Biotechnology and Bioprocess Engineering **2004**, 9: 256-260

Decolorization of Melanin by Lignin Peroxidase from *Phanerochaete chrysosporium*

Sung Hwan Woo, Jeung Suk Cho, Baek Seok Lee, and Eun Ki Kim*

Department of Biological Engineering, Inha University, Incheon 402-751, Korea

Abstract Melanin was decolorized by lignin peroxidase from *Phanerochaete chrysosporium*. This decolorization reaction showed a Michaelis-Mentens type relationship between the decolorization rate and concentration of two substrates: melanin and hydrogen peroxide. Kinetic constants of the decolorization reaction were 0.1 OD_{475}/min (V_{max}) and 99.7 mg/L (K_m) for melanin and 0.08 OD_{475}/min (V_{max}) and 504.9 μM (K_m) for hydrogen peroxide, respectively. Depletion of hydrogen peroxide interrupted the decolorization reaction, indicating the essential requirement of hydrogen peroxide. Pulsewise feeding of hydrogen peroxide continued the decolorizing reaction catalyzed by lignin peroxidase. These results indicate that enzymatic decolorization of melanin has applications in the development of new cosmetic whitening agents.

Keywords: *Phanerochaete chrysosporium*, melanin, decolorization, lignin peroxidase, hydrogen peroxide

INTRODUCTION

Melanin pigments determine the color of the skin, hair, and eye [1]. Melanin may have the biological function of protecting the underlying tissues from harmful ultraviolet radiation [2]. However, having a white face is a deep desire for many hyper-pigmented women, especially in Asia and Africa. Many skin whitening studies on decreasing melanin concentration have focused on UV-ray cut-off using sunscreen agents. Other studies have examined inhibition of tyrosinase activity using arbutin, kojic acid, glucosamine, galactosamine, manosamine, or tunicamycin. Other researchers have looked at activation of cell turn-over using vitamin A, or azealic acid [3]. Whitening by melanin synthesis inhibition, as described above, takes a long time to see satisfying results. In addition, toxic side effects of ingredients were observed.

The basic structural unit of melanin is represented by covalently linked indoles. Although the overall structure is not known, most melanin is believed to be a heterogeneous polymer composed mainly of dihydroxyindole units existing as a mixture of both the catechol and the quinone [1]. This structural feature is similar to lignin or coal, in which polymers are composed of indolic or phenolic subunits.

White rot fungi produce various extracellular oxidases and peroxidases. These enzymes oxidize a broad spectrum of structurally different substrates, including lignin or coal as well as highly toxic phenolic compound and azo dye [4-7]. Ralph and Catcheside showed that the white-rot fungus, *Phanerochaete chrysosporium*, causes decolorization and depolymerization of low-grade coal under culture conditions that facilitate mineralization of lignin [8]. These capabilities of lignin-degrading enzymes make it possible to decolorize melanin, which possesses structures similar to those of coal or lignin. Despite many research studies about these enzymes, there have been no reports about the enzymatic decolorization of melanin.

In this paper, we tried to decolorize melanin by lignin peroxidase produced by *Phanerochaete chrysosporium*. In addition, the effects of hydrogen peroxide and melanin concentration on decolorization rates were investigated to define optimum conditions for the decolorization of melanin by lignin peroxidase.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The white rot fungus *P. chrysosporium* ATCC 24725 was obtained from the Korea Type Culture Collection. This fungal strain was inoculated on YMPG agar plates (Difco Laboratories., Detroit, MI, USA) and incubated at 37℃ until extensive mycelial growth occurred. YMPG-agar plates contained the following components: 3 g/L yeast extract, 3 g/L malt extract, 5 g/L bacteriological-peptone, 20 g/L agar, and 2% glucose (w/w). They were maintained at 4°C with periodic transfer. The growth medium for lignin peroxidase production was prepared according to Tien and Kirk [9].

***Corresponding author**
Tel: +82-32-860-7514 Fax: +82-32-875-0827
e-mail: ekkim@inha.ac.kr

Preparation of Crude Enzyme Solution

Three mycelium blocks (4 mm diameter), excised from agar plates, were transferred to 250-mL Erlenmeyer flasks containing 100 mL of growth medium. The flasks were maintained in an orbital shaker at 200 rpm and 30°C for 6 days. The culture supernatant was obtained by centrifugation at 10,000 g for 5 min at 4°C. The yellow supernatant was concentrated by ultra filtration using a 10-kDa cut off membrane (Satorius, Germany). The concentrated crude enzymes were maintained at 4°C until use.

Lignin Peroxidase Activity

Lignin peroxidase activity was analyzed by spectrophotometer according to Tien and Kirk [9] with some modification, *i.e.* using ABYS(2,2-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt) instead of VA(veratryl alcohol) as a substrate. The assay reaction was carried out in test tubes at 30°C, and the reaction mixture contained 125 mM tartaric acid buffer (pH 2.5), 5 mM ABTS, 400 μM H_2O_2, and 0.1 mL of crude enzyme solution (containing lignin peroxidase) in a final volume of 1 mL. One unit was defined as the amount of enzyme required for the increase in 0.01 absorbance at 734 nm per min. The activities were reported as units/L.

Melanin Decolorization by Lignin Peroxidase

To assess melanin decolorization, *P. chrysosporium* ATCC 24725 was inoculated to growth medium agar plates supplemented with melanin (200 mg/L) and incubated at 30°C.

In a liquid assay, decolorization of melanin was measured as the decrease in the absorbance at 475 nm in 125 mM tartarate buffer solution at pH 2.5 containing 100 mg/L melanin, 772 units/L lignin peroxidase and 80 μM H_2O_2. Decolorization rate was expressed as the decrease of optical density at 475 nm per minute (-ΔOD/min). Kinetic constants of the decolorization reaction were determined by varying concentrations of melanin (10-100 mg/L) or H_2O_2 (0.8-80 μM) in the tartarate buffer (125 mM, pH 2.5) at 475 nm. All the data were the average of two independent measurements.

RESULTS AND DISCUSSION

Decolorization Activity of Fungi and Enzyme

It has been demonstrated that the decolorization (bleaching) of agar media containing colored compound such as coal or humic acids is a suitable and easy method for the rapid selection of fungi having bleaching activity [10]. Melanin is a dark-brown biopolymer. We tested the decolorization ability of *P. chrysosporium* on agar plates containing melanin. *P. chrysosporium* mycelium blocks were inoculated to agar plates containing melanin. Partial decolorization of the agar around growth areas was observed after 3 days. After 6 days, most agar plates were decolorized. This indicates that *P. chrysosporium* was able to decolorize melanin (Fig. 1). Some workers showed that this colored material could affect the inhibitory effect on microbial growth for various fungi as well as bacteria [11-13]. However, melanin did not affect the growth or decolorization of *P. chrysosporium* .

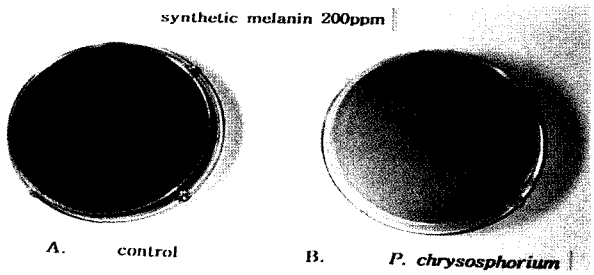

Fig. 1. Decolorization of synthetic melanin by *P. chrysosporium.*

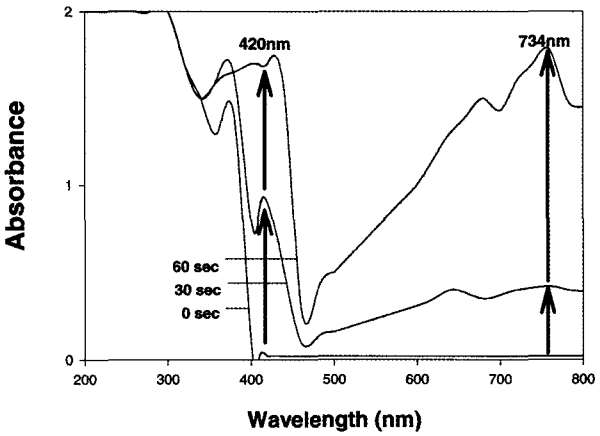

Fig. 2. Time course changes in absorption spectra after addition of H_2O_2 to lignin peroxidase-ABTS: H_2O_2 (80 μM), ABTS (0.5 mM), lignin peroxidase (88 unit/L) in 125 mM tartric buffer. Arrows indicate the increases in absorbance at 420 nm and 734 nm.

The Spectra of Reactants and Oxidation Products

To quantify the amount of melanin and measure lignin peroxidase activity with the spectrophotometric method, we analyzed the spectrophotometric characteristics of the reactants.

Generally, lignin peroxidase activity has been determined by the veratryl alcohol method at 310 nm [9]. However, melanin itself has a dark brown color overlapping this absorbance area. To overcome this problem, we tried to measure the activity of lignin peroxidase using ABTS, which uses oxidation products at 420 and 735 nm instead of VA as substrate. When H_2O_2 was added to the ABTS/lignin peroxidase reaction mixture, the oxidation product showed two distinct absorption peaks at 420 nm and 734 nm (Fig. 2). The linearity of these two substrates

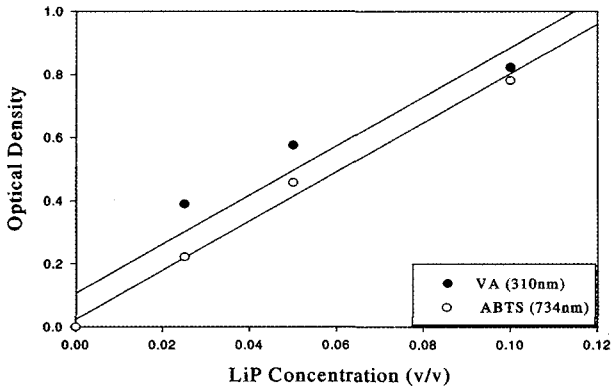

Fig. 3. Linear absorbance of lignin peroxidase-H_2O_2 products at 310 nm and 734 nm.

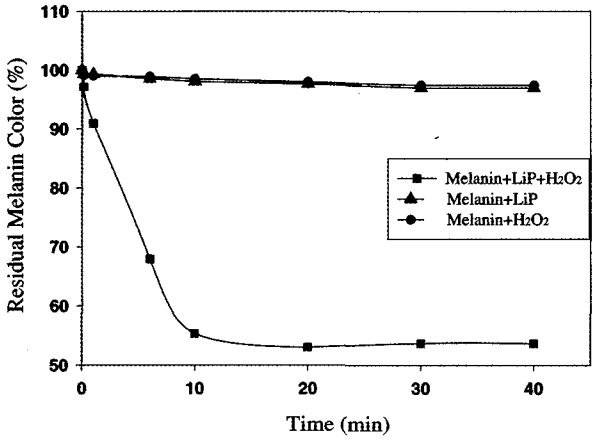

Fig. 4. Effect of lignin peroxidase (LiP) and H_2O_2 on melanin decolorization. 100 mg/L (Melanin, 772 unit/L lignin peroxidase, 800 μM H_2O_2).

for lignin peroxidase was also compared. As shown in Fig. 3, the measurement of lignin peroxidase activities using ABTS was directly proportional to that using VA. Therefore, lignin peroxidase activities could be detected using ABTS at 734 nm.

Characteristics of Melanin Decolorization by Lignin Peroxidase

Melanin decolorization was investigated with 772 units/L lignin peroxidase in crude enzyme solution and 800 μM H_2O_2. Fig. 4 shows a 45% decrease in absorbance at 475 nm in the presence of lignin peroxidase and H_2O_2, which indicates that the melanin was decolorized. In the absence of lignin peroxidase or H_2O_2, the decolorization of melanin was not observed. This indicates that both lignin peroxidase and H_2O_2 are necessary for the decolorization of melanin. Since Wondrack *et al.* [14] first demonstrated the oxidizing effect of lignin peroxidase from *Phanerochaete chrysosporium* on coal polymers in aqueous solution, lignin peroxidases have been intensively studied due to their ability to degrade different recalcitrant xenobiotics and to bleach dyes [15-17]. Despite these studies, our presentation is the first report that melanin was decolorized by lignin peroxidase/H_2O_2.

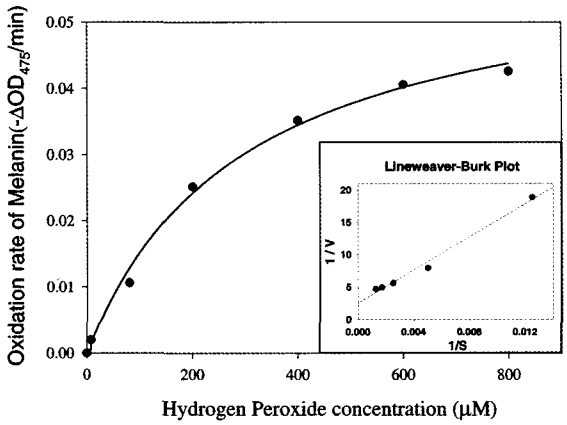

Fig. 5. Decolorization rate of melanin as a function of H_2O_2 concentration (772 unit/L lignin peroxidase, 70 mg/L Melanin, pH 4, 25°C).

The effects of two substrates, melanin and H_2O_2, on melanin decolorization rate were investigated by varying the concentration of one substrate while maintaining the other substrate concentration constant. The effects of H_2O_2 on decolorization rate showed Michaelis-Menten type kinetics. From the Lineweaver-Burk plot, the V_{max} (apparent maximum reaction rate) and K_m (apparent Michaelis constant) were determined as 0.08 $-\Delta OD_{475}$/min and 504.9 μM, respectively (Fig. 5). High concentrations of H_2O_2 (about 30%) can also decolorize melanin, as hydrogen peroxide is itself a strong oxidant [18]. In our experiment, the melanin was not decolorized below 800 μM H_2O_2 without the addition of enzymes (data not shown).

The concentrations of lignin peroxidase and H_2O_2 were constant at 772 units/L and 400 μM. Increasing the melanin concentration increased the decolorization rates. The V_{max} (apparent maximum reaction rate) and K_m (apparent Michaelis constant) were determined as 0.1 OD_{475}/min and 99.7 mg/L (Fig. 6).

Melanin decolorization rate, lignin peroxidase activity, and consumption rate of H_2O_2 were measured in a time course reaction (Fig. 7). Melanin decolorization rate was detected at 475 nm and the consumption rate of H_2O_2 and residual lignin peroxidase activity was measured at 734 nm using ABTS. 48 percent of the melanin was decolorized in 5 min. After 10 min, the melanin-decolorization reaction was interrupted at 52%. Lignin peroxidase activity remained constant but H_2O_2 concentration decreased dramatically during melanin decolorization reactions. Therefore we can assume that the discontinuation of the decolorization reaction was due to depletion of H_2O_2 in the reaction mixture.

To extend the duration of melanin decolorization activity by lignin peroxidase, low concentrations of H_2O_2 were pulse wisely supplied. As shown in Fig. 8, pulse wise

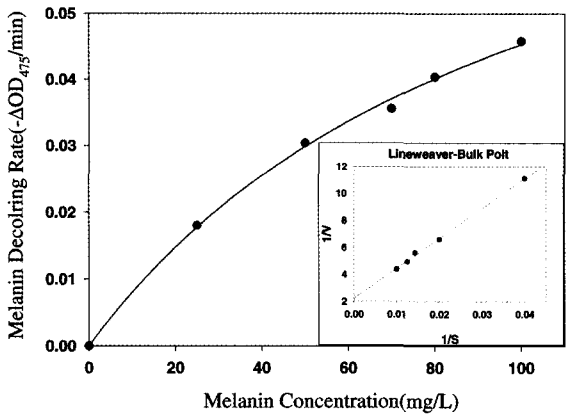

Fig. 6. Decolorization rate of melanin as a function of melanin concentration (772 unit/L lignin peroxidase, 400 μM H_2O_2, pH 4, 25°C).

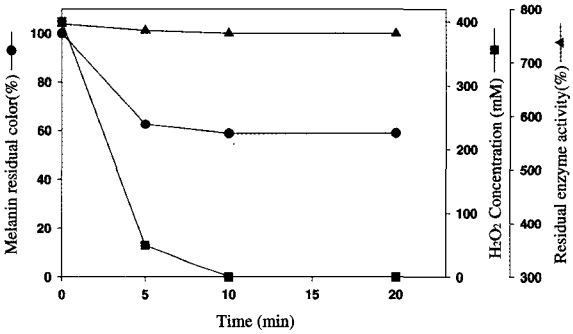

Fig. 7. Time course of melanin decolorization by lignin peroxidase (Melanin 100 mg/L, lignin peroxidase 772 unit/L, H_2O_2 400 μM, Tartaric buffer 125 mM, pH 4, at 25°C).

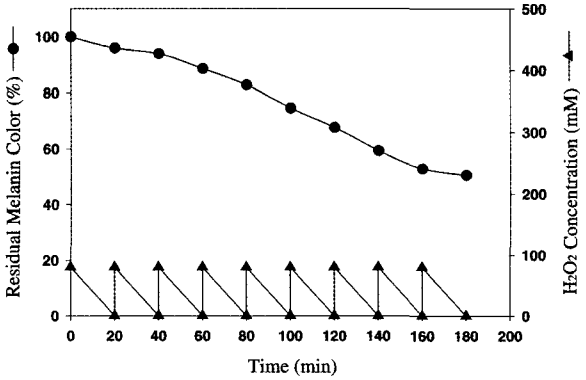

Fig. 8. Melanin decolorization by pulse feeding of H_2O_2 with lignin peroxidase (Lignin peroxidase 386 unit/L, Melanin 100 mg/L, H_2O_2 80 μM pulse feeding every 20 min).

feeding of H_2O_2 every 20 min decolorized the melanin continuously, achieving 32.6% decolorization in 180 min. These results, which proved that lignin peroxidase and H_2O_2 could decolorize melanin, could lead to the development of new skin-whitening ingredients for the cosmetic industry.

In this report, an attempt was made to decolorize the melanin biopolymer by lignin peroxidase and H_2O_2. We isolated and identified the melanin-decolorization fungus. In addition, we demonstrated that crude lignin peroxidase prepared from culture broth, could decolorize melanin. This is the first report showing the decolorization of melanin by lignin peroxidase. Although this work is very preliminary, a different approach for the whitening of human skin can be developed.

Acknowledgements This research was supported by funds from the Ministry of Health Welfare, and, in part, by Inha Biotechnology Funds, for which the authors are grateful.

REFERENCES

[1] Prota, G. (1992) Pigment cell metabolism: Enzymatic and chemical control. pp. 153-184. In: G. Prota (ed.). *Melanins and Melanogenesis.* Academic Press, San Diego, USA.

[2] Krol, E. S. and D. C. Liebler (1998) Photoprotective actions of natural and synthetic melanins. *Chem. Res. Toxicol.* 11: 1434-1440.

[3] Horii, I. (2000) The 21C research trend on cosmeceuticals. *J. Soc. Cos. Sci. Korea* 26: 9-24.

[4] Jeon, J. H. (2002) Purification and Characterization of 2,4-Dichlorophenol Oxidizing Peroxidase from *Streptomyces* sp. AD001. *J. Microbiol. Biotechnol.* 12: 972-977.

[5] Ryu, W. T., M. Y. Jang, and M. H. Cho (2003) The selective visualization of lignin peroxidase, manganese peroxidase and laccase, produced by white rot fungi on solid media. *Biotechnol. Bioprocess Eng.* 8: 130-135.

[6] Tekere, M., A. Y. Mswaka, R. Zvavya, and J. S. Read (2001) Growth, dye degradation, and ligninolytic activity studies on Zimbabwean white-rot fungi. *Enzyme Microb. Technol.* 28: 420-426.

[7] Nakamura, Y. and G. Mtui (2003) Biodegradation of endocrine-disrupting phenolic compounds using laccase followed by activated sludge treatment. *Biotechnol. Bioprocess Eng.* 8: 294-299.

[8] Ralph, J. P. and D. E. A. Catcheside (1994) Decolourisation and depolymerisation of solubilised low lank coal by the white rot basidiomycete *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* 42: 536-542.

[9] Tien, M. and T. K. Kirk (1988) Lignin peroxidase of *Phanerochaete chrysosporium. Method. Enzymol.* 161: 238- 249.

[10] Hofrichter, M. and W. Fritsche (1996) Depolymerization of low-rank coal by extracellular fungal enzyme systems: I. Screening for low-rank-coal-depolymerizing activities. *Appl. Microbiol. Biotechnol.* 46: 220-225.

[11] Flaig, W. and H. L. Schmidt (1957) Uber die Einwirkung von Huminsauren auf das Wachstum einiger Penicilliummarten. *Arch. Microbiol.* 27: 1-32.

[12] Ladd, J. N. and J. H. A. Butler (1969) Inhibition and stimulation of proteolytic enzyme activities by soil humic acids. *Aust. J. Soil Res.* 7: 253-262.

[13] Macor, M. (1979) Decomposition of humic acids from

peat soil by micromycetes. *Acta Fac. Rerum. Microbiol.* 8: 1-23.

[14] Wondrack, L., M. Szanto, and W. A. Wood (1989) Depolymerization of water soluble coal polymer from subbitumious coal and lignite by lignin peroxidase. *Appl. Biochem. Biotechnol.* 20: 765-780.

[15] Ollikka, P., K. Alhonmaki, V. M. Leppanen, T. Glumoff, T. Raijola, and I. Suominen (1993) Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 59: 4010-4016.

[16] Kim, M. S., E. J. Huh, H. J. Kim, and K. W. Moon (1998) Degradation of polycyclic aromatic hydrocarbons by selected white-rot fungi and the influence of lignin peroxidase. *J. Microbiol. Biotechnol.* 8:129-133.

[17] Swamy, J. and J. A. Ramsay (1999) The evaluation of white rot fungi in the decolorization of textile dyes. *Enzyme Microb. Technol.* 24: 130-137.

[18] Agodi, A., S. Stefani, C. Corsaro, F. Campanile, S. Gribaldo, and S. Sichel (1996) Study of a melanic pigment of *Proteus mirabilis. Res. Microbiol.* 147: 167-174.

[Received March 3, 2004; accepted August 16, 2004]