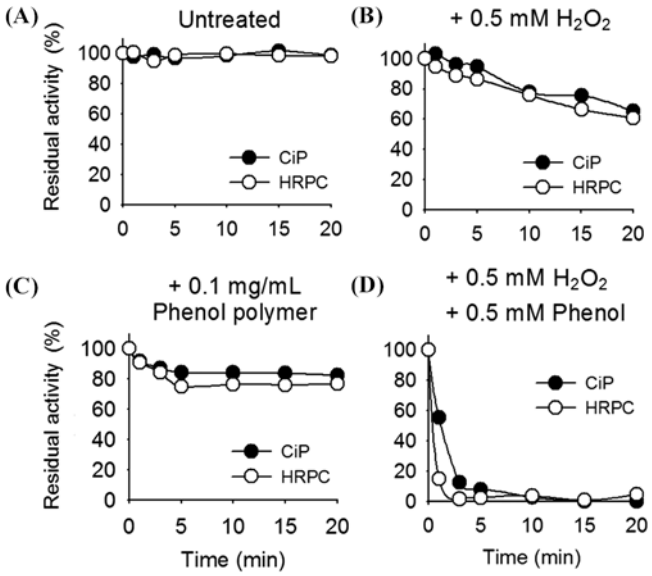
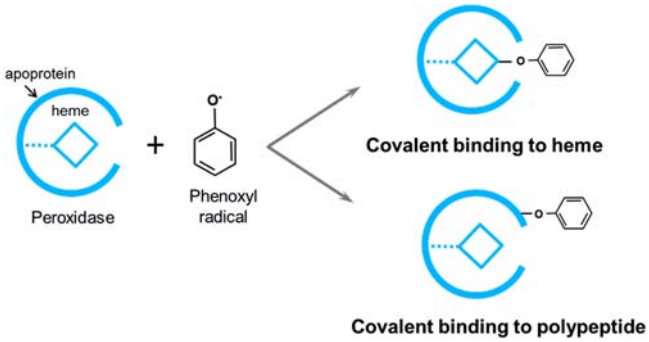


Fig. 1 Peroxidase inactivation in various reaction conditions. CiP and HRPC were incubated in phosphate buffer, pH 7.0 (A); 0.5 mM H_2O_2 (B); 0.1 mg/mL phenol polymer (C); or 0.5 mM H_2O_2 and 0.5 mM phenol (D).



Scheme 1 Inactivation of peroxidase by radical coupling during the phenol oxidation reaction.

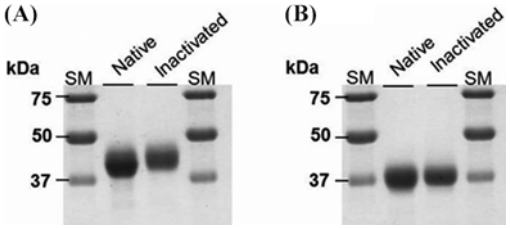


Fig. 2 Molecular weights of inactivated peroxidase polypeptides after phenol oxidation reactions. CiP (A) and HRPC (B) were incubated with 0.5 mM H_2O_2 and 0.5 mM phenol, and the molecular weight of each peroxidase was analyzed by 15% SDS-PAGE. SM; size marker.

factor is dominant during the oxidation of phenol, the inactivation of two types of peroxidases, CiP and HRPC, was observed under various conditions; CiP and HRPC yielded the same result under each reaction condition (Fig. 1). The activity of each peroxidase was decreased by approximately 20% due to H_2O_2-mediated inactivation (Fig. 1B). It is well-known that peroxidases are inactivated by hydrogen peroxide (Nakajima and Yamazaki, 1987; Arnao et al., 1990; Valderrama et al., 2002); however, at a low concentration of hydrogen peroxide (0.5 mM), the contribution of H_2O_2-mediated inactivation was small (Fig. 1B).

After the addition of a phenol polymer, the peroxidases maintained their initial level of activity (Fig. 1C). The inactivating effect of phenol polymer is not obvious unless a large amount (grams per liter) of precipitated polymer product is formed (Nakamoto and Machida, 1992; Huang et al., 2005). The amount of phenol polymers used in the present study was not sufficient to affect peroxidase activity, even though the added phenol polymers were formed with 20-fold higher concentrations of substrates and enzyme. This result indicated that the precipitated polymeric product barely inactivated the peroxidase at a low substrate concentration.

In contrast, in the presence of hydrogen peroxide and phenol, CiP and HRPC were inactivated to a dramatic extent within 5 min (Fig. 1D). The H_2O_2-mediated inactivation of peroxidase is largely suppressed by the addition of a reducing substrate, because it competes with hydrogen peroxide for Compound II (Arnao et al., 1990; Choi et al., 1999). However, phenol did not prevent CiP and HRPC inactivation by hydrogen peroxide, and the peroxidases were more rapidly inactivated by the addition of phenol. Thus, the main factor causing the inactivation of CiP and HRPC was the phenoxyl radical attacking the peroxidase.

The modification of peroxidase polypeptide. Peroxidase can be inactivated by covalent bonding between the peroxidase and a phenoxyl radical (Chang et al., 1999; Huang et al., 2005; Kim et al., 2009). There are two possible outcomes if a radical attacks the peroxidase during the oxidation of phenol (Scheme 1): a critical amino acid residue in the polypeptide is modified or the heme is modified or destroyed via covalent bonding between the phenoxyl radical and the enzyme.

To determine if the polypeptide was modified by a phenoxyl radical, samples of CiP and HRPC that had been inactivated during the phenol oxidation reaction were analyzed by SDS-PAGE, CiP and HRPC were separated from the phenol oxidation reaction mixture and subjected to SDS-PAGE. Fig. 2 shows that the molecular weights of inactivated CiP and HRPC shifted slightly upward compared to those of the native controls, suggesting that the reacted CiP and HRPC polypeptides were modified by phenoxyl radicals. Covalent modifications by a phenolic substrate at the heme edge of HRPC have been identified by Gilfoyle et al. (996). However, the molecular weight changes in CiP and HRPC confirmed by SDS-PAGE were not due to heme modification, because the heme moiety of peroxidase would have been released from the polypeptide during denaturation by sodium dodecyl sulfate. Therefore, the phenoxyl radical was covalently bound to the polypeptide rather than the peroxidase heme moiety.

Table 1 The proportions of intact hemes from CiP and HRPC following phenol oxidation by peroxidase

Peroxidase	Proportion of intact heme (%)		
	Native	H_2O_2 -incubated	Phenoxyl radical-inactivated
CiP	100	86.6	74.6
HRPC	100	72.9	48.3

Heme destruction of peroxidase. To further investigate heme destruction by phenoxyl radical attack, intact hemes from peroxidases were quantified following the peroxidase-catalyzed oxidation of phenol. The proportions of intact heme groups from native, H_2O_2-incubated, and phenoxyl radical-inactivated CiP and HRPC are shown in Table 1. Following treatment with hydrogen peroxide alone, the amount of intact hemes from CiP and HRPC decreased to 87 and 73%, respectively, of those found in native peroxidases. This result is in accord with the stability of peroxidase after treatment with hydrogen peroxide (Fig. 1B). When CiP and HRPC were treated with hydrogen peroxide and phenol, the amount of intact hemes decreased to 75 and 48%, respectively. Although CiP and HRPC were completely inactivated (Fig. 1D), CiP and HRPC maintained a high proportion of intact hemes in the presence of 0.5 mM phenol and hydrogen peroxide (Table 1). The activity loss is substantially greater than that caused by heme destruction during the phenol oxidation reaction. This result suggests that the phenoxyl radicals are associated with the loss of intact heme groups; however, the loss of catalytic activity is primarily caused by a modification of the peroxidase polypeptide rather than heme destruction by radical attack.

In the present study, we showed that two types of peroxidases, CiP and HRPC, catalyzed phenol oxidation and were subject to enzyme inactivation. After incubation with hydrogen peroxide and phenol, CiP and HRPC had slightly higher molecular weights. Although heme destruction occurred, more of enzyme catalytic activity was lost than could be accounted for by heme destruction alone. From these results, we suggest that phenoxyl radicals produced by peroxidase-mediated phenol oxidation were covalently bound to the peroxidase polypeptides and that these covalent attachments were the dominant source of enzyme inactivation (Scheme 1).

During the oxidation of phenolic compounds, peroxidase inactivation can occur as a result of phenoxyl radicals interacting with hydrogen peroxide and a phenolic substrate (Klibanov et al., 1983; Nakamoto and Machida, 1992; Baynton et al., 1994; Huang et al., 2005). HRPC can be inactivated through heme destruction by radical attack. However, neither a direct interaction between a phenoxyl radical and heme nor a phenol-modified heme from a phenolic substrate-reacted HRPC or CiP could be detected by Raman spectrometry (Gilfoyle et al., 1996 Huang et al., 2005; Mao et al., 2013). Although there was a modified heme compound (20-phenyl heme) in phenylhydrazine-treated HRPC, the modified heme constituted less than 10% of the total heme (Gilfoyle et al., 1996).

The substrate radicals generated by oxidation reactions also covalently bind to peptide residues in peroxidases (Chang et al., 1999; Cohen-Yaniv and Dosoretz, 2009; Kim et al., 2009; Chang et al., 2011). The polypeptides in 4-chloroanillin-inactivated HRP, CiP, and lactoperoxidase (LPO) are covalently modified by 4-chloroanillin derivatives (Chang et al., 1999). In addition, a covalent link is generated between the HRP polypeptide and a bromophenol intermediate generated during the oxidation reaction of bromophenol (Cohen-Yaniv and Dosoretz, 2009), and LPO is inactivated by the irreversible covalent binding of genistein to a particular peptide fragment (Chang et al., 2011).

While it is known that peroxidase polypeptides are covalently modified by phenoxyl radicals, the sites of covalent attachment and the molecular inactivation mechanism remain to be elucidated. Further study is required to describe the molecular mechanisms of peroxidase inactivation by phenoxyl radical coupling. Molecular modeling and mass spectrometry analyses will clarify these mechanisms.

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