0273-2289/02/101/0031/$12.50

Oxidation of Kojic Acid Catalyzed by Manganese Peroxidase from *Ceriporiopsis subvermispora* in the Absence of Hydrogen Peroxide

Francisco Bastidas,[1] Ulises Urzúa,[2] and Rafael Vicuña*,[1]

[1]Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile. Casilla 114-D. Santiago, Chile and Millennium Institute for Fundamental and Applied Biology, E-mail: rvicuna@genes.bio.puc.cl; [2]Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile. Casilla 70086. Santiago 7, Chile

Received June 5, 2001; Revised August 16, 2001; Accepted September 1, 2001

Abstract

We have previously reported the oxidation of kojic acid catalyzed by manganese peroxidase (MnP) from *Ceriporiopsis subvermispora*. This reaction is strictly dependent on Mn(II), although it does not require the addition of hydrogen peroxide. We have extended these studies because this reaction can be considered as a model system for the *in situ* generation of hydrogen peroxide in natural environments. We show here that oxidation of kojic acid with horseradish peroxidase (HRP) plus hydrogen peroxide or with manganic acetate rendered a product with identical chromatographic and spectral properties as the one obtained in the reaction catalyzed by MnP. The initial lag observed in the latter reaction decreased significantly upon UV irradiation of the substrate. On the other hand, ascorbic acid increased the lag and did not affect the yield of the reaction. The superoxide anion trapping agents glutathione, nitroblue tetrazolium, and superoxide dismutase markedly affected the reaction. In contrast, addition of the hydroxyl radical scavengers mannitol and salicylic acid had no effect. Based on these results, a mechanism for the MnP-catalyzed reaction is proposed.

Index Entries: Manganese peroxidase; kojic acid; autooxidation; oxygen radicals; *Ceriporiopsis subvermispora*.

*Author to whom all correspondence and reprint requests should be addressed.

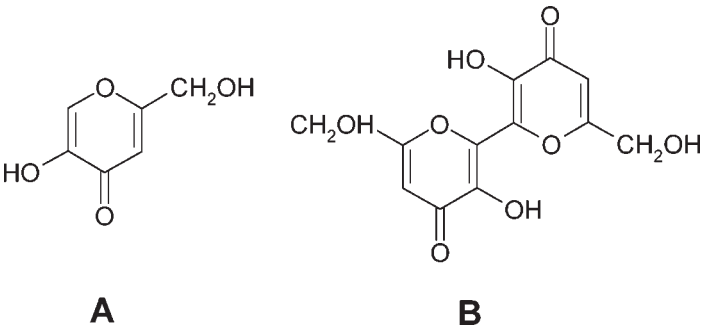

Fig. 1. Structure of kojic acid **(A)** and its oxidation product 6,6'-bis(5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one **(B)**.

Introduction

MnP is a heme-containing enzyme produced by several white-rot fungi that is thought to play an essential role in lignin degradation *(1,2)*. MnP catalyses the oxidation of Mn(II) to Mn(III), which when stabilized by an organic acid chelator can act as a diffusible agent to oxidize a variety of phenolic substrates including lignin model compounds *(3,4)*.

In recent years, our research has focused in the ligninolytic system of the white-rot basidiomycete *Ceriporiopsis subvermispora*, which is composed of MnP and laccase *(5)*. The H_2O_2 required as a co-substrate by MnP is produced through the manganese-dependent oxidation of oxalate and glyoxylate, metabolites that are secreted to the extracellular fluid by the fungus *(6)*. These reactions proceed as follows: trace amounts of Mn(III) oxidize oxalate producing CO_2 and formate radical. The latter reacts with oxygen to give a second molecule of CO_2 and superoxide. Superoxide is then reduced by Mn(II) to give hydrogen peroxide and Mn(III) *(7)*, both of which further accelerate MnP-catalyzed reactions. Similarly, glyoxylate is oxidized by Mn(III) with the production of formate and formate radical from the organic acid *(8)*.

We have previously shown that MnP catalyses the oxidation of kojic acid, a hydroxypyranone metabolite produced by some fungi (Fig. 1A), in the absence of hydrogen peroxide *(9)*. This reaction exhibits a short lag period and has an absolute requirement for Mn(II). When hydrogen peroxide is added at the onset of the reaction, the lag period disappears. On the other hand, inhibition by catalase of the former reaction strongly suggests that hydrogen peroxide is generated *in situ (9)*.

We have pursued our studies of this reaction. In this report, we provide evidence indicating that the product formed by MnP is identical to the one formed by horseradish peroxidase (HRP) in the presence of hydrogen peroxide *(10)*, the structure of which has been recently elucidated using NMR and LC-API-MS *(11)*. Based on the effect of several reduced-oxygen species trapping agents, we herein propose a possible mechanism for this reaction.

Materials and Methods

Chemicals

Kojic acid, hydrogen peroxide, ascorbic acid, salicylic acid, and HRP were purchased from Sigma (St. Louis, MO). Manganic acetate and ^2H-H_2O were from Aldrich (Milwaukee, WI). Nitroblue tetrazolium (NBT), manganese sulfate, reduced gluthation, and mannitol were obtained from Merck (Germany). Bovine liver catalase and bovine erythrocyte superoxide dismutase (SOD) were from Boehringer Mannheim (Germany).

Microorganism and Purification of MnP

The fungus *C. subvermispora* strain FP-105752 was obtained from the Center for Forest Mycology Research (Forest Products Laboratory, Madison, WI). Culture conditions and enzyme purification were performed as previously described *(6,9)*.

Oxidation Reactions

The standard assay mixture (1 mL) to measure oxidation of kojic acid was composed of 10 m*M* of this substrate, 0.1 m*M* $MnSO_4$, and 50 m*M* sodium succinate pH 5.0, plus 0.02 units of MnP or 50 µmol of manganic acetate. Either of the last two reagents was added to start the reaction. Assays with HRP contained, in 1 mL, 10 m*M* kojic acid, 50 m*M* sodium succinate pH 5.0, 0.1 m*M* H_2O_2, and 5 µg of HRP type II (Sigma Chemical Co, St. Louis, MO). In each case, oxidation of kojic acid was measured as an increase in absorbance at 377 nm during 20 min at 30°C *(9)*. When the effect of light on the reaction was tested, the assay mixture was placed in a wide beaker and it was irradiated with an UV-light lamp (254 nm) during the indicated times. Subsequently, the reaction was started by the addition of MnP. To assess the possible involvement of singlet oxygen in the reaction, stock solutions of the assay components and reaction mixtures were prepared using ^2H-H_2O. Thereafter, both chemical and enzymatic assays were conducted as described.

Oxidation of ascorbic acid with manganic acetate was as indicated above for kojic acid, although in this case the incubation mixture contained 10 m*M* ascorbic acid instead of 10 m*M* kojic acid. The reaction was monitored at 262 nm.

HPLC Analysis

Solutions containing the products of enzymatic and chemical reactions were filtered through a polysulfone membrane 10,000 nominal lower molecule weight (NLMW) (Millipore, Bedford, MA). Filtrates were analyzed in a Shimadzu chromatograph equipped with a SCL-6A system controller, a LC-6A pump, a SPD-6A detector, and a Chromatopac C-R3A recorder. Separation was achieved with a 150×4.6 mm 5-µm Lichrosper 100 RP-18 column fitted with a guard cartridge (Merck, Darmstadt, Ger).

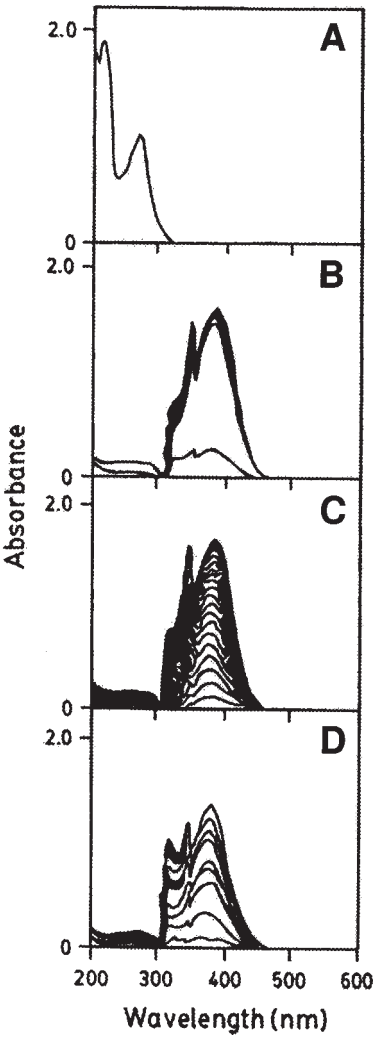

Fig. 2. UV-visible spectra of kojic acid and its oxidized products formed with various catalysts. Separate incubation mixtures containing kojic acid **(A)**, kojic acid, manganese sulfate plus MnP **(B)**, kojic acid, HRP, and hydrogen peroxide **(C)**, and kojic acid plus manganic acetate **(D)**, were recorded between 200 and 600 nm at 30°C at intervals of 1 min over a total period of 20 min. A baseline against kojic acid was constructed to obtain the spectra shown in B, C and D.

Isocratic runs were conducted at a flow rate of 0.4 mL/min using a mobile phase composed of 10% methanol in water adjusted to pH 3.0 with phosphoric acid. The column eluent was monitored at 350 nm.

Results

The first goal of this work was to characterize the products obtained by oxidation of kojic acid with either MnP plus Mn(II) or with manganic

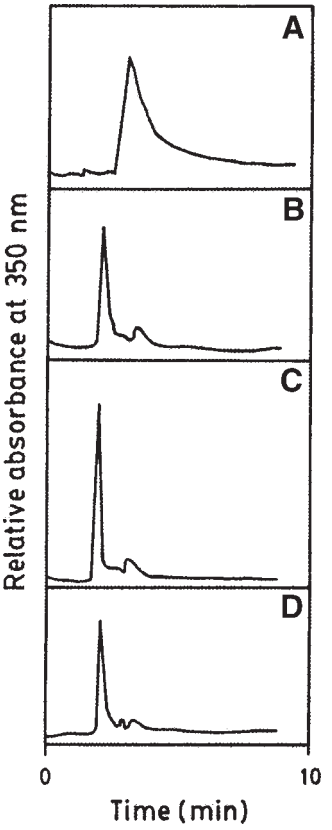

Fig. 3. HPLC analysis of the reaction products. Incubation mixtures described in the legend to Fig. 1 were subjected to HPLC analysis as described in *Methods*.

acetate. Since the oxidation product obtained with HRP plus hydrogen peroxide had recently been described *(11)*, this reaction was also set up for comparative purposes. As illustrated in Fig. 2, all three reactions gave rise to a product with very similar, if not identical, spectra, each with a maximum at 377 nm. A remarkable difference between the reactions catalyzed by MnP and HRP is that the former is about 20-fold faster, in spite of lacking externally added hydrogen peroxide (Fig. 2B and 2C). The products of the same reactions were analyzed by HPLC and the corresponding elution profiles are shown in Fig. 3. Again, MnP, HRP, and manganic acetate generated a product exhibiting an identical retention time (2.68 ± 0.04 min) when monitored at 350 nm. These results strongly suggest that the product is the same in all three reactions, namely, 6,6'-bis(5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, as identified by Zeringue et al. *(11)*.

It has been well established that some organic compounds having weak C–H bonds are susceptible to undergo autooxidation to form hydroperoxides *(12)*. This reaction requires the presence of a free radical initiator and proceeds through a free radical chain mechanism. Based on the mechanism proposed to describe the Mn(II)-dependent oxidation of malon-

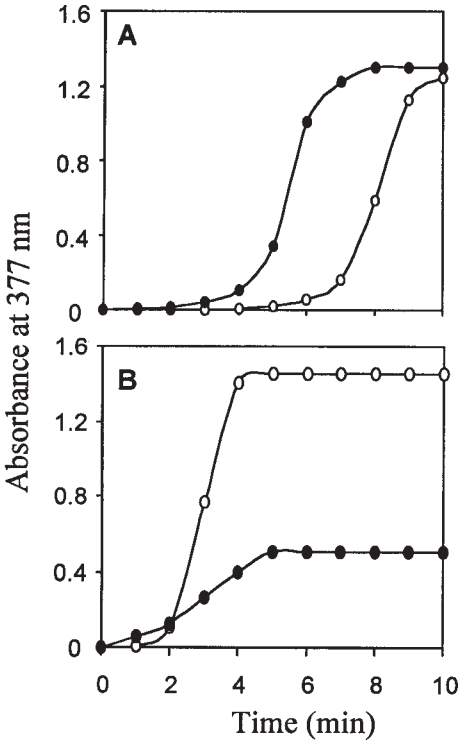

Fig 4. Effect of light and oxygen on kojic acid oxidation by MnP. In **(A)**, the reaction mixture was prepared in the absence of succinate buffer and UV-irradiated during 0 (○) or 5 (●) min. In **(B)**, the buffered assay mixture was bubbled with N_2 during 0 (○) or 5 (●) min.

aldehyde by HRP *(13)*, we hypothesized that light could trigger the initial steps of kojic acid oxidation. Because preliminary experiments had shown that in nonbuffered reactions the duration of the lag was longer, in this case succinate buffer was omitted to increase the sensitivity of the assay. Indeed, as shown in Fig. 4A, a 5-min irradiation pulse significantly decreased the lag phase of the reaction, suggesting that formation of a kojic acid-initiator radical is stimulated. On the other hand, irradiation of kojic acid during 72 h in the absence of MnP and Mn(II) rendered a product with identical spectral and chromatographic properties as those observed with the product obtained with MnP plus Mn(II), although at a much lower yield (about 3%, data not shown).

When air was displaced from the assay mixture using N_2, the reaction catalyzed by MnP was notably inhibited (Fig. 4B), supporting the idea that the reaction is initiated by an autooxidation step. Owing to a technical limitation, the cuvet employed in these assays permitted some degree of air exchange and, therefore, we did not observe an absolute inhibition.

Based on the previous results, the possible involvement of both carbon and oxygen-centered radicals was studied. Compounds that scavenge

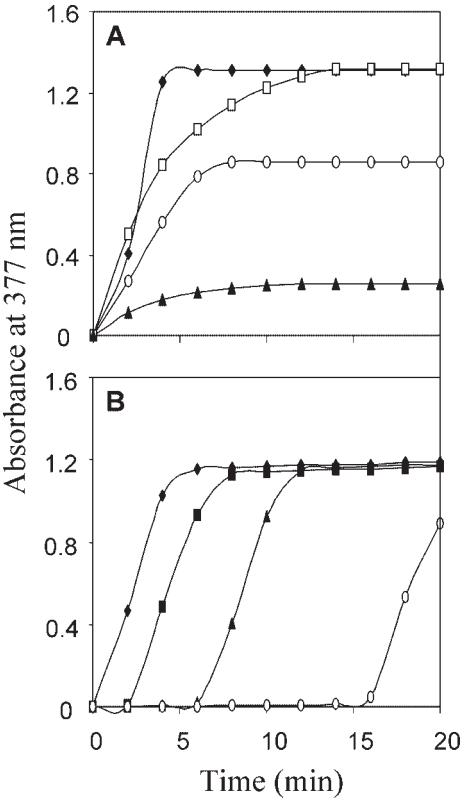

Fig. 5. Effect of radical trapping agents on kojic acid oxidation by MnP. In **(A)**, reaction mixtures contained 10 µg of SOD (□), 50 µ*M* NBT (▲), or 0.5 m*M* GSH (○). In **B**, ascorbic acid was added at 3 (■), 5 (▲) and 8 (○) µ*M* to the standard assay mixture. In both panels, control reactions lacking trapping agents are also shown (◆).

superoxide anion, peroxyl radicals, and hydroxyl radicals were tested in the MnP assay. These agents influenced the kinetics of the reaction in a complex manner, i.e., affecting the lag phase, the kinetic progress of the reaction, or the yield of product. For example, SOD decreased the velocity of the reaction without altering the final yield, whereas reduced glutathione and NBT affected both parameters (Fig. 5A). Hydroxyl radical scavengers such as mannitol and salicylic acid did not affect any reaction parameter (data not shown).

On the other hand, a remarkable effect on the lag phase was observed upon addition of ascorbic acid, even at very low concentrations (Fig. 5B). In separate reactions, we observed that ascorbic acid is readily oxidized by Mn(III) (see *Methods*), a reaction that is not affected by the presence of kojic acid, added at concentrations of up to 5 m*M* (data not shown).

Finally, the possible involvement of singlet oxygen was tested in incubation mixtures containing ^{2}H-H_2O as solvent *(14)*. No effect was observed in these assays with respect to the reaction conducted under standard conditions (data not shown).

Discussion

Reactions catalyzed by peroxidases that do not require the external addition of hydrogen peroxide have been termed peroxidase–oxidase reactions. They have been extensively studied with HRP and other plant peroxidases using substrates such as indole-3-acetic acid *(15)*, dihydrofumaric acid *(16)*, 2-nitropropane *(17)*, and NADH *(18,19)*. In some cases, the presence of manganese stimulates the oxidation of the substrate. We have recently described an example of the physiological importance of this type of reaction. It consists in the generation of extracellular hydrogen peroxide required for MnP activity in cultures of *C. subvermispora* by means of the aerobic oxidation of oxalate and glyoxylate, metabolites which are found in the extracellular fluid *(6)*. A general scheme of a peroxidase-oxidase reaction is as follows:

$$XH + Mn(III)\ (trace) \longrightarrow X^{\bullet} + Mn(II) + H^{+}$$

$$X^{\bullet} + O_2 \longrightarrow O_2^{\bullet -} + X^{+}$$

$$O_2^{\bullet -} + 2H^{+} + Mn(II) \longrightarrow Mn(III) + H_2O_2$$

where $X^{\bullet}$ is a free radical derived from the substrate. The Mn(III) thus produced can oxidize further molecules of the substrate. The enzyme may now promote an amplifying effect on substrate oxidation by catalyzing the following reaction through the usual intermediates compound I and compound II:

$$XH + \tfrac{1}{2}\, H_2O_2 \longrightarrow X^{\bullet} + H_2O$$

A different situation has been observed in the oxidation of malonaldehyde by HRP, in which the enzyme displays the so-called oxygenase behavior *(13)*. In this case, the reaction is initiated by the photooxidation of the substrate to give a free radical. The radical reacts with molecular oxygen and through the action of manganese, an organic peroxide is generated:

$$XH \xrightarrow{h\nu} X^{\bullet} + H^{\bullet}$$

$$X^{\bullet} + O_2 \longrightarrow XOO^{\bullet}$$

$$XOO^{\bullet} + Mn(II) \longrightarrow XOO^{-} + Mn(III)$$

$$XOO^{-} + H^{+} \longrightarrow XOOH$$

This peroxide drives the peroxidase cycle to generate additional free radicals that propagate the consumption of oxygen.

In a previous report, we described the oxidation of kojic acid by MnP in the absence of externally added hydrogen peroxide *(9)*, suggesting that a free radical mechanism might be involved. Based on the results obtained in the present work, we propose that this reaction is triggered by an initial photooxidation of the substrate. Indeed, a decrease in the lag after UV

irradiation (Fig. 4A), as well as a slow UV-induced formation of product in the absence of externally added MnP and Mn(II) (see *Results*), strongly support this hypothesis. Considering that superoxide scavengers as well as catalase *(9)* affect the course of the reaction, a likely order of events could be:

$$KH + O_2 \xrightarrow{h\nu} K^{\bullet} + O_2^{\bullet -} + H^+$$

$$O_2^{\bullet -} + 2H^+ + Mn(II) \longrightarrow Mn(III) + H_2O_2$$

This hydrogen peroxide could then be utilized by MnP to produce further Mn(III), which would contribute to the generation of kojic acid radicals by direct oxidation of this compound:

$$KH + Mn(III) \longrightarrow K^{\bullet} + Mn(II) + H^+$$

We propose that these radicals are centered in carbon 6 and that two of them react forming 6,6'-bis(5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (Fig. 1B), the main product observed in this reaction. The identity of this compound is based on the fact that the spectra obtained in separate reactions containing either MnP, manganic acetate, or HRP were identical (Fig. 2). They resemble the so-called spectral "I-band" typical of plant secondary metabolites such as quercetin and myricetin, in which a pyrone is conjugated with an aromatic moiety *(20)*. The elution pattern in HPLC of the three products is also the same (Fig. 3). Since Zeringue et al. had identified the product obtained with HRP plus H_2O_2 as 6,6'-bis(5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (11), we assume that it is the same as the one obtained with MnP plus Mn(II).

In addition to those indicated above, other reactions may been taking place as well. For example, it is conceivable that the radical of kojic acid may add oxygen to form the peroxyl radical $KOO^{\bullet}$. The latter could then be reduced by Mn(II) to form an organic peroxide *(13)*, which might constitute a substrate for MnP. However, the fact that the main product of the reaction is a dimer of kojic acid suggests that addition of molecular oxygen to the carbon centered radical does not constitute a predominant pathway in this system. The strong inhibitory effect of ascorbate, a well-known trapping agent of peroxyl radicals *(21)*, can be explained its ability to react with the Mn(III) generated in the reaction. Indeed, the fact that ascorbate affects only the lag of kojic acid oxidation by MnP and the lack of interference of kojic acid in the oxidation of ascorbic acid by Mn(III) support this assertion.

Acknowledgments

This work was supported by a grant from Fondecyt (Grant 8990004) to R.V and by the Millennium Institute for Fundamental and Applied Biology. We thank Dr. Eduardo Lissi for his helpful comments.

References

1. Kirk, T. K. and Farrell, R. L. (1987), *Ann. Rev. Microbiol.* **41,** 465–505.
2. Orth, A. B., Royse, D. J., and Tien, M. (1993), *Appl. Environ. Microbiol.* **59,** 4017–4023.
3. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986), *Arch. Biochem. Biophys.* **251,** 688–696.
4. Wariishi, H., Valli, K., and Gold, M. H. (1992), *J. Biol. Chem.* **267,** 23,688–23,495.
5. Lobos, S., Larrain, J., Salas, L., Cullen, D., and Vicuña, R. (1994), *Microbiology* **140,** 2691–2698.
6. Urzúa, U., Kersten, P. J., and Vicuña, R. (1998), *Appl. Environ. Microbiol.* **64,** 68–73.
7. Archibald, F. S. and Fridovich, I. (1982), *Arch. Biochem. Biophys.* **214,** 452–463.
8. Kuan, I. C. and Tien, M. (1993), *Arch. Biochem. Biophys.* **302,** 447–454.
9. Urzúa, U., Larrondo, L., Lobos, S., Larrain, J., and Vicuña, R. (1995), *FEBS Lett.* **371,** 132–136.
10. Zeringue, H. J. and Shih, B. Y. (1998), *J. Agric. Food. Chem.* **46,** 1071–1075.
11. Zeringue, H. J., Shih, B. Y., Maskos, K., and Grimm, D. (1999), *Phytochemistry* **52,** 1391–1397.
12. Schmid, H. (1996), *Organic Chemistry*, Wiley Interscience, New York.
13. McDonald, I. D. and Dunford, H. B. (1989), *Arch. Biochem. Biophys.* **272,** 185–193.
14. Lion, Y., Delmelle, M., and van de Vorst, A. (1976), *Nature* **263,** 442–443.
15. Yamazaki, H. and Yamazaki, I. (1973), *Arch. Biochem. Biophys.* **154,** 147–159.
16. Halliwell, B. (1977), *Biochem. J.* **163,** 441–448.
17. de Rycker, J. and Halliwell, B. (1978), *Biochem. J.* **175,** 601–606.
18. Yamazaki, I. and Yokota, K. (1973), *Mol. Cell. Biochem.* **2,** 39–52.
19. Halliwell, B. (1978), *Planta* **140,** 81–88.
20. Singleton, V. L. (1981), in *Flavonoids*, Childester, C. O., Mrak, E. M., and Stewart, G. F. (eds.), Academic Press, New York, pp. 149–242.
21. Hofrichter, M., Ziegenhagen, D., Vares, T., Friedrich, M., Jäger, M. G., Fritsche, W., and Hatakka, A. (1998), *FEBS Lett.* **434,** 362–366.