Degradation of Cellulose by *Poria placenta* in the Presence of Compounds that Affect Hydrogen Peroxide

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1. Introduction

It has been proposed that certain brown-rot fungi employ a non-protein system involving H_2O_2/Fe to breakdown cellulose in wood (J. W. KOENIGS, 1972 a - b, 1974 a - b). Hydrogen peroxide in the presence of Fe^{++} forms the highly reactive radical (·OH) which initiates oxidative degradation of carbohydrates. Support of this hypothesis was given by T. L. HIGHLEY (1977) who found that the brown-rot fungus, *Poria placenta,* produced oxidized cellulose degradation products. In addition, it was determined that *P. placenta* could not utilize cellulose without the addition of a readily utilizable carbon source, such as glucose (T. L. HIGHLEY, 1975 a, 1977). These results suggested that a metabolic product, such as H_2O_2, may indeed be produced during the utilization of the alternative carbon source that attacks the cellulose and permits it to be utilized. Hydrogen peroxide may be involved in the initial breakdown of the cellulose molecule by brown-rot fungi which results in smaller fragments that may then be acted on by carbohydrate-degrading enzymes to produce monomer sugars. For the hydrogen peroxide system to be operative, an oxidative enzyme must be produced which generates hydrogen peroxide by oxidation of an alternate carbon source. These enzymes have been found in many wood-decaying basidiomycetes as well as other types of fungi (J. W. KOENIGS, 1972 b).

To further clarify the role of H_2O_2 in brown rot, cellulose degradation by *P. placenta* was observed in the presence of various materials that

[1] Maintained at Madison, Wis., in cooperation with the University of Wisconsin

might effect levels of H_2O_2: (a) oxidizing enzyme inhibitors, (b) chelators, (c) ·OH and H_2O_2 quenching agents, (d) protein-synthesizing inhibitors, (e) thiol reagents, (f) salts, (g) phenolics and wood extracts, and (h) miscellaneous.

2. Materials[2] and Methods

Pieces of sterile cellulose (50 mg, purified, Herculus) were soaked in solutions of the test materials sterilized with a Millipore filter (0.45 μm). Hot water wood extracts were prepared by a previously described method (T. L. HIGHLEY. 1975 b). After air drying under aseptic conditions the cellulose was exposed to attack by P. placenta using the standard ASTM soil-block method previously described (T. L. HIGHLEY, 1977). Treated cellulose was also exposed in soil-block test without P. placenta to determine nonbiological breakdown.

After 12 weeks measurements were made of weight loss and degree of polymerization (DP). Percentage weight loss was determined by comparing weights of cellulose, conditioned at 27° C and 70 percent relative humidity, before and after exposure to P. placenta. Average DP was determined viscosimetrically with cupriethylenediamine hydroxide solvent (Ecusta Paper Corp.) (T. L. HIGHLEY, 1977).

With the above test method, it is not possible to determine if a material acts by inhibiting the cellulose-degrading system or if it acts on some other system that prevents growth. Thus, to determine their toxicity, *P. placenta* was grown in the presence of the materials in liquid culture with glucose as the carbon source.

3. Results and Discussion

Table 1 presents the effect of the various materials on cellulose depolymerization, cellulose utilization by *P. placenta*, and its growth in liquid culture with glucose as the carbon source.

If the brown-rot fungus, *P. placenta*, employs H_2O_2 to depolymerize cellulose in wood, the fungus must first produce an oxidative enzyme that oxidizes a substrate such as simple sugars, hemicelluloses, phenolics, or Lignin, yielding oxidized substrate and H_2O_2. Inhibition of the oxidase should therefore prevent cellulose breakdown. In this work most of the antioxidants did not inhibit cellulose utilization. The only antioxidants that prevented cellulose utilization, butylated hydroxy toluene and propyl gallate, were toxic to the fungus with glucose as the carbon source; thus, their effect was probably not on the cellulolytic system.

Many of the reported oxidases require metals as cofactors and, therefore, chelators should render these enzymes inactive. The chelators

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Table 1. Degradation of cellulose by *Poria placenta* in the presence of materials influencing levels of H_2O_2

Treatment	Conc.	Weight loss (% of untreated control)	DP (% of un-inoculated)	Growth on glucose liquid media[a)]
	Antioxidants			
Diethyl dithiocarbamate	10^{-2} M	100	< 10.1[c)]	+
Sodium dithionate	10^{-2}	160	< 9.5	++
Sodium metabisulfite ..	10^{-2}	100	< 9.5	++
Sodium azide	10^{-2}	175	< 9.5	0
Ethylene diamine tetra-acetate (Na)	10^{-2}	150	13.3	++
Glutathione	10^{-2}	100	20.0	++
Hydroquinone	10^{-2}	200	< 9.5	++
Quinacrine	10^{-2}	65	5.0	+
Hydroxylamine	10^{-2}	100	< 9.5	0
Cysteine	10^{-2}	100	10.6	0
Butylated hydroxy anisole	10^{-2}	100	< 9.5	0
Butylated hydroxy toluene	10^{-2}	0	75.3	0
Propyl gallate	10^{-2}	0	75.2	0
	H_2O_2 Stabilizers			
Acetanilide	10^{-3} M	86	42.4	++
Acetanilide	10^{-4}	78	23.6	++
Quinine sulfate	10^{-4}	56	7.5	++
Amino triazole	10^{-4}	115	13.5	++
Amino triazole	10^{-5}	110	15.8	++
Urea	10^{-2}	118	14.5	++
Sodium bisulfate	10^{-4}	63	13.5	++
	Chelators			
$MgCO_3$	0.5 %	109	< 9.5	++
MgOH	.5 %	109	< 9.5	0
Sodium silicate	.1 %	82	10.0	0
Sodium silicate	.5 %	0	23.0	0
Phenanthroline	10^{-2} M	0	37.0	0
Phenanthroline	10^{-4}	110	8.1	0
Bathophenanthroline-disulfonic acid (BPS)	10^{-3}	0	68.9	0
BPS	10^{-5}	100	< 9.5	++
Dipyridyl	0.01 %	100	9.9	0
Dipyridyl	.1 %	110	10.7	0
Dipyridyl	.5 %	122	10.1	0
Gluconate	.5 %	0	12.3	++
Gluconate	1.0 %	0	61.8	++

Treatment	Conc.	Weight loss (% of untreated control)	DP (% of uninoculated)	Growth on glucose liquid media[a]
·OH and H_2O_2 Quenching Agents				
KI	10^{-1} M	0	100.0	0
KI	10^{-2}	0	23.3	++
KI	10^{-3}	30	10.0	++
$KMnO_4$	0.05 %	0	31.6	0
Sodium pyruvate	.5 %	30	20.8	++
Sodium pyruvate	1.0 %	0	100.0	++
Thiourea	10^{-2} M	0	44.0	+
Thiourea	10^{-4}	100	< 9.5	++
Mannitol	0.1 %	0	78.2	++
Mannitol	0.5 %	35	11.7	++
Formic acid	0.05 %	35	19.2	++
Formic acid	0.05 %	0	80.3	0
Sodium formate	10^{-1} M	0	100.0	+
Sodium formate	10^{-3}	0	100.0	++
Thiol Reagents				
p-chloromercuribenzoate	10^{-3} M	0	82.6	0
p-chloromercuribenzoate	10^{-4}	0	31.0	0
Iodoacetate	10^{-2}	0	87.5	0
Iodoacetate	10^{-4}	110	11.4	++
Indole	10^{-2} M	0	89.8	0
Indole	10^{-4}	140	18.1	++
Iodosuccinimide	10^{-2}	0	41.2	0
Iodosuccinimide	10^{-4}	125	12.1	+
Iodoacetamide	10^{-2}	25	< 9.5	0
Iodoacetamide	10^{-3}	85	< 9.5	0
Phenolics and Wood Extracts				
Tannic acid	10^{-2} M	0	66.4	0
Tannic acid + PO[d]	10^{-2}	0	66.3	0
Ferulic acid	10^{-2}	0	66.9	0
Ferulic acid + PO	10^{-2}	0	83.2	0
Pyrogallol	10^{-2}	0	65.4	0
Pyrogallol + PO	10^{-2}	0	115.2	0
Catechol	10^{-2}	0	25.3	0
Catechol + PO	10^{-2}	0	62.8	0
White oak	—[b]	50	18.5	—
White oak + PO	—	125	33.5	—
Redwood	—	0	75.1	—
Redwood + PO	—	50	14.0	—
Douglas-fir	—	25	23.5	—
Douglas-fir + PO	—	40	19.1	—

Treatment	Conc.	Weight loss (% of untreated control)	DP (% of un-inoculated)	Growth on glucose liquid media[a]
		Salts		
NH_4SO_4	0.5 %	0	75.6	++
NH_4NO_3	.5	0	66.2	++
NH_4NO_3 + glucose	.5	0	48.4	++
Na arsenate	.5	0	100.0	0
$ZnSO_4$	.5	0	78.9	0
$MgSO_4 \cdot 7H_2O$	.5	80	12.8	++
Na bifluoride	.5	0	80.5	0
KCl	.5	100	< 9.5	++
$CaCl_2$	.5	113	< 9.5	++
$NaNO_3$	.5	100	< 9.5	++
$NaNO_2$	.5	100	< 9.5	0
		Protein-Synthesizing Inhibitors		
8-Azaquanine	10^{-2} M	68	15.5	0
8-Azaquanine	10^{-4}	108	12.8	+
Chloroamphenicol	10^{-4}	0	17.8	0
Chloroamphenicol	10^{-5}	74	15.8	++
Cycloheximide	10^{-5}	0	78.1	0
Cycloheximide	10^{-6}	51	11.6	++
Guanazolo	10^{-3}	36	13.6	++
Guanazolo	10^{-4}	92	7.3	++
Fluorouracil	10^{-4}	0	43.7	0
Fluorouracil	10^{-2}	0	80.2	0
Fluorouracil	10^{-3}	0	68.2	0
Puromycin	10^{-3}	50	18.4	++
Puromycin	10^{-4}	25	14.4	++
Puromycin	10^{-2}	0	60.0	+
		Miscellaneous		
δ-Gluconolactone	10 %	130	< 9.5	++
Peptone	.5	100	< 9.5	++
Malt extract	.5	100	< 9.5	++
Casein amino acids	.5	112	< 9.5	++
Yeast extract	.5	105	< 9.5	++
Glycolic acid	.5	100	< 9.5	++

a) 0, No growth.
+, Growth but less than controls without chemical.
++, Growth approximately equal to controls.
b) −−, Not determined.
c) Flow in viscosimeter too fast to determine minimum DP with the concentration of cellulose used.
d) Phenoloxidase.

EDTA and diethyl dithiocarbamate. often reported as inhibitors of oxidases, were ineffective in preventing cellulose utilization. It is difficult, however, to determine the action of these chelators because they depend on pH for maximum effectiveness. For example, diethyl dithiocarbamate, which is specific for copper-containing enzymes, is unstable at low pH's. *Poria placenta* lowers the pH of the substrate during growth and, therefore, may render the chelator ineffective.

Cellulose was degraded about the same with or without the presence of H_2O_2 stabilizers. Depending on how H_2O_2 is stabilized, cellulose degradation could either be enhanced or inhibited by these stabilizers. If, for example, the stabilizer prevents destruction of H_2O_2 by inhibiting catalase, degradation should be enhanced. Aminotriazole acts in this way, and did slightly enhance utilization. On the other hand, if the stabilizer acts by preventing radical formation, degradation would be inhibited. Transition metals such as Fe, Cu, and Co promote free radical generation by catalyzing the decomposition of peroxides to radicals, resulting in higher oxidation rates and a more extensive oxidative depolymerization of cellulose. Thus, materials that deactivate these metals should stabilize cellulose.

Magnesium compounds retard cellulose depolymerization in alkaline oxygen bleaching by deactivating the transition metal catalysts (G. E. PAVLOVOVA and W.H. RAPSON, 1973). In this work Mg did not prevent cellulose decomposition by P. placenta because it has been shown to have a negligible effect on metal deactivation at pH's below 9 or 10 (J. L. MINOR and N. SANYER, 1974). Silicates also tie-up metals required for OH formation. They were effective in preventing utilization at the two highest concentrations but not depolymerization. However, growth in liquid culture was inhibited at these concentrations, indicating that the effect may be toxicity toward the fungus. At the lowest concentration of silicate (0.1%), cellulose was depolymerized and utilized even though silicate prevented growth in liquid culture. Phenanthroline (10^{-2} M) prevented cellulose utilization but not depolymerization. At 10^{-4} M phenanthroline permitted both depolymerization and utilization although growth was inhibited in liquid culture. Dipyridyl, a powerful iron chelator, prevented growth in liquid culture at all concentrations, but did not inhibit cellulose utilization or depolymerization. Bathophenanthroline-disulfonic acid (B. HALLIWELL, 1978), another iron chelator, inhibited utilization and depolymerization at 10^{-3} M. However, this material was toxic to *P. placenta* in liquid culture at that concentration. Gluconate was not toxic to *P. placenta* in liquid culture but prevented cellulose utilization and inhibited depolymerization at 1.0% conc.

Complexing agents should stabilize cellulose by tying up metals. In this work not all of the chelators prevented cellulose utilization by *P. placenta*, but some did, indicating the important role of metals in cellulose metabolism by this fungus. However, without strict pH control, it is difficult to discuss the effects of complexing or chelating agents on specific metals. The effectiveness of a complexing agent on metal deactivation (or activation) depends on the stability constant of the particular complex at a specific pH.

The effect of the metal complexing agents on the cellulose-utilizing system of P. placenta might be better determined under a system free of wood and soil and with better pH control. Degradation of cellulose treated with chelators may not be inhibited because of of ·OH radical formation on the wood below the treated cellulose. Use of an agar system without wood might eliminate this problem.

Potassium iodide, potassium permanganate, and sodium pyruvate quench radicals and H_2O_2 (P. S. HOFFMAN et al., 1979; J. L. MINOR and N. SANYER, 1974; M. K. RAYMAN, 1978). Potassium iodide is used in oxygen pulping to destroy H_2O_2 and pyruvate and permanganate in certain bacteriological media to quench H_2O_2 (J. L. MINOR and N. SANYER, 1974; M. K. RAYMAN, 1978). Depolymerization und utilization were reduced in the presence of these materials at the higher concentrations. Furthermore, the .OH scavengers, mannitol, formate, and thiourea, inhibited cellulose utilization and depolymerization. Thus, the results with the ·OH and H_2O_2 quenching agents support the H_2O_2/Fe^{++} system of cellulose degradation by P. placenta.

Most of the protein-synthesizing inhibitors were very toxic to P. placenta and, therefore, had to be used at very low concentrations to determine their effect on cellulose utilization. At concentrations not toxic to P. placenta, puromycin (10^{-2} M), guanazolo (10^{-3} M), and cycloheximide (10^{-6} M) inhibited cellulose utilization. However, these compounds had little inhibitory effect on cellulose depolymerization. It is interesting that chloroamphenicol (10^{-4} M) completely inhibited cellulose utilization while depolymerization remained extensive. This finding suggests that two distinct systems are involved in cellulose utilization by the brown-rot fungus, *P. placenta.* The protein-inhibiting materials could inhibit the formation of an enzyme, such as a B-D-glucosidase, necessary for the breakdown of small chains to metabolizable products, while not affecting the system responsible for initial depolymerization.

Cellulose was utilized at concentrations of thiol reagents not inhibitory to growth in liquid culture. Iodoacetamide inhibited growth in liquid culture but cellulose was depolymerized and utilized at the concentrations used. Thus, sulfhydryl-requiring enzymes evidently are not involved in cellulose utilization by P. placenta. In an earlier study (T. L.

HIGHLEY, 1975 b), these reagents were also inactive toward cellulase activity (carboxymethylcellulose) of *P. placenta.*

Sodium arsenate, sodium bifluoride, and zinc sulfate inhibited cellulose utilization and growth in liquid culture with glucose. It is interesting that the ammonium nitrogen salts inhibited cellulose depolymerization and utilization. These salts are usually employed as nitrogenous sources in liquid culture studies and could be a factor in the inability of P. placenta to utilize cellulose in liquid culture.

D. PARK (1976) found cellulolytic fungi to vary considerably in their response to nitrogen levels on cellulose decomposition. Decomposition was decreased by some fungi as nitrogen concentration increased. H. SCHMITZ and F. KAUFERT (1936) and W. P. K. FINDLAY (1934) report decreased decay rates in wood when ammonium nitrate levels over 0.1 percent were used. Organic nitrogen sources were not inhibitory to decay of wood. In this study organic nitrogen also did not inhibit cellulose decomposition by P. placenta. The ammonium salt nitrogen could be inhibitory to a key enzyme necessary for cellulose utilization. For example, T. NAKAMATSU et al. (1975) found that *Penicillium purpurogenum* did not produce glucose oxidase activity on media containing ammonium salts.

The wood extracts generally reduced cellulose utilization but, except for redwood, they were not as effective as the phenolics. Although cellulose was not utilized in the presence of the phenolics, some chain-shortening did occur, particularly in the presence of catechol. This reresult is further evidence that two distinct systems are operative in the cellulose-degrading system of P. placenta. It is not possible to speculate as to the effect the phenolics might have on the cellulose-degrading system of *P. placenta* because of the toxicity of the compounds as evidenced by their effect on growth in liquid cultures with glucose.

This study did not fully clarify the question of H_2O_2 involvement in cellulose degradation by *P. placenta* primarily because toxicity of many materials made it difficult to separate effects on growth from effects on cellulose degradation. It does appear evident, however, that because a number of materials stopped utilization but not depolymerization, two distinct systems are involved in the complete utilization of cellulose. Further evidence that H_2O_2 might be required for cellulose depolymerization was obtained by decreased cellulose breakdown in the presence of ·OH and H_2O_2 quenching agents. The results with antioxidants and chelators were less clear, but some of the interpretation problems with results from these materials have already been discussed. The inability of some of these materials to inhibit cellulose utilization might also be due to differences in the H_2O_2-generating oxidases of *P. placenta* from those of other fungi. Perhaps the next step should be to isolate

and characterize H_2O_2-generating oxidase(s) from *P. placenta*. The effect of different nitrogen sources and concentrations on production of these enzymes deserves attention. With this information cellulose degradation could be studied in the presence of materials inhibitory to the oxidase(s) of *P. placenta*.

4. Summary

Cellulose depolymerization and utilization by the brown-rot fungus, Poria placenta, was studied in the presence of materials that manipulate H_2O_2. Because a number of materials stopped utilization but not depolymerization, two distinct systems apparently are involved in the complete utilization of cellulose. Evidence that H_2O_2 might be involved in cellulose breakdown by P. placenta was obtained by decreased cellulose degradation in the presence of ·OH and H_2O_2 quenching agents. The results with other materials were less clear.

Zusammenfassung

Abbau von Cellulose durch *Poria placenta* in Gegenwart von Verbindungen, die auf Wasserstoffperoxid einwirken

Die Hydrolyse und Verwertung von Cellulose durch den Braunfäulepilz *Poria placenta* wurde in Zusammerthang mit Substanzen untersucht, die mit H_2O_2 reagieren. Da eine Reihe von Substanzen die Verwertbarkeit, aber nicht die Hydrolyse, aufhob, sind offensichtlich 2 verschiedene Systeme an dem vollständigen Aufschluß der Cellulose beteiligt. Der Nachweis, daß H_2O_2 am Abbau von Cellulose durch *P. placenta* beteiligt sein könnte, wurde durch einen verminderten Abbau der Cellulose in Gegenwart von .OH und H_2O_2-bindenden Mitteln erbracht. Die Ergebnisse mit anderen Substanzen waren weniger eindeutig.

Résumé

Degradation de la cellulose par le *Poria placenta* en présence de composés qui ont une influence sur le peroxyde d'hydrogène

La dépolymérisation de la cellulose et son utitisation par le champignon de pourriture brune *Poria placenta,* ont été étudiées en présence de substances qui agissent sur H_2O_2. Comme de nombreuses substances arrêtent l'utilisation mais non la dépolymérisation, deux systèmes distincts sont apparemment impliqués dans l'utilisation complete de la cellulose. L'evidence que H_2O_2 puisse être impliquée dans la dégradation de la cellulose par *P. placenta* a été obtenue par la diminution de la degradation de la cellulose en présence d'agents d'extinction tels que ·OH et H_2O_2. Les résultats avec d'autres substances étaient moins évidents.

Resumen

Degradación de la celulosa por *Poria placenta* en presencia de compuestos que actúan sobre el peróxido de hidrógeno

Se estudió la hidrólisis y la utilización de la celulosa por el hongo de la pudrición parda, *Poria placenta,* en presencia de sustancias que reaccionan con H_2O_2. Dado que una serie de sustancias detuvieron la utilización, mas

no la hidrólisis, es evidente que en la desintegración total de la celulosa participan dos sistemas diferentes. Prueba de que H_2O_2 podría estar interviniendo en la degradación de la celulosa por *Poria placenta* se obtuvo al reducirse tal degradación en presencia de agentes que combinan con ·OH y H_2O_2 Los resultados obtenidos con otras susiancias fueron menos patentes

References

FINDLAY, W. P. K.: Studies in the physiology of wood-destroying fungi. I. The effect of nitrogen content upon the rate of decay of timber. Ann. of Bot. *48* (1934) 109 - 117.

HALLIWELL, B.: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: its role in degradation of hyaluronic acid by a superoxide-generating system. FEBS Lett. *96* (1978) 238 - 242.

HIGHLEY, T. L.: Can wood-rot fungi degrade cellulose without other wood constituents? For. Prod. J. *25* (1975 a) 38 - 39.

— Inhibition of cellulases of wood-decay fungi. USDA For. Serv. Res. Pap. FPL 247. For. Prod. Lab., Madison, Wis. 1975 b.

— Requirements for cellulose degradation by a brown-rot fungus. Material u. Organismen *12* (1977) 25 - 36.

HOFFMANN, P. S., H. A. GEORGE, N. R. KRIEG, and R. M. SMIBERT: Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. Can. J. Microbiol. *25* (1979) 8 - 16.

KOENIGS, J. W.: Effects of hydrogen peroxide on cellulose and its susceptibility to cellulase. Material u. Organismen *7* (1972 a) 133 - 147.

— Production of extracellular hydrogen peroxide and peroxidase by wood-rotting fungi. Phytopathology *62* (1972 b) 100 - 110.

— Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood and Fiber *6* (1974 a) 66 - 79.

— Production of hydrogen peroxide by wood-decaying fungi in wood and its correlation with weight loss, depolymerization, and pH changes. Arch. Mikrobiol. *99* (1974 b) 129 - 145.

MINOR, J. L., and N. SANYER: Carbohydrate stabilization with iodide in oxygen bleaching of kraft pulps. Tappi *57* (1974) 109 - 112.

NAKAMATSU, T. T. AKAMATSU, R. MIYAJIMA, and I. SHIIO: Microbial production of glucose oxidase. Agric. Biol. Chem. *39* (1975) 1803 - 1811.

PARK, D.: Nitrogen level and cellulose decomposition by fungi. Int. Biodetn. Bull. *12* (1976) 95 - 99.

PAVLOVOVA, G. E., and W. H. RAPSON: Mechanism of magnesium retardation cf cellulose degradation during oxygen bleaching. Tappi *56* (1973) 95 - 99.

RAYMAN, M. K., B. AVIS, and H. B. DEREA: The effect of compounds which degrade hydrogen peroxide on the enumeration of heat-stressed cells of *Salmonella senftenberg*. Can. J. of Microbiol. *24* (1978) 803 - 885.

SCHMITZ, H., and F. KAUFERT: The effect of certain nitrogenous compounds on the rate of decay of wood. Am. J. Bot. *23* (1936) 577 - 634.

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