

Degradation of the antifouling compound Irgarol 1051 by manganese
peroxidase from the white rot fungus *Phanerochaete chrysosporium*

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Abstract

Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine), a derivative of *s*-triazine herbicide, is an antifouling compound used as an alternative to organotin. The compound is highly persistent and is known to be biodegraded only by the white rot fungus, *Phanerochaete chrysosporium*. We used partially purified manganese peroxidase (MnP) prepared from *P. chrysosporium* to evaluate its capacity to degrade Irgarol 1051. MnP degraded Irgarol 1051 to two major products, one identified as M1 (identical to GS26575, 2-methylthio-4-*tert*-butylamino-6-amino-*s*-triazine) and the other not identified but with same mass spectrum as M1 and a different ultraviolet spectrum. This report clearly demonstrates that this ligninolytic enzyme is involved in the degradation of Irgarol 1051.

Keywords: Antifouling compound; Biodegradation; Irgarol 1051; Manganese peroxidase; *Phanerochaete chrysosporium*; White rot fungi

1. Introduction

New antifouling compounds are required to protect ship hulls and fishing nets from biofouling, because organotin compounds have become strictly regulated internationally. Currently, approximately 18 compounds are used as antifouling biocides worldwide, and the environmental fates and behaviors of the eight biocides in common use have been reviewed (Thomas 2001). Of these eight biocides, Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine), an analogue of *s*-triazine herbicides, has been used worldwide, and its residues have been detected in many coastal waters, lake waters, and sediments. Irgarol residues are frequently found in seawater along with the degradation product known as M1 (identical to GS26575, 2-methylthio-4-*tert*-butylamino-6-amino-*s*-triazine) because of their high persistence in aquatic systems (Ferrer and Barcelo 2001; Okamura et al., 2000; Thomas et al., 2000). Three mechanisms of Irgarol degradation have been described to date: biodegradation by the white rot fungus (Liu et al., 1997), mercuric ion-catalyzed hydrolysis (Liu et al., 1999), and photodegradation by sunlight (Okamura et al., 1999). The appearance of M1 as the degradation product of the parent compound occurs in all three pathways.

A previous study of the biodegradation of Irgarol using the white rot fungus *Phanerochaete chrysosporium* suggested that the ligninolytic enzymes of the fungus

were not involved but that constitutive enzymes possibly were (Liu et al. 1997). There currently exists great interest in investigating the lignin-degrading white rot fungi and their ligninolytic enzymes because of their recognized potential for degrading and detoxifying recalcitrant environmental pollutants, including dioxins (Bumpus et al., 1985), chlorophenols (Joshi and Gold, 1993), polycyclic aromatic hydrocarbons (Bezalel et al., 1996; Collins et al., 1996), and dyes (Ollikka et al., 1993). Manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase are extracellular enzymes that are secreted by white rot fungi and have been demonstrated to be involved in the degradation of lignin and these pollutants. The present study was undertaken to clarify whether MnP prepared from cultures of *P. chrysosporium* is capable of degrading Irgarol.

2. Materials and methods

2.1 Test chemicals

Irgarol 1051 (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) and M1 (identical to GS26575, 2-methylthio-4-*tert*-butylamino-6-amino-*s*-triazine) were gifts from Ciba-Specialty Chemicals, KK, Japan. Both compounds were dissolved in acetonitrile (HPLC grade, Kanto Chemicals) to a stock solution concentration of

10,000 mg/l. All other chemicals were extrapure grade and were obtained from commercial sources.

2.2 Preparation of manganese peroxidase (MnP)

Partially purified MnP was prepared from cultures of *P. chrysosporium* ME-446 as described in our previous report (Katagiri et al., 1997). Laccase and LiP activities were not detected in MnP preparations.

MnP activity was assayed by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 30°C at 470 nm. The reaction mixture contained 1 mM DMP, 0.1 mM MnSO₄, 0.2 mM hydrogen peroxide (H₂O₂), and 50 mM malonate buffer (pH 4.5). One kat of MnP activity was defined as the amount of enzyme required to produce 1 mol quinone dimer from DMP per second (Wariishi et al., 1992).

2.3 Treatment of Irgarol with MnP

The reaction mixture consisted of 20 µg/ml Irgarol, partially purified MnP (50 nkat/ml), 50 mM malonate buffer (pH 4.5), 50 µM MnSO₄, 25 mM glucose, and 3.33 nkat/ml glucose oxidase (Wako, Osaka, Japan) to supply H₂O₂. The reaction was performed at 30°C as it was stirred at 150 rpm in the dark. The MnP reaction mixture

(100 μ l) then was subjected directly to high performance liquid chromatography (HPLC). HPLC analytical conditions were a Wakosil-II 5C18HG column (4.6 mm \times 150 mm; Wako), a mobile phase of water (X) and acetonitrile (Y), elution by a linear gradient of 30% to 100% Y in 30 minutes, a flow rate of 1.0 ml/min, and detection at 250 nm.

The reaction mixture was subjected to a preparative HPLC to isolate the Irgarol reaction products derived from MnP catalysis. The preparative HPLC conditions were the same as the analytical conditions. Two fractions (A and B), each containing one major peak, were collected and evaporated to dryness *in vacuo*. The two fractions were dissolved in acetonitrile and analyzed by gas chromatography-mass spectrometry (GC-MS) and ultraviolet (UV) spectrometry (UV-2400PC; Simadzu) to identify the MnP reaction products. Mass spectral analysis was carried out with a QP 5050 mass spectrometer (Shimadzu, Japan) equipped with a TC-1 capillary column (0.25 μ m \times 0.25 mm \times 30 m; GL Science) under the following chromatographic conditions: an injector temperature of 230°C and a column temperature program of 80°C for 2 minutes, 80°C to 150°C at 5°C/min, 150°C to 250°C at 2°C/min, and 250°C to 300°C at 8°C/min. Helium at 14 psi was used as the carrier gas. The interface was kept at 270°C. The mass spectra were obtained by electron impact positive mode at 70 eV in full scan

mode.

3. Results and Discussion

MnP is a heme peroxidase produced by white rot fungi that catalyzes the oxidation of Mn(II) to Mn(III) in the presence of H₂O₂. Malonate, oxalate, and α -hydroxy acids such as malate, lactate, and tartrate chelate the generated Mn(III) and release Mn(III) from the manganese binding site of MnP. The released Mn(III)-organic acid complex in turn oxidizes various phenolic compounds, including lignin (Wariishi et al., 1992). Recently, we demonstrated that MnP effectively degrades polyethylene and nylon (Deguchi et al., 1998; Iiyoshi et al., 1998) and removes the estrogenic activities of bisphenol A (BPA) and nonylphenol (NP) (Tsutsumi et al., 2001). In this study, Irgarol was treated with MnP prepared from *P. chrysosporium* cultures.

The HPLC data demonstrate that the concentrations of Irgarol in the reaction mixture decreased by 16%, 26%, and 37% after 6-, 12-, and 24-hour MnP treatments, respectively (Fig. 1). Two chromatographic peaks (A and B) were simultaneously produced in the same reaction after 12 hours of incubation (Fig. 2), and these peaks were suspected to be Irgarol degradation products. Neither the degradation of Irgarol nor the formation of degradation products was observed when heat-denatured MnP

preparations were used. These results strongly support the hypothesis that MnP is involved in Irgarol biodegradation, although Liu et al. (1997) did not try to detect the ligninolytic enzymes such as MnP and LiP, but estimated that the enzymes were not involved in the degradation by *P. chrysosporium* under the high-nitrogen and high-carbon (HN-HC) culture conditions that these investigators had used. One of the important characteristics of *P. chrysosporium* metabolism is that its ligninolytic activity appears as a secondary metabolic event and nitrogen limitation permits extensive lignin degradation (Keyser et al., 1978; Kirk et al., 1978). In a previous report, we examined the relationship between the degradation of residual lignin in unbleached kraft pulp (UKP) and the activities of ligninolytic enzymes produced by *P. chrysosporium* in a solid-state fermentation system using different culture media (Katagiri et al. 1995). The production of ligninolytic enzymes, which are correlated with the degradation of residual lignin in UKP, was also observed under HN-HC conditions. Thus, it is probable that the ligninolytic enzymes produced during the fungal treatment under HN-HC conditions may have participated in the degradation of Irgarol reported by Liu et al. (1997).

One of the degradation products, peak A, was identified by a comparison of the GC-MS mass spectra and retention time with those of authentic compound. Fig. 3

demonstrates that peak A had a molecular ion at m/z 213 and with major fragment ions at m/z 198, 157, 111, 83, and 68. This molecular ion and the pattern of major fragmentations are completely coincident with those of M1 (Fig. 3), resulting from *N*-dealkylation at the Irgarol cyclopropylamino group. Furthermore, both the retention times in HPLC and the total ion chromatograms (TIC) of peak A and M1 were identical. This is the first report to describe the degradation and transformation of Irgarol to M1 by the ligninolytic enzyme MnP.

GC-MS analysis was also used in an attempt to identify another degradation product, peak B. The molecular ion, the pattern of major fragmentations, and the TIC of peak B were the same as those of M1 and peak A (Fig.3). However, the retention time in the HPLC analysis and the UV spectrum of peak B were not coincident with those of M1 (Figs. 2 and 4). Furthermore, mass spectra and retention time results for peak B did not coincide with those of Desmetryn (2-isopropylamino-4-methylamino-6-methylthio-1,3,5-triazine), which has a molecular ion at m/z 213. These observations demonstrate the chemical structural similarities of peak B and M1. The identification of the compound should be a subject of further studies.

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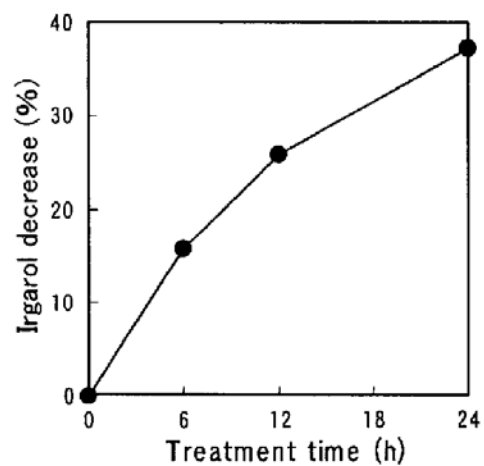


Fig. 1 Decrease in Irgarol concentration by manganese peroxidase treatment.

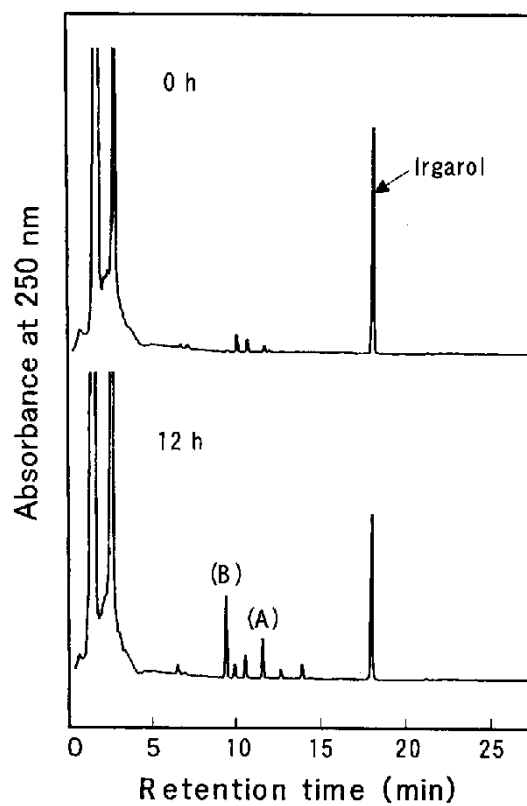


Fig. 2 HPLC analyses of Irgarol treated with manganese peroxidase.

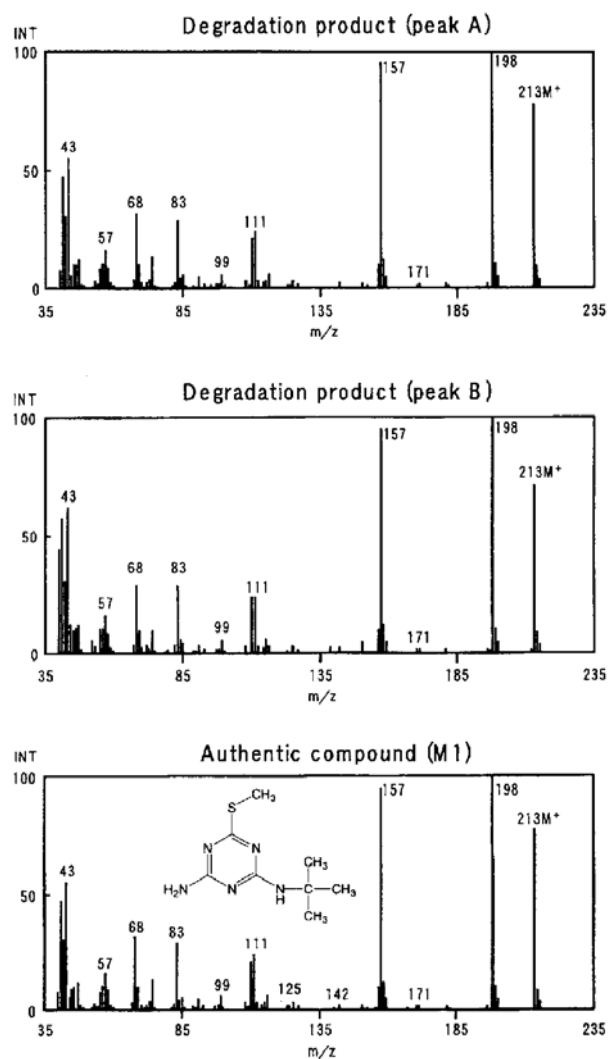


Fig. 3 Mass spectra of the Irgarol degradation products (peak A and B) and authentic compound (M1).

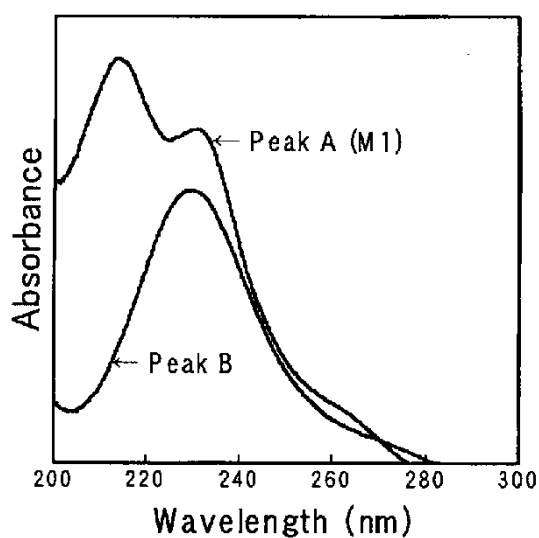


Fig. 4 UV spectra of Irgarol degradation products dissolved in acetonitrile.