OXALATE METABOLISM IN LIQUID CULTURES OF *Ceriporiopsis subvermispora:* A POSSIBLE PATHWAY FOR EXTRACELLULAR H_2O_2 PRODUCTION

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ABSTRACT

In this work, the source of extracellular hydrogen peroxide in cultures of *Ceriporiopsis subvermispora* was investigated. A thorough search for the presence in the growth medium of oxidases known to be produced by other fungi gave negative results. We therefore explored the prospect that H_2O_2 might arise from the oxidation of organic acids by MnP. Both oxalate and glyoxylate were found in the extracellular fluid of *C. subvermispora* grown in salt medium, at concentrations of 2.5 and 0.24 mM, respectively. MnP titers correlated with the mineralization of [^{14} C]-oxalate in cultures maintained at constant pH. *In vitro* assays confirmed the Mn-dependent oxidation of oxalate by MnP in the absence of externally added H_2O_2, as evidenced by the formation of MnIII-oxalate complex and by $^{14}CO_2$ evolution from [^{14}C]-oxalate. This reaction was stimulated by physiological concentrations of glyoxylate and was inhibited by superoxide dismutase. In addition, both organic acids supported phenol red oxidation by MnP without adding H_2O_2, glyoxylate being more reactive than oxalate. Based on the above evidence, a model is proposed for the production of extracellular H_2O_2 by *C. subvermispora.* other hand, an oxidase activity responsible for intracellular degradation of oxalate has been identified in this fungus. This enzyme, highly specific for oxalate. has a native molecular mass of 407 kDa and migrates as a single band of 65 kDa in SDS-PAGE

INTRODUCTION

The extracellular production of hydrogen peroxide, a cosubstrate of lignin peroxidase (LiP) and manganese peroxidase (MnP), constitutes an essential step of the lignin breakdown process. Several oxidases have been proposed to accomplish this task These include pyranose oxidase [l], methanol oxidase [2], aryl alcohol oxidase [3] and glyoxal oxidase (GLOX) [4]. The fact that GLOX is secreted by *Phanerochaete chrysosporium* and is activated by LiP and its corresponding aromatic substrate [5], strongly suggests that GLOX plays a key role in regulation as well as production of extracellular hydrogen peroxide by *P. chrysosporium,* the most-studied ligninolytic basidiomycete.

In recent years we have been characterizing the ligninolytic system of the basidiomycete *Ceriporiopsis subvermispora,* which is composed of MnP and lactase [6]. These activities are secreted as two families of isoenzymes with isoelectric points that vary with the composition of the growth medium [7,8]. In addition to the absence of LiP, *C.* subvermispora also differs from *P. chrysosporium* in that it does not produce GLOX [6]. To our knowledge. the mechanism utilized by this fungus to produce extracellular hydrogen peroxide has not been studied.

Preliminary work showed that the extracellular fluid of cultures of *C. subvermispora* apparently lacks a H_2O_2 generating oxidase activity. Therefore, we explored the prospect that hydrogen peroxide could arise from reactions involving free radicals derived from the oxidation of organic acids by MnP. We report here that *C. subvermispora* secretes glyoxylate and oxalate to the culture fluid and provide evidence suggesting that their oxidation by MnP may constitute a physiological source of extracellular hydrogen peroxide for this fungus Some of these results have been recently published [9].

MATERIALS AND METHODS

Fungus and cultivation. *C. subvermispora* strain FP-105752 was obtained from the Center for Forest Mycology Research of the Forest Product Laboratory, Madison, WI. The fungus was maintained on agar slants of potato dextrose agar medium (Difco). Liquid cultures of *C. subvermispora* were grown in 125-ml Erlenmeyer flasks as previously described [6].

Extracellular oxidase activity. Aliquots were withdrawn from cultures every two days and they were clarified by centrifugation for 15 min at 9,000 x g The supernatant was assayed for oxidase activity as follows: 100 µl of sample were added to 100 µl of a reaction mixture containing 50 mM sodium succinate pH 5.0 and 10 mM of substrate These mixtures were incubated at 30°C for 30 min and then filtered through polysulfone membranes (Ultrafree-MC; Millipore [NMWL, 10000]). The H_2O_2 present in the filrate was determined by the method of Bernt and Bergmeyer [10].

Intracellular oxalate oxidase. The fungal extract was prepared by grinding the mycelium with a pestle in a chilled mortar. Thereafter, this enzyme was fractionated by a procedure to be published elsewhere. Enzymatic activity was measured in a coupled assay employing HRP and phenol red [11].

Determination of oxalate. Aliquots (0.5 ml) withdrawn from the cultures were filtered through polysulfone membranes (ultrafree-MC; Millipore [NMWL, l0,000]) and they were analyzed by ion exclusion HPLC with an SCL-6A system controller (Shimadzu) equipped with a RT 300-6,5 Polyspher OAHY pre-packed column (Merck), a LC-6A pump, a SPD-6A detector and a Chromatopac C-R3A recorder. Compounds were eluted with 0.01 N H_2SO_4 pumped at a flow rate of 0.4 ml/min and absorbances measured at 210 nm.

Determination of glyoxylate. Culture aliquots (0.5 ml) were reacted with 0.5 ml of 1 mM 2,4-dinitrophenylhydrazine in 0.32 N HCl for 1 h at room temperature. The hydrazone derivatives were analyzed by a HPLC instrument equipped with a mBondapak C-18 column The mobile phase of 10 mM NaH_2PO_4, pH 6.8 in 15% methanol was pumped at a flow rate of 1 ml/min and the absorbance recorded at 366 nm.

***In vivo* $^{14}CO_2$, evolution from [^{14}C]oxalic acid.** The concentration of oxalate in culture fluid was first determined by HPLC as indicated above and "C-labeled oxalate (Sigma, 10^7 dpm/mmol) was added to the cultures to achieve a final specific activity of 17,000 cpm/mmol. The cotton plugs of the flasks were then replaced by gas-tight rubber stoppers. After 8 h of further incubation at 30 °C, the flasks were flushed with air for 10 min to trap the $^{14}CO_2$ in an ethanolamine-containing scintillation fluid.

MnP activity and *in vitro* oxidation of [^{14}C]oxalic acid. MnP was routinely assayed with vanillylacetone as substrate. *In vitro* oxidation of [^{14}C]oxalate was conducted in gas-tight 50 ml tubes containing 1 ml reaction mixtures including 0.04 units of MnP, 1 mM [^{14}C]oxalate (6,0 x 10^5 cpm/mmol), 0.1 mM $MnSO_4$ and 50 mM sodium succinate pH 5.0. Incubations were at 30 °C and the kinetics of $^{14}CO_2$ evolution were followed by trapping the $^{14}CO_2$ released as indicated above. Where mentioned, anaerobic experiments were conducted by purging the reaction mixtures for 10 min. with nitrogen. *In* vitro oxidation of glyoxylate by MnP was assayed in open tubes containing 0.04 units of the enzyme, 0.25 mM glyoxylate, 0.1 mM $MnSO0_4$ and 50 mM sodium succinate pH 5.0 in 1 ml reaction mixtures Formate generated in this reaction was quantitated using formate dehydrogenase [12]. Phenol red oxidation was assayed in 10 ml mixtures containing 0.4 units of MnP, 1 mM oxalate or 0.25 mM glyoxylate, 0.03 mM phenol red, 0.1 $MnSO_4$ and 50 mM sodium succinate pH 5.0. Aliquots of 0.7 ml were removed at the indicated times and 50 µl of 5 N NaOH were added to halt the reaction. Absorbance values at 610 nm were measured.

MnIII-oxalate formation assay. The formation of the complex was measured as an increase in absorbance at 270 nm (ε_{270} = 5634 M^{-1} cm^{-1}) [13].

RESULTS

Aliquots of the culture fluid were withdrawn at various times of the growth period and assayed as indicated in Methods, with the following substrates: methylglyoxal, glyoxal, formaldehyde, glycolaldehyde, di-hydroxyacetone, methanol, ethanol, anisyl alcohol, oxalic acid glucose and galactose. After repeated attempts with samples from different cultures, none of these compounds promoted the generation of the hydrogen peroxide required by HRP in the coupled assay.

It is known that white-rot fungi secrete acids and their *in vitro* decomposition by MnP has been documented. Indeed, Mn-dependent oxidation of both oxalate and glyoxylate by MnP proceeds with the transient formation of superoxide. The latter oxidizes Mn(II) to Mn(III) with the concomitant production of hydrogen peroxide [14-16]. The Mn(III) produced can further accelerate MnP-catalyzed reactions. Therefore, we looked for the presence of these metabolites in the growth medium. Glyoxylate and oxalate were identified and quantitated by HPLC in the extracellular fluid of *C. subvermispora* cultures grown in both standard medium (with 10 mM ammonium tartrate) and in low nitrogen (1 mM ammonium tartrate) medium Glyoxylate reached a maximal concentration of 0.24 mM on day five, whereas the concentration of oxalate was maximal on day 10, reaching levels near ten fold higher than those of glyoxylate. In cultures containing low nitrogen, glyoxylate reached maximal concentration of 0.025 mM on day 6, whereas oxalate reached a maximum of 1.5 mM at days eight and sixteen. No other carboxylic acids were identified in either early or late cultures in standard salt medium.

The *in vitro* oxidation of both oxalate and glyoxylate that is catalyzed by MnP from *P. chrysosporium suggested* that *the* corresponding enzymatic activity might be responsible for the metabolism of *these organic* acids *in vivo* by *C. subvermispora.* To test this hypothesis, the MnP titers were determined throughout the growth period of *C. subvermispora* in standard medium, with and without Mn (II), and in low nitrogen medium. In standard medium, MnP activity increases continually up to about 1.0 U/ml on day twelve. MnP activity was lower in cultures with low nitrogen and was virtually null in cultures lacking Mn(II) [6,17].

Additions of [^{14}C]-labeled oxalate were made to parallel cultures and $^{14}CO_2$ evolution was determined as a measure of oxalate mineralization *in vivo.* To facilitate interpretation of the data, the specific activity of oxalate at the times of addition was kept constant (calculations based on oxalate concentrations in culture). In standard cultures, oxidation of oxalate is most active between days four and ten, with a maximum at day six. This profile does not correspond to MnP titers or to maximum oxalate concentrations but, significantly, has a better correlation with glyoxylate concentrations In low nitrogen cultures, mineralization of oxalate is low in early cultures, although it proceeds throughout late cultures with a profile roughly mirroring MnP titers and oxalate concentrations. In medium lacking Mn(II), $^{14}CO_2$ evolution from labeled oxalate was negligible. Interpretation of these results must take into consideration that MnP activity in cultures containing high nitrogen is inhibited by a steady increase in the pH of the medium [17]. This effect is not evidenced by *in vitro* assays of MnP that are buffered with 100 mM sodium tartrate pH 5.0. Therefore the apparent lack of correlation between oxlate oxidation, MnP titers, and oxalate concentration might be explained if the MnP is increasingly inactive due to pH effects in culture.

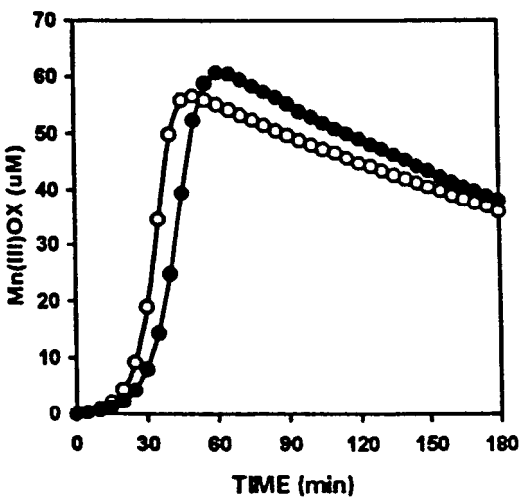

Fig 1. Kinetics of the Mn(III)-oxalate complex in standard assay with MnP. Organic acids present in the reaction mixture are oxalate (●) or oxalate plus glyoxylate (○).

Day 6 cultures grown in standard medium are of particular interest because of the dynamic changes ocurring: oxalate concentration is increasing glyoxylate is near maximal and beginning to decrease, oxalate oxidation is maximal and MnP is at early onset To explore the possible connection between MnP activity and oxalate and glyoxylate metabolism in culture, *in vitro* experiments were tested using the approximate physiological concentrations of these organic acids (1 mM for oxalate and 0.25 mM for glyoxylate) in day-6 cultures. We determined that MnP from *C. subvermispora* catalyzed the oxidation of Mn(II) to Mn(III), which thereafter

oxidizes both glyoxylate and oxalate. As stated in Materials and Methods, the formation of the Mn(II)-oxalate complex was monitored at 270 nm. As observed in Fig 1, the complex is formed after a distinc lag, which is shortened when the reaction mixture is supplemented with glyoxylate. On the other hand, mineralization of oxalate was determined by evolution of $^{14}CO_2$ from [^{14}C]-labelled oxalate, while the oxidation of glyoxylate was monitored by the appearance of formate. As observed in the previous reactions, the oxidation of oxalate exhibits a lag which decreases when glyoxylate is simultaneously added to the incubation mixture. Trace amounts of exogenous hydrogen peroxide or Mn(III) acetate greatly stimulate the reaction Mineral&ion of labeled oxalate by MnP in the absence of hydrogen peroxide decreased to about 5-10% when the reaction was conducted under nitrogen, or in the presence of 0.5 mM glutathione or 0.5 mM nitro-blue tetrazolium. Glyoxylate is more suspectible than oxalate to oxidation by MnP. In this case, trace amounts of Mn(III) acetate, but not of hydrogen peroxide, increased the rate of the reaction, while the addition of oxalate inhibited the oxidation of glyoxylate.

A useful assay to monitor MnP activity employs phenol red as substrate; the formation of oxidized phenol red is monitored at 610 nm. This assay has been used to characterize the oxidations of MnP from *P. chrysosporium* with glyoxylate and oxalate [15,16]. We found that both organic acids support oxidation of phenol red with the MnP of *C. subvermispora* at physiological concentrations for the organic acids. As expected, glyoxylate is more reactive than oxalate even at a four-fold lower concentration These reactions have an absolute requirement for Mn(II).

Based on the above results, we propose the following scheme for the production of extracellular peroxide by *C. subvermispora* (Fig 2): trace amounts of Mn(III) can be produced by MnP using hydrogen peroxide as oxidant, and this peroxide might originate from the mycelium. The intracellular oxidation of oxalate itself may contribute to this purpose (see below). Alternatively, slow autoxidation of an extracellular fungal metabolite may spark the initial formation of peroxide. This Mn(III) is then amplified by the action of MnP in the presence of organic acids and oxygen. In early cultures, the Mn(III) generated would oxidize glyoxylate since it appears earlier and is more reactive than oxalate. MnP oxidized by the peroxide generated in this reaction would give rise to more Mn(III), which could react with oxalate as its concentration increases in the cultures thus producing an amplifying effect. Alternative, Mn(III) could react directly with lignin, if present, or with glyoxylate still remaining in the medium

As mentioned above, we have also been studying the intracellular metabolism of oxalate. A crude extract prepared by homogenization of the mycelium in a chilled mortar and then subjected to chromatography on Q-Sepharose pH 7.5 followed by precipitation at pH 3.0 and then to chromatography on phosphocellulose at pH 3.0, leads to the purification of an oxalate oxidase activity that migrates as a single band in SDS-PAGE with a molecular mass of 65 kDa. This enzyme exhibits a native molecular mass of 407,000, as determined by gel permeation in Sephadex G-200. Its pH optimum is 3.5 and it is highly specific for oxalate (Km app = 50) µM). We are presently attempting to identify the subcellular location of oxalate oxidase by transmission electron microscopy.

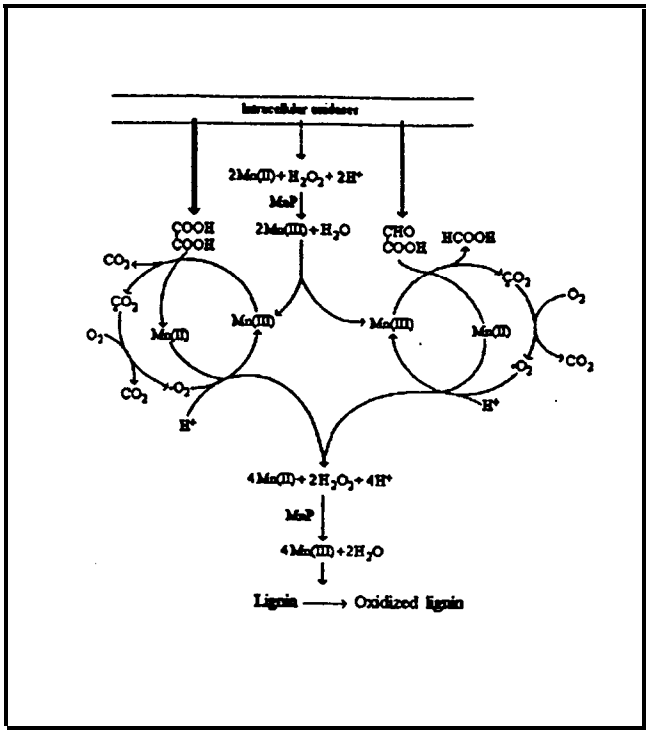

Fig 2. Proposed scheme for the production of extracellular production of hydrogen peroxide by *C. subvermispora.*

CONCLUSIONS

- *C. subvermispora* secretes both oxalate and glyoxylate to the extracellular medium.
- MnP titers correlated with the mineralization of [^{14}C]-oxalate in cultures maintained at constant pH
- *In vitro,* MnP oxidizes oxalate and glyoxylate in the absence of externally added hydrogen peroxide.
- In the former reaction, a complex Mn(III)-oxalate is first formed and it then decomposes generating CO_2 and H_2O_2.
- Based on these results, we propose a scheme for the production of extracellular H_2O_2 by *C. subvermispora.*

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