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GENERATION OF HYDROXYL RADICAL AND ITS INVOLVEMENT IN LIGNIN DEGRADATION BY *Phanerochaete chrysosporium*

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Cultures of *Phanerochaete chrysosporium* produced ethylene from methional and 2-keto-4-thiomethyl butyric acid (KTBA) only under conditions when the organism was competent to degrade [^{14}C]-lignin to $^{14}CO_2$. The ability of several mutant strains to produce ethylene reflected their ability to degrade lignin. Hydroxyl radical scavengers including thiourea, salicylate, mannitol, 4-O-methylisoeugenol, as well as catalase, inhibited fungal lignin degradation, fungal ethylene production from methional and KTBA, as well as ethylene generation from KTBA via Fenton's reagent and γ-irradiation. In addition, methional inhibited fungal lignin degradation and lignin inhibited ethylene generation from methional. All of these results indicate that hydroxyl radical plays an important role in lignin degradation by *P. chrysosporium*.

Although a variety of white rot basidiomycetes are capable of degrading lignin to CO_2 (1), the specific catalysts involved have not been determined. A number of studies with the polymer (2), polymeric (3), and dimeric model compounds (4-6), however, indicate that at least some of the catalyst(s) may be very nonspecific. Additional chemical (7-9) and physiological studies (10-12) indicate that the fungal degradation of lignin and lignin model compounds is an oxidative process. These results, coupled with the fact that no extracellular enzymes involved in lignin degradation have been isolated, led to the postulate that a form of activated oxygen may be involved in the initial depolymerization of lignin by white rot fungi (13).

The aim of this study was to investigate the possible activated oxygen species involved in lignin degradation. We have now shown that *Phanerochaete*

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Abbreviations used: 2-keto-4-thiomethyl butyric acid, KTBA; hydroxyl radical, •OH.

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chrysosporium produces significant quantities of ethylene from methional (14) and KTBA (15,16) under a variety of physiological conditions which parallel those required for the onset of secondary metabolism and lignin degradation. These results indicate that this organism produces •OH under these conditions. Further, studies with •OH scavengers indicate that it is indeed this radical which we are measuring and that •OH is probably involved in the fungal degradation of lignin. A recently presented report (17) appears to confirm some of these findings.

MATERIALS AND METHODS

Chemicals

3-Thiomethyl propanal (methional), KTBA, and catalase were obtained from Sigma. Authentic ethylene and Porapak N were obtained from Supelco. D-[^{14}C-(U)]-glucose was obtained from New England Nuclear. [^{14}C]-methoxy labeled lignin was synthesized as described previously (18).

P. chrysosporium ME 446 and mutant strains were maintained on slants as described previously (19). Stationary cultures in 250 ml flasks were prepared using 25 ml of a medium containing 2% glucose, 1.2 mM $(NH_4)_2$ tartrate, 20 mM Na 2,2-dimethylsuccinate buffer and salts as previously described (18,20).

Assay of lignin and glucose metabolism

An aqueous solution of [^{14}C]-glucose (1 mCi/mmole, 2×10^5 cpm/flask) or [^{14}C]-lignin (3×10^5 cpm/mg, 5.0×10^4 cpm/flask in DMF) were added to cultures on day 5 or as indicated. $^{14}CO_2$ was purged periodically and trapped in a basic scintillation fluid as previously described (18,21). Radioactivity was measured in a Beckman LS-3133P spectrometer.

Assay of ethylene production

Methional or KTBA was added to 5-day-old cultures or as indicated to a final concentration of 10 mM and 1 mM respectively. Hydroxyl radical scavengers were added simultaneously, as indicated, and the flasks were sealed with a rubber septum. After 3 days of incubation, 2.5 ml of gas was removed from each culture. Ethylene concentration was monitored directly by gas chromatography at 60°C in the isothermal mode using a column containing Porapak N.

γ-Irradiation

Solutions of KTBA (1 mM) and •OH scavengers as indicated in 1 ml of water were transferred to a 10 ml vial, sealed with a serum cap and irradiated for 12 hours with a ^{137}Cs γ-ray source (24.4 krads/hr). Gas samples (1 ml) were removed from the head space and ethylene was monitored as described above.

Fenton's reagent

Reactions were carried out in 25 ml flasks at 30°C for 1 hr. Reaction mixtures (5 ml) contained 10 mM potassium phosphate, pH 7.5, 200 mM H_2O_2, 2 mM $FeSO_4$, 2 mM EDTA and 1 mM KTBA. Gas samples (2.5 ml) were removed and ethylene was monitored as described above.

Table 1. Effect of culture conditions and mutant strains on the fungal generation of ethylene from methional and KTBA[a]

	Substrates			
	Methional (10 mM) C_2H_4 produced (nmol)	%	KTBA (1 mM) C_2H_4 produced (μmol)	%
Standard conditions	290	100	3.6	100
Minus substrate	0	0	0	0
Agitated cultures	13	4.5	0.12	3.3
High nitrogen cultures	2.6	0.9	0.005	0.14
Strain 104-2	0	0	0	0
Strain 424-2	68	23	2.8	78

[a] 5-Day old ligninolytic cultures of *Phanerochaete chrysosporium* ME 446 or cultures of mutant strains were incubated with methional or KTBA, purged with 100% O_2 and incubated for an additional 3 days. Evolved ethylene was determined as described in the text. High nitrogen cultures contained excess levels of nitrogen (12 mM $(NH_4)_2$ tartrate). In experiments with agitated cultures, flasks were placed on a New Brunswick G-10 shaker operating at a speed of 150 rpm and describing a 2-inch circle.

RESULTS

Effect of culture parameters and genetic mutations on the fungal production of •OH

The results in Table 1 indicate that 5- to 8-day-old stationary cultures of *P. chrysosporium* produce ethylene from exogenously added methional and KTBA. No ethylene was produced when these substrates were absent. The amount of ethylene produced in agitated cultures or in cultures containing an excess of $(NH_4)_2$ tartrate (12 mM) was less than 5% of that produced under the standard conditions. A lignin degradation mutant 104-2 (18) was unable to produce ethylene from either methional or KTBA under the standard conditions while a phenotypic revertant 424-2 strain partially regained this capacity.

The evolution of ethylene from KTBA and $^{14}CO_2$ from [^{14}C]-lignin by cultures of *P. chrysosporium* ME 446 is shown in Figure 1. Both ethylene and $^{14}CO_2$ evolution started after an initial lag of approximately 3 days and continued through the 8-day course of this experiment. Growth of the organism in stationary culture as measured by dry weight (data not shown) was essentially complete after 2 days, confirming earlier experimentation (12,20).

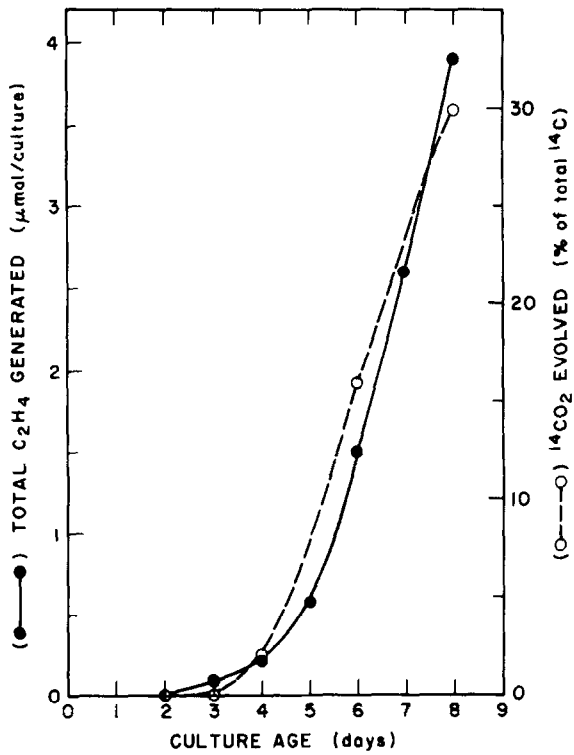

Figure 1. Dependence of the evolution of ethylene from KTBA and of lignin degradation on culture age. Stationary cultures were prepared as described in the text. To measure lignin degradation, [^{14}C]-methoxy labeled lignin was added at zero time, the flasks were purged with 100% O_2 and evolved $^{14}CO_2$ was trapped and determined. To measure ethylene production, KTBA (final concentration 1 mM) was added to individual flasks at days 2,3,4,5,6 and 7, and ethylene generation was determined after an additional 24 hours of incubation as described in the text.

Effect of radical scavengers on ligninolytic activity and ethylene production by *P. chrysosporium*

The results in Table 2 show that a variety of •OH radical scavengers have a significant inhibitory effect on both ligninolytic activity and ethylene production by *P. chrysosporium*. Thiourea, a potent scavenger of •OH (22,23), had the strongest inhibitory effect on both $^{14}CO_2$ evolution and ethylene production. Salicylate, which can be hydroxylated and/or decarboxylated by •OH (24), was also an effective inhibitor of the evolution of both gases. In addition, mannitol, another •OH scavenger, had a lesser, although still significant, effect. Finally, the olefin 1-(3',4'-dimethoxyphenyl)1,2-propene(4-O-methylisoeugenol) which is hydroxylated by the fungus under ligninolytic conditions (unpublished data) to form 1-(3',4'-dimethoxyphenyl)1,2-dihyroxypropane, also inhibited $^{14}CO_2$ and ethylene evolution under these conditions.

Table 2. Effect of hydroxyl radical scavengers on fungal ligninolytic activity and on the chemical and fungal generation of ethylene.

•OH scavengers	*Phanerochaete chrysosporium*[a]						Fenton's reagent[b]		γ-irradiation[c]	
	$^{14}CO_2$		C_2H_4(methional)		C_2H_4(KTBA)		C_2H_4(KTBA)		C_2H_4(KTBA)	
	cpm	%	nmol	%	μmol	%	nmol	%	nmol	%
Standard conditions	4390	100	350	100	3.6	100	140	100	370	100
Thiourea 1 mM	180	4.1	38	11	2.9	81	90	64	120	32
10 mM	141	3.2	5.0	1.4	0.065	1.8	24	17	5.2	1.4
Na-Salicylate 1 mM	4330	99	45	13	1.3	36	70	50	200	54
10 mM	2730	62	3.8	1.1	0.080	2.2	25	18	11	3.0
4-O-Methyl- 1 mM	2620	60	230	66	1.0	28	70	50	110	30
isoeugenol 10 mM	1880	43	7.0	2.0	0.55	15	42	30	140	38
Mannitol 50 mM	4270	97	350	100	4.4	122	100	71	41	11
100 mM	2550	58	180	51	2.9	81	77	55	12	3.2
Catalase[d] (1 mg/culture)	2960	61	260	74	2.2	61				
BSA (1 mg/culture)	4960	113	320	91	3.9	108				
Methional 1 mM	4130	94	---	---	---	---				
10 mM	1630	37	---	---	---	---				
Lignin (DHP) 1%	---	---	238	68	---	---				

[a]Ligninolytic stationary cultures (5 days old) of *Phanerochaete chrysosporium* were incubated with [^{14}C]-methoxy labeled lignin, KTBA or methional and •OH scavengers as indicated, purged with 100% oxygen and incubated for an additional 3 days. Evolved $^{14}CO_2$ or ethylene were determined as described in the text.
[b]Reactions with Fenton's reagent, KTBA and •OH scavengers as indicated were as described in the text.
[c]γ-Irradiation of aqueous solutions of KTBA and •OH scavengers were as described in the text.
[d]1 mg of catalase (2.5×10^3 units/mg) was added as indicated.

The generation of •OH by Fenton's reagent and γ-irradiation of water has been well documented (23,25). As shown in Table 2, when KTBA was exposed to either of those systems, ethylene was generated. In addition, as shown in Table 2, the inhibitory effect of the radical scavengers on the generation of ethylene from KTBA was confirmed when γ-irradiation and Fenton's reagent were used for •OH formation. The results in Table 2 also show that exogenously added catalase--but not BSA--had a significant inhibitory effect on ethylene production and $^{14}CO_2$ evolution by the organism.

The inhibitory effect of methional on [^{14}C]-labeled lignin degradation is also shown in Table 2. In contrast, neither KTBA nor methional had a significant effect on $^{14}CO_2$ evolution when [^{14}C]-glucose was used as the labeled substrate under identical conditions (data not shown). Finally the inhibitory effect of lignin on the fungal production of ethylene is also shown in Table 2.

DISCUSSION

The results reported herein provide several indications that •OH is involved in lignin degradation by *P. chrysosporium*. Using a previously described assay (14,15) for •OH formation, we have shown that ethylene is generated from methional and KTBA by the fungus only after the cessation of primary growth and that ethylene generation parallels the appearance of ligninolytic activity. In addition, ethylene production and ligninolytic activity (12,20) are both repressed in agitated cultures and in cultures growing in the presence of excess concentrations of nitrogen. Further, the amount of ethylene production in a lignin degradation mutant (18) and a phenotypic revertant parallel the capacity of these strains to degrade lignin. All of these results suggest that •OH radical is produced as an integral part of the lignin degradation system.

H_2O_2, superoxide radical and singlet oxygen were not effective at generating ethylene (data not shown). Although it has been noted that alkoxy radicals also react with methional, their reactivity was only 10% that of •OH (16). Further, the more common occurrence of •OH in biological systems and the inhibi-

tory effect of catalase on ethylene production (implicating H_2O_2 as a •OH precursor) indicate that •OH is being produced under the conditions.

The effect of •OH scavengers on ethylene production and ligninolytic activity were examined in order to test this hypothesis. Thiourea, salicylate, mannitol, 4-O-methylisoeugenol, and catalase all had similar inhibitory effects on ethylene production and ligninolytic activity. Parallel effects of these •OH scavengers on ethylene generation were also observed when Fenton's reagent and γ-irradiation were used as sources of •OH. Finally, lignin inhibited ethylene generation and methional inhibited lignin degradation by the fungus.

Hydroxyl radical meets the criteria for a ligninolytic catalyst; it is nonspecific and oxygen derived. Its known reactivity, hydroxylation of aromatic rings (24) and demethylation of methoxylated aromatic rings (26) is also suggestive. Its importance in other relatively nonspecific biological systems including the microbicidal activity of phagocytes (27,28), where it was assayed using the ethylene generation assay (29), and in cellular lipid peroxidation (30,31) had been previously documented. Recently, we have determined that •OH generating systems such as UV/H_2O_2 (24) have profound degradative activity towards polymeric lignin and several lignin model compounds (In preparation). Further studies are in progress on the reactivity of •OH with lignin and on the biochemistry of its origin in *P. chrysosporium*.

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