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Ultrastructural Localization of Hydrogen Peroxide Production in Ligninolytic *Phanerochaete chrysosporium* Cells†

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Previous studies have shown that the hydroxyl radical derived from hydrogen peroxide (H_2O_2) is involved in lignin degradation by *Phanerochaete chrysosporium*. In the present study, the ultrastructural sites of H_2O_2 production in ligninolytic cells of *P. chrysosporium* were demonstrated by cytochemically staining cells with 3,3′-diaminobenzidine (DAB). Hydrogen peroxide production, as evidenced by the presence of oxidized DAB deposits, appeared to be localized in the periplasmic space of cells from ligninolytic cultures grown for 14 days in nitrogen-limited medium. When identical cells were treated with DAB in the presence of aminotriazole, periplasmic deposits of oxidized DAB were not observed, suggesting that the deposits resulted from the H_2O_2-dependent peroxidatic oxidation of DAB by catalase. Cells from cultures grown for 3 or 6 days in nitrogen-limited medium or for 14 days in nitrogen-sufficient medium had little ligninolytic activity and low specific activity for H_2O_2 production and did not contain periplasmic oxidized DAB deposits. The results suggest that in cultures grown in nitrogen-limited medium, there is a positive correlation between the occurrence of oxidized DAB deposits, the specific activity for H_2O_2 production in cell extracts, and ligninolytic activity.

Previous studies have shown that the ligninolytic system of *Phanerochaete chrysosporium* is synthesized after the cessation of primary growth and in response to nitrogen starvation, apparently as a part of secondary metabolism (an idiophasic event) (13). Furthermore, a temporal relationship between the appearance of ligninolytic activity and hydrogen peroxide (H_2O_2) production activity in nitrogen-limited cultures of *P. chrysosporium* has been demonstrated (L. J. Forney and C. A. Reddy, J. Biol. Chem., in press). The hydrogen peroxide-derived hydroxyl radical (· OH) plays an integral role in lignin degradation, as evidenced by the inhibition of lignin degradation by benzoate and mannitol, which scavenge · OH (Forney and Reddy, in press).

The hydroxyl radical is highly reactive and causes damage to cellular constituents such as proteins, lipids, and DNA (2, 6, 16). In many cells these effects are minimized by compartmentalizing the H_2O_2 production activity and catalase within subcellular organelles such as peroxisomes and glyoxysomes (8, 22). In light of the observation that high levels of H_2O_2 are produced by cell extracts of ligninolytic cultures of *P. chrysosporium* (Forney and Reddy, in press), in contrast to minimal levels of H_2O_2 being produced by non-ligninolytic cultures, it was of interest to determine how H_2O_2 production is localized in *P. chrysosporium* in such a way as to minimize its cytotoxic effects.

The diaminobenzidine (DAB) procedure is extensively used in the cytochemical demonstration of peroxidatic activity of heme-containing enzymes such as catalase (12). In the presence of exogenously added or endogenously produced H_2O_2, the peroxidatic activity of catalase catalyses the oxidation of DAB into an osmiophilic polymer which reduces osmium tetroxide to osmium black (12, 21). Sites of catalase are, thus, indicated by dark deposits of reduced osmium tetroxide. Aminotriazole, which is a known inhibitor of catalase activity (9, 18, 19) completely inhibits the DAB staining reaction attributed to the activity of endogenous catalase (7, 24).

The results of this study show that H_2O_2 production activity, as evidenced by the presence of oxidized DAB deposits, is localized in the periplasmic space of the cells from ligninolytic cultures, but not in cells from non-ligninolytic cultures.

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* Burdsall ME-446 (ATCC 34541) was obtained from

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TABLE 1. Effect of culture age and nitrogen concentration on DAB reaction, hydrogen peroxide production, and synthetic [^{14}C]lignin degradation by *P. chrysosporium*[a]

Culture age (days)	Nitrogen concn (mM)	DAB reaction[b]	H_2O_2 production[c]	% [^{14}C]lignin degraded[d]
3	2.4	−	2.9	0.1
	24	−	12.5	0.2
6	2.4	−	3.1	0.7
	24	−	ND[e]	0.4
10	2.4	+	22.1	5.1
	24	−	ND	0.6
14	2.4	+++	52.3	11.8
	24	−	6.1	0.9

[a] Cultures were incubated in air at 39°C without agitation in either nitrogen-limited (2.4 mM N) or high-nitrogen (24 mM N) medium.

[b] −, No oxidized DAB deposits; +, moderate oxidation of DAB; +++, extensive oxidation of DAB.

[c] Expressed as nanomoles of H_2O_2 minute^{-1} milligram of protein^{-1}.

[d] [^{14}C]lignin recovered as $^{14}CO_2$.

[e] ND, Not determined.

T. K. Kirk (Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wis.) and was maintained by periodic transfers on malt extract agar slopes as previously described (14).

The composition of the nitrogen-limited basal medium used for these experiments has been described previously (14) and contained 0.6 mM asparagine plus 0.6 mM NH_4NO_3 (2.4 mM N). The high-nitrogen medium was identical to the basal medium except that it contained 6 mM asparagine plus 6 mM NH_4NO_3 (24 mM N). The medium (50 ml) was dispensed into 500-ml Erlenmeyer flasks, which were then foam stoppered and autoclaved for 15 min at 121°C. The inoculum consisted of a conidial suspension of *P. chrysosporium* in water prepared as previously described (14). The cultures were incubated in air at 39°C without agitation for various periods of time as indicated below.

Cytochemical staining techniques. Hydrogen peroxide production activity in cells of *P. chrysosporium* was demonstrated by cytochemical staining with 3,3′-DAB tetrahydrochloride (Aldrich Chemical Co., Milwaukee, Wis.) by a procedure modified from that of Van Dijken and Veenhuis (23). The cells were harvested by centrifugation at 12,000 × *g* for 10 min and washed once with 50 mM sodium phosphate (pH 5.5). The pellet was resuspended in the same buffer, homogenized with a glass tissue homogenizer, and centrifuged as described above. The cells were fixed in 3% glutaraldehyde–0.1 mM cacodylate buffer (pH 7.0) since glutaraldehyde treatment greatly enhances peroxidatic oxidation of DAB by catalase (10, 12). The cells were then treated with aerated DAB solutions (2 mg of DAB and 100 μmol of glucose per ml of sodium phosphate buffer [70 mM, pH 6.6] at 39°C for 1 h). In a control experiment glutaraldehyde-fixed cells were preincubated for 30 min in phosphate buffer (described above) containing 50 μmol of 3-amino-1,2,4-triazole (aminotriazole) (Sigma Chemical Co., St. Louis, Mo.) per ml. These cells were treated with a DAB solution identical to that described above except that it contained an additional 50 μmol of aminotriazole per ml. After 30 min, the cells from both treatments were transferred to fresh DAB solutions. These DAB-treated cells were postfixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.0) at room temperature for 45 min, washed with the same buffer, and held in 1% aqueous uranyl acetate overnight. The uranyl acetate was decanted, and the cells were embedded in 1% Noble agar. A thin layer of solidified agar was cut into 2-mm squares, dehydrated in a graded alcohol series and propylene oxide, and then embedded in Poly-bed (Polysciences, Warrington, Pa.). After being sectioned, the specimens were poststained for 10 min with 2% uranyl acetate and for 3 min with lead citrate and examined with a Philips EM 300 electron microscope.

H_2O_2 production. The specific activity for H_2O_2 production in cell extracts of *P. chrysosporium* was determined by using a modified catalase-aminotriazole assay as previously described (4; Forney and Reddy, in press).

Degradation of synthetic [^{14}C]lignin. The degradation of synthetic [2′-^{14}C]lignin (10^5 dpm mg^{-1}) to $^{14}CO_2$ was determined as described previously (Forney and Reddy, in press).

RESULTS

Correlation between occurrence of oxidized DAB deposits, specific activity for H_2O_2 production, and ligninolytic activity. Experiments were conducted to determine the relationship between levels of ligninolytic activity, H_2O_2 production, and the presence of deposits of oxidized DAB within the cells. Less than 1% of the synthetic [^{14}C]lignin was degraded during the first 6 days of incubation by cells grown in nitrogen-limited medium, whereas 5.1 and 11.8% of the added [^{14}C]lignin was degraded after 10 and 14 days of incubation, respectively (Table 1). Furthermore, extracts prepared from cells grown for 3, 6, 10, and 14 days in nitrogen-limited medium had specific activities for H_2O_2 production (in nanomoles of H_2O_2 minute^{-1} milligram of protein^{-1}) of 2.9, 3.1, 22.1, and 52.3, respectively (Table 1). Thus, only cells from 10- and 14-day-old cultures of *P. chrysosporium*, which were ligninolytic, had significant levels of H_2O_2 production activity, whereas 6-day-old cultures did not have appreciable levels of ligninolytic activity or H_2O_2 production activity. When cells from 10- and 14-day-old nitrogen-limited cultures were stained with DAB, electron-dense deposits of oxidized DAB were observed between the cell wall and the cytoplasmic membrane of the fungal cells (Fig. 1A and C). In contrast, in cells from 3- and 6-day-old cultures, these periplasmic deposits were not seen (Fig. 1D and E). The deposits of oxidized

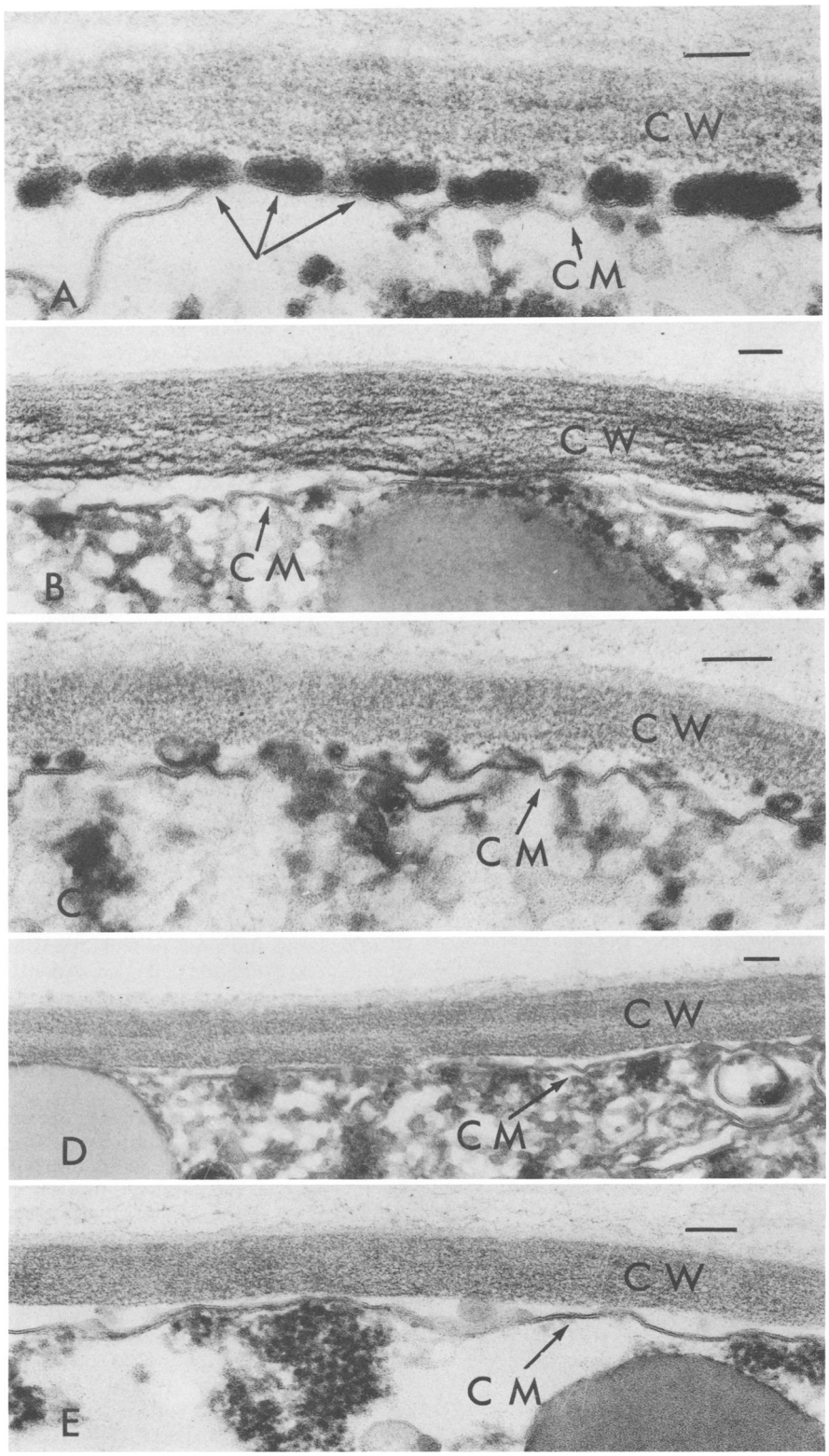

FIG. 1. Transmission electron micrographs of *P. chrysosporium*, showing details of cell wall (CW), periplasmic area, and cytoplasmic membrane (CM). Deposits of oxidized DAB (see text for details) are indicated by arrows. Bar = 0.1 μm. Cells were grown in nitrogen-limited medium for 14 (A), 10 (C), and 6 (D) days or in medium not limited in nitrogen for 14 days (E). (B) is identical to (A) except for the addition of aminotriazole to the staining solution. Magnifications: A, 90,100×; B, 60,000×; C, 113,400×; D, 56,700×; E, 80,000×.

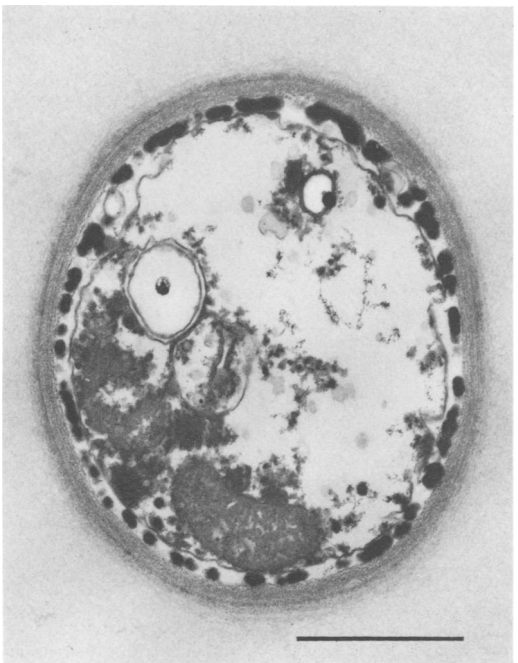

FIG. 2. Transmission electron micrograph of a transverse section of a cell of *P. chrysosporium* grown for 14 days in nitrogen-limited medium. Note the fairly even distribution of the deposits of oxidized DAB in the periplasmic space. Magnification, 21,500×; bar = 1 μm.

DAB deposits were distributed rather evenly in the periplasmic space in the cells from 14-day-old cultures (Fig. 2). The H_2O_2 production in 10-day-old cells grown in nitrogen-limited medium was less than half of that in 14-day-old cells (Table 1). As one might expect, the staining intensity and number of deposits of oxidized DAB in the periplasmic space of 10-day-old cells were qualitatively less than those in 14-day-old cells (Fig. 1C). These data suggest a positive correlation between the numbers of periplasmic deposits of oxidized DAB, which appear to be the sites of H_2O_2 production, and the ligninolytic activity of *P. chrysosporium*.

Little lignin degradation occurred in cultures of *P. chrysosporium* grown in high-nitrogen medium (Table 1). Also, the specific activity for H_2O_2 production decreased in these cultures from 12.5 on day 3 to 6.1 on day 14. Furthermore, in these 14-day-old cultures the specific activity for H_2O_2 production was approximately eightfold lower than that observed in similar cells grown in nitrogen-limited medium. If there was a positive correlation between the number of periplasmic deposits of oxidized DAB and ligninolytic activity, as indicated by the results described above, then one would expect few or no periplasmic deposits in cells from 14-day-old non-ligninolytic cultures of *P. chrysosporium* grown in high-nitrogen medium. The results showed that this was the case (Fig. 1E).

Inhibition of DAB staining reaction by aminotriazole. Aminotriazole is a known inhibitor of catalase activity (3, 9, 18, 19) and completely inhibits the DAB staining reaction which is due to peroxidative oxidation of DAB by catalase (7, 12, 24). Therefore, when cells from 10- and 14-day-old nitrogen-limited cultures were incubated with DAB in the presence of aminotriazole, deposits of DAB would not be expected to be formed if only catalase (and no peroxidase) was present in the periplasmic space. The results (Fig. 1B) showed that this was the case, indicating that the electron-dense regions observed in the electron micrographs (Fig. 1A and C) were due to the peroxidatic oxidation of DAB by catalase.

DISCUSSION

Lignin was not degraded during the first 6 days of incubation in cultures of *P. chrysosporium* grown in nitrogen-limited medium (2.4 mM N), and the H_2O_2 production activity of these cells was also low. Similarly, cells from 14-day-old cultures of *P. chrysosporium* grown in high-nitrogen medium (24 mM N) degraded less than 1% of the added synthetic [^{14}C]lignin and exhibited low levels of H_2O_2 production activity. The most recent results of Kelley and Reddy (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982. K115, p. 155) show that various nutritional parameters which are known to affect lignin degradation (13, 14) by *P. chrysosporium* apparently do so by affecting H_2O_2 production.

Deposits of oxidized DAB were observed in the periplasmic space of cells from ligninolytic cultures but not in non-ligninolytic cultures. Furthermore, the development of ligninolytic activity in nitrogen-limited cultures was concomitant with an increase in the specific activity for H_2O_2 production, which was in turn correlated with the appearance of periplasmic deposits of oxidized DAB. There was no noticeable difference in the number of cytoplasmic oxidized DAB-positive microbodies between cells from ligninolytic cultures and cells from non-ligninolytic cultures. These observations suggest that the H_2O_2 production activity associated with lignin degradation is localized in the subcellular structures located in the periplasmic space of cells from ligninolytic cultures. Most, but not all, of the hyphae examined from 14-day-old cultures contained deposits of oxidized DAB in the periplasmic space. In cells from 10-day-old cultures, the deposits of oxidized DAB were observed in a lower proportion of the cells examined and, when present, were less numer-

ous and smaller (Fig. 1C) than those in 14-day-old cells.

In the presence of H_2O_2, the oxidation of DAB can be accomplished by several enzymes, including peroxidase and catalase (12, 17, 21). When cells containing high specific activities for H_2O_2 production were treated with DAB in the presence of aminotriazole, deposits of oxidized DAB were not observed in the periplasmic space of the cells. Since aminotriazole inhibits catalase (3, 9, 18–20), these data indicate that peroxidatic oxidation of DAB was most likely effected by catalase and was not an artifact of the staining procedure. Furthermore, if significant levels of peroxidase had been present in the periplasmic space, deposits of oxidized DAB would have been observed in the presence of aminotriazole. This was not the case, indicating that little peroxidase was present in the periplasmic space.

Wood-decomposing fungi produce numerous enzymes, including various carbohydrate oxidases (15), cellobiose-quinone oxidoreductase (25), and aromatic alcohol oxidases (5, 11), which oxidize various substrates with the concomitant production of H_2O_2. Hydrogen peroxide could also be produced through the enzymatic or chemical dismutation of superoxide radicals (1, 2).

To our knowledge, these results represent the first time that H_2O_2 production activity and catalase have been demonstrated to be localized in the periplasmic subcellular structures of a fungus. This unusual arrangement would seem to be advantageous to *P. chrysosporium*. With the H_2O_2 production activity located outside the cytoplasmic membrane, the fungus is able to avoid high levels of H_2O_2 in the cytoplasm, where it could participate in cytotoxic processes, yet the enzymes responsible for the production of H_2O_2 are retained by the cell. Furthermore, the H_2O_2 could readily diffuse from the cells whereupon it could undergo reductive cleavage to produce $\cdot OH$, which is known to be involved in lignin degradation.

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