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Production of H_2O_2 in *Phanerochaete chrysosporium* during lignin degradation

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Evidence in support of an essential role for H_2O_2 in lignin degradation by the white-rot fungus *Phanerochaete chrysosporium* has been presented by several laboratories. H_2O_2 is formed simultaneously with the ligninolytic system, and when it is degraded by catalase the lignin-degrading capacity is also reduced.

We have now identified, purified and characterized a sugar-oxidizing enzyme that produces H_2O_2 during glucose starvation in *P. chrysosporium*. The enzyme oxidizes glucose at the 2-carbon position to yield glucosone, but δ-D-gluconolactone and xylose are also oxidized at significant rates.

Another H_2O_2-producing enzyme in *P. chrysosporium*, methanol oxidase, has also been identified, purified and characterized in this laboratory. Methanol is formed from the methoxyl groups in lignin. Hydrogen peroxide, necessary for further degradation of lignin, is formed by enzyme-catalysed oxidation of the lignin-derived methanol.

Induction and repression of the H_2O_2-producing enzymes is discussed, as well as ways for the fungus to control the glucose level in its environment.

One of the reasons for the presence of lignin in wood and other plant material is to increase their resistance to microbial degradation. The lignin polymer serves as a barrier that must be partly removed or at least morphologically changed before the wood polysaccharides can be attacked by the enzymes specific for these substrates.

The normal way for white-rot fungi to degrade wood is by a simultaneous attack on the polysaccharides and the lignin. It seems that an absolutely specific attack on the lignin is probably impossible for the microorganisms tested so far. The fungi seem to need polysaccharides or low molecular mass sugars to degrade lignin. The sugars thus derived are necessary to provide energy for growth and cell metabolism. They are also essential because it is the oxidation of these sugars that provides most of the hydrogen peroxide necessary for lignin degradation (Forney *et al.* 1982; Faison & Kirk 1983; Leisola *et al.* 1984; Ander & Eriksson 1985).

Several other physiological demands for lignin degradation by the white-rot fungus *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*) have been discovered and revealed. Thus, lignin metabolism occurs only during secondary metabolism (Kirk *et al.* 1978; Keyser *et al.* 1978). It has also been shown that the 'ligninolytic system' is 'turned on' by carbon, sulphur or particularly nitrogen limitation (Jeffries *et al.* 1981). It has recently been found that both lignin and veratryl alcohol increase ligninase production and lignin degradation by *P. chrysosporium* (Leisola *et al.* 1984; Faison & Kirk 1985). These findings suggest that there is a physiological connection between the biosynthesis of veratryl alcohol and lignin biodegradation, thus explaining why a *de novo* synthesis of veratryl alcohol takes place in *P. chrysosporium* even when grown only on glucose (Lundquist & Kirk 1978; Harvey *et al.* 1985).

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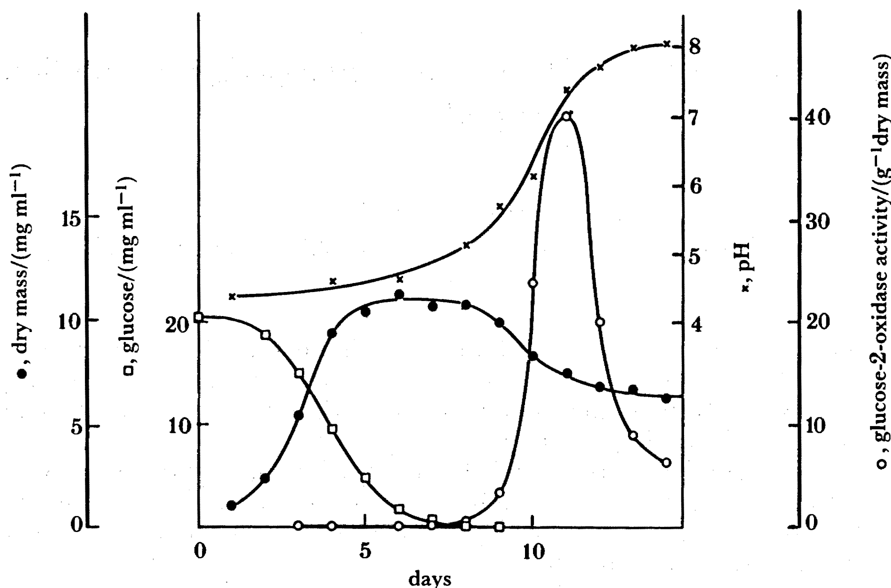

FIGURE 1. Glucose-2-oxidase production by *P. chrysosporium*. Symbols: □, glucose concentration in the culture medium; ●, mycelial dry mass; ○, glucose-2-oxidase activity; ×, pH values. (From Eriksson *et al.* (1986).)

Many enzymes participating in lignin metabolism have now been identified, purified and characterized (Eriksson 1983; Kirk 1985). Particularly, phenol oxidases (e.g. laccase) and peroxidases have been shown to be of importance for lignin degradation (Ander & Eriksson 1976). Recently, the so-called ligninases have been discovered in American laboratories (Tien & Kirk 1983; Glenn *et al.* 1983). These enzymes split the lignin polymer (C_α–C_β-cleavage) and depend on H_2O_2 for their activity. H_2O_2 is formed simultaneously with the ligninolytic system, and when destroyed by catalase the lignin-degrading capacity is also reduced (Faison & Kirk 1983).

An important question in this context is: how is the necessary H_2O_2 produced? Forney *et al.* (1982) and Reddy *et al.* (1983) suggested that glucose oxidase might be the major source of H_2O_2 production. However, the type of glucose oxidase and the reaction products were not identified by these authors.

In our laboratory, we have recently purified and characterized a sugar-oxidizing enzyme that produces H_2O_2 during glucose starvation in the white-rot fungus *P. chrysosporium*.

This enzyme, glucose-2-oxidase, primarily oxidizes glucose to 2-keto-glucose + H_2O_2 but is much less specific than glucose-1-oxidase, also being able to oxidize xylose. It seems likely that this is the enzyme studied by Reddy *et al.* (1983) because their enzyme, but not glucose-1-oxidase, is able to use xylose as a substrate. The glucose-2-oxidase enzyme also oxidizes δ-gluconolactone, considerably increasing its possibilities for H_2O_2 production compared with glucose-1-oxidase. If both glucose-1-oxidase and glucose-2-oxidase are produced, two moles of H_2O_2 can be produced from each mole of glucose. However, simultaneous occurrence of glucose-2-oxidase and glucose-1-oxidase activities in a fungus have not been reported so far. The substrate specificity of glucose-2-oxidase is evident from table 1.

The discovery of the glucose-2-oxidase enzyme seems to explain why a xylan-degrading Cel^- mutant of *P. chrysosporium* (*Sporotrichum pulverulentum*) degrades lignin much more efficiently than a Cel^- mutant lacking xylanase activity (Eriksson *et al.* 1983; Johnsrud & Eriksson 1985). Xylan is degraded to xylose, which is oxidized with simultaneous production of H_2O_2.

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TABLE 1. SUBSTRATE SPECIFICITY OF GLUCOSE-2-OXIDASE

(From Eriksson *et al.* (1986).)

substrate	relative activity	substrate	relative activity
D-glucose	100	L-arabinose	< 5
6-deoxy-D-glucose	73	L-fucose	< 5
δ-D-gluconolactone†	60	D-lyxose	< 5
L-sorbose	52	*N*-acetyl-D-glucosamine	< 5
D-xylose	37	lactose	< 5
2-deoxy-D-glucose	18	maltose	< 5
D-galactose	5	cellobiose	< 5
D-mannose	< 5	methyl-β-D-glucoside	< 5
D-fructose	< 5	methyl-α-D-glucoside	< 5
D-arabinose	< 5		

† A freshly prepared solution was used for the measurement.

We have recently found that the glucose concentration in itself can be of importance for H_2O_2 production. It thus seems as if the glucose concentration has a pronounced effect on the induction of glucose-oxidase activity. A high glucose concentration appears to repress the production of glucose-oxidizing enzymes. At low nitrogen concentrations, the fungus is unable to metabolize glucose. To regulate the glucose level in its surroundings, the fungus produces an extracellular, slimy polysaccharide, the nature of which seems to be a branched β-1,3-,β-1,6-glucan. During secondary metabolism, this polysaccharide is again degraded by a 1,3-β-glucanase produced by the fungus. This enzyme has been purified and characterized in our laboratory. The 1,3-β-glucanase enzyme provides glucose for oxidation and simultaneous production of H_2O_2 (B. Bes, B. Pettersson, H. Lennholm, T. Iversen & K.-E. Eriksson, in preparation).

We have recently found another important H_2O_2-producing enzyme that is also produced under secondary metabolism by *P. chrysosporium*, namely methanol oxidase. This enzyme is the more interesting because it produces hydrogen peroxide during the metabolism of lignin itself. We have earlier demonstrated (Ander *et al.* 1983; Ander & Eriksson 1985; Ander *et al.* 1985) that considerable amounts of methanol are formed from lignin and lignin degradation products in culture solutions of *P. chrysosporium*. This methanol is oxidized to formaldehyde by the enzyme methanol oxidase and as a by-product of this oxidation H_2O_2 is formed (A. Nishida & K.-E. Eriksson, in preparation). It can be added that the importance of H_2O_2 itself for the demethoxylation of lignin and lignin degradation products has also been demonstrated recently (Ander & Eriksson 1985; Ander *et al.* 1985).

The enzyme methanol oxidase is synthesized when the fungus is grown on glucose alone, but a higher production is obtained if vanillate is present in the medium. The enzyme is not absolutely specific for methanol but also oxidizes ethanol, *n*-propanol and *n*-butanol, although at considerably reduced rates, as is evident from table 2. The relation between oxygen uptake and formaldehyde formation is demonstrated in table 3. It can be seen that slightly more formaldehyde is formed compared with that expected for the uptake of oxygen. The reason for this slight discrepancy is not known. It has also been found that slightly more hydrogen peroxide is formed compared with the oxygen uptake. A consumption of 44.5 nmol oxygen min^{-1} results in the formation of 49.4 nmol H_2O_2 min^{-1}. The reason for this is not known either. The alcohol oxidase has been isolated, purified and characterized. It has been found to have a molecular mass of approximately 310000. When it is run on SDS gels, the enzyme

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TABLE 2. SUBSTRATE SPECIFICITY OF METHANOL OXIDASE

(From A. Nishida & K.-E. Eriksson, in preparation.)

substrate	amount/μmol	enzyme/ml	relative activity (%)
methanol	300	0.1	100
ethanol	500	0.1	91.9
n-propanol	500	0.1	34.4
n-butanol	300	0.2	10.6
isopropanol	500	0.5	1.7
ethylene glycol	500	0.5	3.8
ethylene glycol-monomethylether	500	0.5	10.0
glycelin	500	0.5	0.0
vanillyl alcohol	1.0	0.2	0.0
formaldehyde	500	0.4	0.0

TABLE 3. RELATION BETWEEN OXYGEN UPTAKE AND FORMALDEHYDE FORMATION

(From A. Nishida & K.-E. Eriksson, in preparation.)

Methanol/μmol	O_2-uptake/nmol	formaldehyde formation/nmol	alcohol oxidase/ml
300	178	226	0.10
—	292	342	0.15
—	391	413	0.20

yields subunits with a molecular mass of 75000 each, suggesting that the enzyme is composed of four such subunits. The enzyme has also been shown to carry FAD as a prosthetic group. The FAD content was calculated from spectrophotometric data to be 5.62 mol per mol of enzyme.

This alcohol oxidase, which produces H_2O_2 during secondary metabolism, seems to be of great importance for lignin degradation. It is thus interesting to conclude that the fungus can contribute to its ability to degrade lignin by producing the necessary H_2O_2 from the lignin itself.

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Discussion

C. Evans (*Thames Polytechnic, London, U.K.*). Is there any evidence that the slime is binding monomeric sugars from the culture medium? In our studies with *Coriolus versicolor*, the mucilage layer round the hyphae increases partly because sugars from the growth medium are bound within the polymer. Removing these sugars from isolated mucilage is difficult, requiring extensive dialysis or repeated gel-filtration.

K.-E. Eriksson. We have not studied whether the slime is binding monomeric sugars. It seems more likely to me that the monomeric sugars are released from the slime by the β-1,3-glucanase degrading the polysaccharide.