A novel alcohol oxidase (AOX) has been purified from mycelial pellets of the wood-degrading basidiomycete *Gloeophyllum trabeum* and characterized as a homo-octameric nonglycosylated protein with native and subunit molecular masses of 628 and 72.4 kDa, containing noncovalently bonded flavin adenine dinucleotide. The isolated AOX cDNA contained an open reading frame of 1,953 bp translating into a polypeptide of 651 amino acids displaying 51 to 53% identity with other published fungal AOX amino acid sequences. The enzyme catalyzed the oxidation of short-chain primary aliphatic alcohols with a preference for methanol (K_m = 2.3 mM, k_cat = 15.6 s^{-1}). Using polyclonal antibodies and immunofluorescence staining, AOX was localized to liquid culture hyphae and extracellular slime in sections from degraded wood and on cotton fibers. Transmission electron microscopy immunogold labeling localized the enzyme in the hyphal periplasmic space and wall and on extracellular tripartite membranes and slime, while there was no labeling of hyphal peroxisomes. AOX was further shown to be associated with membranous or slime structures secreted by hyphae in wood fiber lumina and within the secondary cell walls of degraded wood fibers. The differences in AOX targeting compared to the known yeast peroxisomal localization were traced to a unique C-terminal sequence of the *G. trabeum* oxidase, which is apparently responsible for the protein’s different translocation. The extracellular distribution and the enzyme’s abundance and preference for methanol, potentially available from the demethylation of lignin, all point to a possible role for AOX as a major source of H_2O_2, a component of Fenton’s reagent implicated in the generally accepted mechanisms for brown rot through the production of highly destructive hydroxyl radicals.

The basidiomycete *Gloeophyllum trabeum* is a representative of the brown rot fungi, which are characterized by a special pattern of wood decay during which they oxidatively degrade wood cell wall components, causing a rapid loss of wood strength (18). Brown rot fungi are the principal cause of the destructive form of decay observed in wooden constructions, particularly in temperate geographic areas (21). In addition, *G. trabeum* and *Serpula lacrymans* are possibly the most reported fungal species causing wood decay in houses. The molecular mechanisms and enzymatic systems these fungi use during the rapid and selective depolymerization of wood cellulose with incipient decay has been highly debated and researched for many years but remain controversial and not fully understood. In current decay models, a central role for a low-molecular-weight immediate depolymerizing agent that can substantially modify this polymer by efficient demethylation (15, 16), thus producing methanol, a preferred physiological substrate of AOX. Because methanol is unlikely to be of any

*Corresponding author. Mailing address: Department of Forest Products/Wood Science, Swedish University of Agricultural Sciences, P.O. Box 7008, SE-750 07 Uppsala, Sweden. Phone: 46-18672489. Fax: 46-18673489. E-mail: geoffrey.daniel@sprod.slu.se.
† G.D. and J.V. contributed equally to this study.
*Published ahead of print on 27 July 2007.

Received 1 May 2007/Accepted 23 July 2007

Vol. 73, No. 19

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2007, p. 6241–6253

© 2007, American Society for Microbiology. All Rights Reserved.
nutritional value for this fungus (in contrast to that known for methanol-utilizing yeasts), we suppose that AOX is more likely related to oxidative wood decay through $H_2O_2$ produced during its catalysis.

In this context, we analyzed the production of this enzyme in laboratory mycelial cultures and attacked wood; characterized some molecular, molecular-genetic and catalytic properties of the purified protein; and studied its ultrastructural localization in hyphae and degraded wood. Our findings of the extracellular localization of the enzyme associated with periplasmic and external membranous structures (i.e., tripartite membranes) strongly indicate that *G. trabeum* AOX is a component of the biodegradative system used by this fungus during brown rot decay of wood. The properties of this enzyme are further compared to AOXs from methanol-utilizing yeasts and molds.

**MATERIALS AND METHODS**

**Fungal strain.** The brown-rot fungus *G. trabeum* (Persoon; Fries) Karsten strain M617 (ATCC 11539) was obtained from the U.S. Department of Agriculture Forest Products Laboratory (Madison, WI). The fungus was routinely maintained on 2.5% (wt/vol) malt agar plates.

**Liquid cultures.** The fungus was grown in darkness at 27°C in 500-ml Erlenmeyer flasks containing 80 ml of complex medium (2% glucose, 1.5% corn steep, 0.15% MgSO$_4$·$7H_2O$ in tap water) under reciprocal shaking (8). The medium was adjusted to pH 5.5 with 5 M NaOH before autoclaving. Mildly homogenized cultures derived from malt-agar stock cultures, showing sufficient growth on the same liquid medium, were used for inoculations (6%). Fungal spherical colonies (2- to 8-mm mycelial pellets) for microscopy were harvested 10 and 14 days after inoculation.

**Cultures on wood.** Four wood blocks (3.0 by 1.5 by 0.5 cm) of birch (*Betula verrucosa* Ehr.) or pine (*Pinus sylvestris* L.) were placed on two pine feeder strips (6.0 by 2.0 by 0.5 cm) previously placed in 500-ml glass bottles (Jena ER Glass) containing 20 mM HCl [pH 7.5], 150 mM NaCl buffer) supplemented with 5% nonfat milk and 0.05% Tween 20 (Sigma). The enzyme was dissolved in 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. The protein concentration (0.03%) was measured by determining the absorbance at 280 nm. The UV-visible absorption spectra of the purified AOX (2 mg ml$^{-1}$ in 20 mM sodium phosphate [pH 7.5]) were recorded at 25°C on a DU 7400 spectrophotometer (Beckman, Fullerton, CA).

**Enzyme purification.** To assess the possible role of AOX during the fungal attack of pure cellulose, extracted cotton (soaked overnight in benzene-acetone to remove wax) was placed between the wood blocks and feeder strips in some flasks.

**Enzyme assay.** The AOX activity was determined by spectrophotometric measurement ($\text{E}$_{340} = 42.3 M$$^{-1}$ cm$^{-1}$)$^{56}$ of the production of hydrogen peroxide using a coupled peroxidase reaction with 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS)·$^-$ (Serva, Heidelberg, Germany) as the chromogen. Reaction mixtures (2 ml) at 25°C contained 200 μM of methanol, 2 μM of AOX, 40 μg of horseradish peroxidase (Sigma P-8250), and a suitable amount of ABTS in 50 mM air-saturated sodium phosphate (pH 7.5). One unit of AOX was defined as the amount of enzyme activity producing 1 μM of ABTS (2 μM of radical cation ABTS$^+$) per min under the assay conditions. To assess effect of pH on stability of AOX, the enzyme (0.1 mg ml$^{-1}$) was kept at 25°C (or 1 week) at various pH values, and the remaining activity was measured. The following buffers (50 mM) were used: pH 2 to 6, sodium malate; pH 7 to 9, bisTris propane; and pH 10 to 12, glycine-HCl buffer. The same buffers were used for determining pH optimum of the AOX activity under otherwise standard assay conditions.

**Protein concentrations** were determined by the bichoronic acid method (Sigma procedure TPRO 562) using bovine serum albumin (Sigma) as the standard.

**Molecular characterization.** The enzyme native molecular mass was assayed by sedimentation equilibrium ultracentrifugation method according to Yphantis (59) by using a Beckman Spinco model E analytical ultracentrifuge equipped with interference optics, rotor Ah6-TI, and 12-mm cuvette (9,341 rpm, 9-h run, 20°C). The enzyme was dissolved in 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl. The protein concentration (0.03%) was measured by determining the absorbance at 280 nm. The UV-visible absorption spectra of the purified AOX (2 mg ml$^{-1}$ in 20 mM sodium phosphate [pH 7.5]) were recorded at 25°C on a DU 7400 spectrophotometer (Beckman, Fullerton, CA).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** according to the method of Laemmli was carried out on a Hoefer SE 250 apparatus (Hoefer SE, Richmond, CA) in 10% gels. Analytical isoelectric focusing of the purified enzyme (2 μg) was performed in 1.5% agarose gels (0.5 mm) containing 7.8% Bio-Lyte 3/10 Ampholyte (Bio-Rad, Hercules, CA) by using a Multiphor II system (Amersham) and low-pI standards. Protein bands were stained with Coomassie blue.

**Western analysis.** Proteins separated by SDS-PAGE were transferred overnight onto nitrocellulose membranes by wet electroblotting (30 V, 90 mA). Membranes were treated with blocking solution containing TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl buffer) supplemented with 5% nonfat milk and 0.05% Tween 20 (Sigma). The blots were then incubated with anti-AOX anti-serum (1:20,000 in the blocking solution) for 1 h at room temperature. After being washed in TBS (three times for 10 min each time), the membranes were treated with horseradish peroxidase conjugated to donkey anti-rabbit immuno-globulin G (IgG [Amersham]; 1:10,000 in the blocking solution, 45 min). Visualization was carried out with enhanced chemiluminescence (ECL) reagent (Super-Signal West Pico ECL substrate; Pierce, Rockford, IL) on Medical X-ray film (Foma, Hradec Královo, Czech Republic).

**Enzyme glycosylation** was tested by dansyl hydrazine fluorescence staining for carbohydrate (12) using AOX samples from the final purification step on SDS-PAGE gels. Ovalbumin was used as a positive control.

**Enzymatic in-gel digestion for mass spectrometry (MS).** Coomassie blue-stained protein bands were excised from the SDS-PAGE gel, cut into small pieces, and washed several times with 10 ml dithiothreitol-0.1 M 4-ethylmorpholine acetate (pH 8.1) in aqueous 50% acetonitrile. After complete destaining, the protein was reduced with 30 μl tris(2-carboxyethyl)phosphine hydrochlo-roide) (TCEP) at 65°C for 30 min and alkylated by treatment with 30 mM iodoacetamide (all from Sigma) for 60 min in the dark. The gel pieces were further washed with water, shrunk by dehydration in acetonitrile, and reswelled again in water. The supernatants were removed, and the samples were partly dried in a SpeedVac concentrator. The gel pieces were then rehydrated in a cleanup buffer containing 0.01% 2-mercaptoethanol, 50 mM 4-ethylmorpholine acetate, 10% acetonitrile, 50% of H$_2$O$_2$ (Sigma), and 1 μl of protease (50 ng/μl for sequencing-grade trypsin [Promega, Madison, WI] and chymotrypsin; 10 ng/μl for sequencing-grade Asp-N [both from Roche Diagnostics, Mannheim, Germany]). After overnight digestion at 37°C, the resulting peptide mixtures were acidified with 5% acetic acid, and aliquots were purified and concentrated by using C18 ZipTips (Millipore) prior to MS analysis.

**MALDI-MS.** Positive-ion mass spectra were measured on a Bruker BIFLEX II time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a SCOUT 26 sample inlet and a 337-nm nitrogen laser (Laser Science, Cambridge, MA). The spectrum of the intact protein was calibrated externally using [M+H]$^+$ and [M+2H]$^2+$ ions of protein standards (trypsinogen,

**Downloaded from aem.asm.org on September 22, 2017 by guest**
protein A, and bovine serum albumin [BSA; Bruker] resulting in the mass accuracy of <0.2%. Sinapinic acid (Bruker) in aqueous 80% acetonitrile–0.1% trifluoroacetic acid (TFA; 10 mg/ml) was used as a matrix-assisted laser desorption ionization (MALDI) matrix. The spectra of the peptides obtained after proteolytic digestion were measured in reflecton mode by using α-cyano-4-hydroxycinnamic acid (Sigma) in aqueous 40% acetonitrile–0.2% TFA (10 mg/ml) as a MALDI matrix. The spectrometer was calibrated externally using the peptide standards angiotensin II and insulin B oxidized form (Bruker) with [M+H]+ ions of 1,046.5 Da and 3,496.4 Da, respectively.

High-pressure liquid chromatography-electrospray ionization MS/MS. Peptide mixtures were loaded onto a homemade capillary column (0.18 by 100 mm) packed with MAGIC C18 (5 μm, 200 Å; Michrom BioResources, Auburn, CA) reversed-phase resin and separated by using a gradient from 5% acetonitrile–0.5% acetic acid to 40% acetonitrile–0.5% acetic acid at 50 ml/min. This column was connected to an LCQDECA ion trap mass spectrometer (Thermo, San Jose, CA) equipped with a nanoelectrospray ion source. Full scan spectra were recorded in positive mode over the mass range 350 to 2,000 Da. Tandem MS (MS/MS) data were automatically acquired on the three most intense precursor ions in each full scan spectrum and interpreted manually.

MS characterization of progestogen group. The colostrum was released by heating the protein (4 mg ml−1) at 90°C for 10 min in 25 mM Tris-HCl (pH 8.0). The reaction mixture was filtered by using Microcon YM 10 centrifuge filter device (Millipore), and the ultrafiltrate was analyzed by MALDI-MS using ferulic acid (10 mg/ml in 50% acetonitrile–0.2% TFA; Sigma) as a MALDI matrix. Post-source decay (PSD) fragmentation spectra were recorded in 10 segments, with each succeeding segment representing a 20% reduction in reflector voltage. The segments were pasted, calibrated, and smoothed under computer control by using the Bruker flexcontrol software.

Molecular cloning and sequencing of a cDNA fragment encoding for the AOX protein. Mycelium from G. trabeum was harvested from glucose-corn steep medium after 8 to 12 days of cultivation and immediately frozen in liquid nitrogen. Total RNA was isolated from 100-kg (wet-weight) portions of the crushed powder of mycelia by using RNAeasy plant minikit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. First-strand cDNA synthesis was done with 5 μg of total RNA and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 50-μl reaction. anchored oligonucleotide d(T)23 VN primer (NEB). Amplification of the partial cDNA sequence of AOX was done using degenegrate forward primer GTRNTE RMWF (5′-ATG GTI CCI CCT GCI GAR GT-3′) derived from N-terminal fragment sequence MVHPEE (Fig. 3, sequence 1) and the degenerate reverse primer GTRINT1G (5′-GC1 CKI GTT GAC ATT GCL TT-3′) or GTRINT3RE (5′-GTY CIG TIC CRA IRT T-3′) derived from internal peptide fragment sequences FNMYTRAY and NVNGTEY, respectively (Fig. 3, sequences 2 and 3). Alternatively, another forward primer GTRINT4FW (5′-AAR TAY ATG ACI ATG TTY CA-3′) derived from N-terminal fragment sequence of AOX was done using degenegrate forward primer GTRINT1RE (5′-GTRINT4RE-GTRINT3RE, and a 1.8-kb fragment with the primer pair GTRINT1RE-GTRINT3RE, a 0.5-kb fragment with the primer pair GTRNTERMFW-GTRINT1RE, and a 0.3-kb fragment sequence MVHPEE (see Fig. 3, sequence 1) and the degenerate reverse primer KYMTMFQY (Fig. 3, sequence 2). The resulting PCR products were cloned into pBluescript SK(−) vector (Stratagene) and further propagated in E. coli TOP10F(−) cells (Invitrogen, Carlsbad, CA) equipped with a nanoelectrospray ion source. Full scan spectra were automatically acquired on the three most intense precursor ions in each full scan spectrum and interpreted manually.

Sample preparation for TEM. For TEM, small spherical G. trabeum mycelium colonies removed from 10- and 14-day-old agitated liquid cultures or small wood slivers removed from degraded wood blocks (2 or 4 months) by using a razor blade were fixed for 3 h in 3% (vol/vol) glutaraldehyde containing 2% (vol/vol) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. Samples were then washed three times in the buffer (for 15 min each time) and postfixed in 1% (vol/vol) osmium tetroxide in 0.1 M buffer for 1 h at room temperature. After further washes in the buffer and distilled water, the samples were dehydrated in an ethanol series (20 to 100% ethanol; 10% steps, 10 min each time), infiltrated with London resin (London Resin Co., Basingstoke, United Kingdom), and polymerized overnight at 60°C. Additional samples were fixed for 3 h in 4% (vol/vol) paraformaldehyde containing 0.1% (vol/vol) glutaraldehyde in the same buffer and thereafter dehydrated and infiltrated with resin as described above. Ultrathin sections of selected material were cut (primarily transverse sections) by using a Reichert FC4 ultramicrotome, and sections were collected on nickel grids stained with ethanolic (50%) 4% (vol/vol) uranyl acetate. Observations were made using a Philips CM12 transmission electron microscope operated at 60 or 80 kV.

TEM-immunocytochemistry studies. Ultrathin sections for immunolabeling were processed as described previously (7). Nickel grids bearing sections were incubated in anti-AOX (1:100 or 1:500) in PBS (pH 7.4) containing 1% (wt/vol) BSA and 0.05% (vol/vol) Tween 20 and left overnight at 4°C. After primary AOX incubation, sections were washed in PBS-BSA-Tween 20, followed by Tris-HCl–BSA–Tween 20 (pH 8.4), and subsequently gold labeled with goat anti-rabbit immunoglobulin G conjugated to 15-nm-diameter Au probes (Janssen Life Science Products, Beerse, Belgium). Postlabeling was performed as described above. Controls included the omission of the AOX primary or secondary antibody labeling stages.

FIG. 1. G. trabeum growth curve on 2% glucose-corn steep medium and time profiles for AOX. Enzyme activity was assayed with methanol as the substrate by measuring H2O2 production through a coupled reaction with peroxidase and chromogen. Symbols: ▲, growth (mg of mycelial dry weight ml−1); □, production of AOX (U per mycelial mass in 1 ml of culture); ○, AOX specific activity (U mg of protein−1); ■, pH of the culture liquid. All data points were obtained for samples taken from quadruplicate cultures harvested and combined at the same time.

RESULTS

AOX production and purification. Figure 1 shows the typical growth pattern of G. trabeum in submerged liquid cultures and AOX production. Synthesis of this enzyme was induced in the stationary growth phase after ~9 days of cultivation when the pH, after an initial decline, began rising above its original value of pH 4.8. AOX was isolated from extracts of mycelia harvested from the 14-day-old submerged cultures, when AOX activity had reached its maximum of ~0.2 U ml of culture−1 and represented a major protein band in the protein pattern, expressed by the fungus (Fig. 2, lane 4).

Results from a representative AOX purification procedure are summarized in Table 1. The enzyme was purified 8.6-fold from the crude mycelial extract with an overall yield of ~51% and a specific activity of 12 U mg of protein−1 under standard assay conditions. The highly purified protein gave a single band on SDS-PAGE (Fig. 2, lanes 2 and 3). One-week storage of the sterile-filtered purified enzyme in 20 mM sodium phosphate (pH 7.5) at 4°C resulted in a 68% loss of activity. The activity was then relatively stable for an additional 2 weeks (~3% loss of activity). Rapid freezing at ~80°C and thawing had no significant effect on the enzyme’s activity.

Molecular properties. The AOX’s native molecular mass of 628 ± 19 kDa was calculated from equilibrium ultracentrifugation data, assuming a partial specific volume of 0.75 cm3 g−1, which compares well with the value of 610 ± 5 kDa estimated for Poria contigua AOX using the same partial specific volume (3). SDS-PAGE gave a band at 73 kDa (Fig. 2) in agreement with the subunit molecular mass determined by MALDI-MS of 72.4 kDa, suggesting that the enzyme is an octamer. Analytical isoelectric focusing of the purified protein confirmed its apparent homogeneity and gave an isoelectric point of 5.3.

Solutions of purified AOX (2 mg of protein ml−1 in 20 mM sodium phosphate [pH 7.5]) had a brownish color and exhibited a UV-visible absorption spectrum with maxima at 280, 390, 404, and 458 nm and a shoulder at 477 nm. The A390/A404 maximum ratio was concentration dependent, increasing its value with the enzyme dilution. The A390/A458 ratio was 15.7. Treatment of purified AOX with 5% trichloroacetic acid, followed by heating, released the flavin moiety from the holoenzyme, suggesting its noncovalent attachment to the polypeptide. The cofactor was also released by heating the protein for 10 min in 25 mM Tris-HCl. MALDI-MS analysis of the released cofactor in the ultrafiltrate of the latter reaction mixture gave an intense peak at m/z 786 corresponding to [M+H]+ ion of unmodified flavin adenine dinucleotide (FAD). The presence of standard FAD was further confirmed by fragmentation using PSD analysis. The PSD spectrum was dominated by peaks of complementary ions m/z 348 (AMP fragment) and m/z 439 consisting of ribitol and isoalloxazine ring. The ions

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U mg−1)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>58</td>
<td>81</td>
<td>1.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>PEG precipitation</td>
<td>40</td>
<td>73</td>
<td>1.8</td>
<td>90</td>
<td>1.3</td>
</tr>
<tr>
<td>DEAE Sephacel</td>
<td>13</td>
<td>71</td>
<td>5.5</td>
<td>88</td>
<td>3.9</td>
</tr>
<tr>
<td>300-kDa UF</td>
<td>8.2</td>
<td>69</td>
<td>8.4</td>
<td>85</td>
<td>6.0</td>
</tr>
<tr>
<td>MonoQ</td>
<td>3.3</td>
<td>41</td>
<td>12</td>
<td>51</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* The initial total protein was measured by using the Bradford method. Protein in samples of (partially) purified AOX was determined using the bicinchoninic assay. For the AOX activity assay, see Materials and Methods.
m/z 250 and 136 comprising ribose plus adenine and adenine alone were also observed (data not shown).

G. trabeum AOX is apparently not a glycoprotein, as was demonstrated by negative staining using the dansyl hydrazine method (data not shown). This is supported by good agreement of experimental (72.4/110060.14 kDa) and theoretical molecular mass based on the primary structure of the mature protein (72.382 kDa). Similar negative findings were reported for Polyporus obtusus and Peniophora gigantea AOX analyzed for carbohydrate content by the anthrone method (29) and concanavalin A precipitation test (11), respectively.

**Catalytic properties.** The purified G. trabeum AOX exhibited a broad substrate tolerance toward oxidation of short-chain primary alcohols (Table 2). Some polyols were also significantly oxidized, whereas secondary alcohols and aryl alcohols were very poor substrates. The apparent kinetic constants determined for methanol under air-saturated (−0.23 mM O2 at 25°C [57]) conditions were $K_m = 2.3$ mM, $V_{max} = 12.9$ µmol of H2O2 min−1 mg of protein−1, $k_{cat} = 15.6$ s−1, and $k_{cat}/K_m = 6.8$ s−1 mM−1. Comparison of the catalytic efficiencies ($k_{cat}/K_m$) for five major AOX substrates (Table 2) indicates that methanol is clearly the preferred AOX substrate.

AOX had a pH stability optimum on the alkaline side in the range of pH 7 to 11 (>80% maximum stability at pH 9). A broad optimum pH range with activity almost constant at pH 6 to 10 (>96% maximum activity at pH 6.5) was followed by a rapid decrease of activity on both alkaline and acidic side, with 16% of activity left at pH 5.5 and no activity detectable at pH 5.0 and 12. The decrease on the acidic side is clearly due to the high instability of the enzyme at pHs of ≤5.5 and not due to the coupled peroxidase assay used with horseradish peroxidase activity optimum at pH 6 (BRENDA database).

**AOX primary sequence.** With PCR primers based on the available N terminus of the purified mature protein and internal peptide sequences determined by high-pressure liquid chromatography-electrospray ionization MS/MS (Fig. 3), AOX cDNA fragments were obtained from G. trabeum mRNA by reverse transcription-PCR using degenerate primers as described above, and the missing 5' and 3' ends of the cDNA were characterized by means of the RACE method (see Materials and Methods). The complete cDNA sequence compiled from independent overlapping sequence fragments codes for 651 amino acids (Fig. 3).

As evidenced by Edman degradation and MS sequencing data (Fig. 3), the initiator methionine was removed cotransla-

### TABLE 2. Substrate specificity of AOX from G. trabeum with respect to electron donors (100 mM)$^a$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>100</td>
<td>6.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>94</td>
<td>1.1</td>
</tr>
<tr>
<td>Allyl alcohol</td>
<td>93</td>
<td>0.53</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>73</td>
<td>0.26</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>54</td>
<td>0.07</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>22</td>
<td>0.03</td>
</tr>
<tr>
<td>2-Methyl-1-propanol</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2-Propanol</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>tert-Butanol</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>(±)-2-Butanol</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>3-Pentanol</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>2-Butene-1,4-diol</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Benzy alcohol</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxybenzyl alcohol</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>d-Arabinitol</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>d-Glucose</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>&lt;4</td>
<td></td>
</tr>
</tbody>
</table>

$a$ Activities are given relative to methanol. Catalytic efficiency constants ($k_{cat}/K_m$) are given for the six major substrates.

**FIG. 3.** Amino acid sequence of G. trabeum AOX derived from cDNA. The sequence stretches used for the construction of degenerate PCR primers are numbered (see Materials and Methods). Sequence confirmation using both peptide mass mapping and tandem mass spectrometry (boldface), peptide mass mapping only (underlined), and automated Edman degradation (double underlined) is labeled. In the mature protein, the initiator methionine is removed from the N terminus by methionine aminopeptidase. Potential sites of N glycosylation are marked with asterisks.
tionally by the action of methionine aminopeptidase, and it was not present in the isolated mature protein. Furthermore, no cleavage of signal peptide from the N terminus during protein posttranslation processing and translocation was observed. The putative C-terminal peroxisome targeting signal 1 (PTS1) of methylotropic yeast/mold AOXs interacting with the Pex5p receptor protein to direct reporter proteins into the peroxisomal matrix (55) was missing (Fig. 4).

**FIG. 4.** Multiple sequence alignment of N and C termini of *G. trabeum* (Gtr) AOX with the termini of yeast and mold AOXs. Known AOXs: Pme, *Pichia methanolica*; Ppi, *P. pinus*; Cvi, *Cochliobolus victoriae*; Cfu, *Cladosporium fulvum*. Two predicted AOXs: Pch, *Phanerochaete chrysosporium*; Cci, *Coprinus cinereus*. The unique sequence of the last 27 C-terminal amino acids of *G. trabeum* AOX is underlined.

**FIG. 5.** AOX immunofluorescent localization on whole mycelia and hyphae from 10- and 14-day-old liquid cultures of *G. trabeum*. (A and B) Control double labeling of 10-day-old culture by DAPI (A) and anti-rabbit IgG-FITC (B); (C and D) double labeling of 10-day-old culture by DAPI (C) and anti-AOX-IgG-FITC (D). All hyphae were strongly labeled by AOX. (E and F) Double labeling of 10-day-old culture by DAPI (E) and anti-AOX-IgG-FITC (F). The signal for AOX was strong on the hyphal wall (yellow arrows) often in regions where DAPI showed a strong signal for nuclei. (G and H) Double labeling of 14-day-old culture using DAPI (G) and anti-AOX-IgG-TRITC (H). Extracellular slime materials (stars) within the *G. trabeum* pellets showed a strong reaction with the AOX antibody. Bars, 50.0 μm.
microscopy (AOX-FITC or AOX-TRITC labeling), *G. trabeum* hyphae from liquid cultures (10 and 14 days) labeled strongly for the presence of AOX (Fig. 5). AOX was located along the fungal cell wall and associated with extracellular slime materials coating hyphae and present within the main slime matrix of the mycelial pellets (ca. 2 to 3 mm in width) produced during culturing (Fig. 5C to H). The enzyme was associated with both active (i.e., indicated by presence of positive staining nuclei with DAPI) and necrotic hyphae lacking nuclei (Fig. 5C to F). Strong AOX labeling was noted on the cut ends (i.e., through sectioning) of hyphae within pellets, as well as small localized zones on the hyphal surface (Fig. 5D and F, arrows). Little difference in labeling was noted between the 10- and 14-day-old cultures, although the mycelia from the latter cultures showed a stronger labeling and a positive response of all mycelia and associated slime materials.

TEM-immunogold labeling of sectioned hyphae from 14-day-old cultures showed strong labeling of the hyphal cell wall (arrowhead in panel B), intrahyphal materials (arrows in panel B), and periplasmic space and electron-dense extracellular slime materials associated with the outer hyphal wall (arrows in panel C). (D to G) Strong AOX labeling of extracellular tripartite membrane structures emanating from the outer hyphal cell wall. (E, H, and I) High magnification images showing the extracellular tripartite membrane structure with AOX mostly associated with the electron dense materials coating the membranes (arrows). (F) Longitudinal section through a hypha showing strong positive AOX labeling of extracellular materials on tripartite membranes (arrows, right side) and negative labeling of a cytoplasmic peroxisome (asterisk, left side). Bars: A, D, E, F, and G, 0.5 μm; B, C, and H, 0.25 μm; I, 0.1 μm.

Immunofluorescence and immunogold-TEM analyses of AOX in *G. trabeum* hyphae grown on wood and cotton. Immunofluorescence labeling of wood sections from birch and pine was carried out after degradation for various time periods ranging from 2 to 4 months, i.e., at a time when decay and mass loss for wood blocks lies in the region of ca. 30 to 65% for birch and ca. 20 to 55% for pine. Labeling of both transverse radial and tangential longitudinal sections was carried out, and most fluorescence labeling was done with anti-AOX, followed by IgG conjugated to FITC rather than TRITC because of the
better differentiation of the wood cell walls (i.e., they appear yellowy with FITC because of the autofluorescence of lignin; Fig. 7B) and G. trabeum hyphae. Observations on fresh sections showed strong labeling of green hyphae within the cell lumina of both the birch and the pine samples studied, showing the presence of AOX (Fig. 7C to H). Hyphae were often distinct against the lumen wall, and the presence of extracellular AOX was demonstrated by green fluorescence of localized neighboring wood fiber cell wall regions (Fig. 7C to H). This was not apparent in control samples (Fig. 7A and B). Fluorescence labeling was also carried out on colonized and degraded cotton fibers sandwiched between wood blocks and incubated for periods of 2 weeks to 2 months. Cotton fibers removed after 2 months of colonization by G. trabeum showed hyphae directly attached and associated with the degraded cotton fibers to be positively labeled for AOX similar to the hyphae from the liquid cultures and those colonizing wood blocks (Fig. 7K to N).
Decay of wood by *G. trabeum* is thought to occur by attack and dissolution of the surrounding wall celluloses and hemicelluloses by hyphae localized in the cell lumina. In the present study we also found *G. trabeum* hyphae to be not only associated with the luminal surface of fibers and parenchymatous cells but also present within the actual secondary S2 wall layer (i.e., the dominating wood cell wall layer) as well as “sunken” into the S2 wall from the lumen (Fig. 8A). Details concerning the ultrastructural aspects of cell wall decay by *G. trabeum* will be published elsewhere. Figure 8 shows typical AOX labeling of hyphae associated with highly degraded birch fibers in transverse sections. The fiber wall shows advanced decay, and the S2 cell wall has a very open structure consistent with the removal of much of the cellulose and hemicellulose components. Positive AOX labeling was found on the hyphal cell walls and associated extracellular materials, both situated within the fiber S2 wall layer (Fig. 8B); results consistent with the immunofluorescence observations (Fig. 7F). Evidence for the presence of AOX was also found in highly degraded wood cell wall regions remote from hyphae (Fig. 8). Control labeling of degraded wood and cotton fibers (i.e., omission of the antibody stage) were all negative (not shown).

**DISCUSSION**

**Enzyme properties.** Production of AOX in laboratory cultures of *G. trabeum* was reported as early as 1969 (34), but the enzyme has not been characterized previously. The enzyme we purified from this brown rot fungus shares most of the basic biochemical characteristics of AOXs (alcohol:oxygen oxidoreductase EC 1.1.3.13) reported previously for a number of yeasts and filamentous fungi (44). Like other enzymes of the glucose-methanol-choline oxidoreductase family, it is an inducible nonglycosylated homooligomeric O2-dependent FAD flavoprotein oxidizing short-chain primary aliphatic alcohols into the corresponding aldehydes with concomitant production of H2O2. Methanol is the preferred substrate, as found for methylotropic yeast AOXs, as well as the AOXs thus far partially characterized from the basidiomycete fungi *Poria contigua* (3), *Peniophora gigantea* (11), *P. obtusus* (29), and *Phanerochaete chrysosporium* (14). It is well documented that during wood deterioration by these fungi, methanol is produced upon enzymatic demethylation of lignin substructures (1, 16). Interestingly, relatively high activity of AOX toward allyl alcohol may also be related to lignin substructures (based on methox[hydroy]phenylallyl alcohol), specifically allyl alcohol side groups exposed after initial lignin demethylation and partial depolymerization.

*G. trabeum* AOX seems to be strictly bound to the demonstrated extraneous membrane structures and polysaccharide hyphal sheath because we could not detect significant activities in culture filtrates. This may also be due to the instability of the enzyme at pHs of <7, which is the case of the laboratory liquid cultures under study. However, its activity was readily released on applying the relatively mild extraction procedure at pH 7.5 using Ultra-Turrax homogenization (see Materials and Methods). Unlike the AOX from *P. gigantea* that displayed a covalently bound FAD (11), the FAD cofactor of *G. trabeum* AOX was easily dissociated by heat treatment similar to that thus far characterized for yeast AOXs (44).

**Sequence analysis and alignments.** Genes encoding AOX have thus far been identified, and cDNAs have been cloned from several yeasts (38, 44, 51) and mold fungi (27, 44, 48, 49). Here we present for the first time the primary structure of AOX from a basidiomycete fungus that compared to the yeast and/or mold enzymes exhibited some unique features.

The deduced amino acid sequence displayed 51 to 53% identity and 67 to 70% similarity to other AOX sequences when comparisons were made with the known protein se-
quences in GenBank using the BLAST algorithm (for the organisms for alignments of yeast-yeast and mold-yeast AOXs were, in contrast, significantly higher (72 to 99% and 65 to 70%, respectively), indicating a certain developmental divergence. This is consistent with the earlier-reported immunological incompatibility of AOX from *Portia placenta* with yeasts AOX and the deduced differences in protein primary structures (3).

Based on genome database searches, predicted sequences for AOX were also obtained for two other basidiomycete fungi: *Phanerochaete chrysosporium* (JGI Eukaryotic Genomics project; http://genome.jgi-psf.org) and *Coprinus cinereus* (Southeast Fungal Biology Group; http://genome.semo.edu). Their comparison with the *G. trabeum* AOX interestingly showed much higher sequence identities of 88 and 85%, respectively, against the above yeast and mold sequences.

In general, N termini of the known AOXs show high similarities (Fig. 4), whereas the sequence for the last 27 C-terminal amino acids of *G. trabeum*’s AOX is characteristic of this enzyme and homologous only to the C termini of the predicted AOXs from the related lignocellulosylsabidiozyme fungi discussed above (Fig. 4), suggesting a similar role for the enzyme. Unlike the corresponding carboxy-terminal sequences of yeast and mold AOXs, the C termini of the three basidiomycete AOXs do not contain the PTS1 LARF-(like) tetrapeptide shown previously to direct reporter proteins to peroxisomes (55). Absence of this motif is consistent with our findings of the extracellular localization of *G. trabeum* AOX using immunofluorescence and TEM labeling. Deletion of the C-terminal PTS1 in the mutant yeast AOX gene had, however, no (23) or limited (55) effect on protein sorting, which suggested that the yeast AOX contains another, internal PTS recognized by HpPex5p (PTS1 receptor). The proposed novel HpPex5p-dependent AOX import pathway does not require PTS1 and the C-terminal TRP motif of HpPex5p. Instead, only the N-terminal part of HpPex5p and a thus-far-unidentified internal PTS (putative PTS3) activated upon FAD binding are essential to mediate AOX translocation (23). This was, among others, shown by mislocalization to the cytosol of *Hansenula polymorpha* AOX mutated in the dinucleotide binding fold. Signaling adaptation that has changed the interaction of the yeast AOX with Pex5p into the above novel mode of binding dependence on FAD-internal PTS3 is thought developed as a physiological advantage specifically by methylophic yeasts (23). Similarly, from an evolutionary point of view, a proper developmental modification at the C-terminal PTS1 motif of *G. trabeum* AOX may thus divert its targeting from peroxisomes to physiologically more advantageous extracellular location enabling, through H$_2$O$_2$ production, use of the enzyme in external mechanisms for the recovery of nutritional resources from decayed wood. The proposed modification resulting in putative PTS3 of methylytrophic yeasts is apparently missing from the *G. trabeum* AOX sequence.

As expected, the N-terminal region of the *G. trabeum* AOX sequence contains the common ADP-binding motif (ββ) present in most FAD-binding proteins containing the characteristic nucleotide-binding site GxGxxG, specifically GGG-PAG for amino acids 13 to 18, which compares with the corresponding sequence GGGSTG (amino acids 13 to 18) of yeast AOXs (44, 57). Other conserved sequence regions of the FAD-binding domain that are found in flavoproteins of the glucose-methanol-choline oxidoreductase family are also present (not shown). The sequence of the enzyme includes two theoretical N-glycosylation sites conforming to the consensus N-X-S/T sequence for Asn residues at positions 141 and 323, both being predicted by the NetNGlyc 1.0 server (www.cbs.dtu.dk/services/NetNGlyc) to be modified with only low N-glycosylation potential (0.399 and 0.545). This is in accord with negative staining for AOX’s glycomoiety using the dansyl hydrazine method.

**Ultrastructural localization.** In yeasts, AOX activity is regulated at the transcriptional level by repression and/or derepression (44). The enzyme is synthesized as an inactive monomer in the cytosol and posttranslationally imported into peroxisomes, where the active homo-octameric FAD-containing enzyme is assembled (20). Ultrastructural localization of AOX in basidiomycete fungi has not been demonstrated to date. Here we present a marked difference in ultrastructural localization of *G. trabeum* AOX compared to the methylotrophic yeasts, the enzyme in this fungus being apparently translocated to the periplasmic and extracellular space. During our TEM labeling studies we were, however, unable to localize *G. trabeum* AOX in peroxisomes, a result consistent with the molecular studies.

Immunofluorescence and TEM-immunogold labeling studies showed AOX to be associated with *G. trabeum* hyphal cell walls (periplasmic space and intrahyphal spaces), extracellular slime, and tripartite membranes emanating from hyphae. This was shown for agitated liquid cultures at different stages of AOX production and on degraded birch and pine wood blocks and on cotton fibers. Immunofluorescence provided a broad overview of positive AOX labeling on hyphal walls and extracellular slime present in pellets and with hyphae growing on cotton fibers or in wood fibers at different stages of decay. AOX has been previously reported from yeasts, fungi imperfecti, and white rot fungi (44), but an extracellular distribution of the enzyme has not been reported previously for fungi. The exception is a structurally unrelated extracellular AOX recently isolated from the thermophilic ascomycete fungus *Thermosascus aurantiacus* and characterized as a hetero-octamer flavoglycoprotein composed of two types of subunits, oxidizing in addition to primary aliphatic alcohols also secondary and aromatic alcohols (36).

**Implications for wood decay.** One of the most important aspects for improved understanding of brown rot decay of wood is knowledge on the involvement and spatial distribution(s) of polysaccharide-degrading agents, including enzymes and nonenzymatic systems in situ. Since the known polysaccharide-degrading enzymes produced by brown rot fungi are considered too large to explain the rapid penetration and attack of wood cell wall components distant to luminal hyphae in wood cells, several systems based on low-molecular-weight active chemicals have been proposed and reviewed recently by Goodell (18). However, in order for these systems to operate in situ, an extracellular source of H$_2$O$_2$ is required.

A possible role of AOX as a source of H$_2$O$_2$ used in fungal wood decay has already been suggested previously for this enzyme purified from the white rot fungus *P. chrysosporium*.
(14, 43). The enzyme was, however, considered an intracellular protein, which was controversial for its implication in fungal decay processes. Extracellular localization of AOX in our present study thus underlines the possible role of AOX to serve as an important extracellular source of H$_2$O$_2$ during brown rot and perhaps also white rot decay. The enzyme may thus significantly contribute to the efficiency of the Fenton chemistry for production of highly biodegradative ·OH radicals in degraded wood.

Apparently controversial to the proposed role in wood decay is the instability of AOX at acidic pH conditions that are characteristic of brown rot wood decay. Although this is true for the soluble purified enzyme, its localization in the periplasmic space and “immobilization” in the hyphal polysaccharide sheath may account for the enzyme’s physiological functionality, providing the fungus can control pH at the hyphal surface and keep it close to neutral (e.g., through oxalate decarboxylation or neutralization in the form of crystalline calcium oxalate, often observed along hyphae). A possible pH gradient may even have a physiological role in preventing hyphae from damage by active radical species formed at low pHs while suppressed at higher pHs in close proximity of hyphae due to higher rate of ferrous auto-oxidation (52). This assumption is contrary to the model of a reciprocal protective pH gradient for hyphae proposed for the brown rot (Coniophora puteana) mechanism by Hyde and Wood (28) assuming a pH as low as 2 at the hyphal surface. Furthermore, it is highly possible that the actual pH along a particular hypha growing in wood need not necessarily be constant. For example, it may be first low at the actively growing apical part as the hypha acidifies its environment by oxalate secretion and then become higher when the specific part is aging. There is also considerable variability in the decay environment, since some brown rot fungi strongly reduce the pH of the substrate (e.g., Postia spp.), while other species such as the G. trabeum used in our study retain wood at a slightly acidic pH (50).

The second reaction product of AOX with methanol, toxic formaldehyde, can either undergo (in hydrated form) subsequent oxidation by the same enzyme (11) thus producing additional H$_2$O$_2$, or be oxidized by ·OH radicals from the Fenton reaction to ·HC(OH)$_2$, which can subsequently interact with molecular oxygen (39). In this way (possibly together with other enzymes acting on formaldehyde intracellularly), its accumulation at toxic concentrations deleterious to the fungus may be prevented.

The present results also indicate that the association of AOX with secreted extracellular membranous or slime structures could provide a means for transporting a high molecular enzyme to sites of attack remote from hyphae, i.e., on the lumen wall of degraded wood cells. In addition, AOX associated this way with hyphal cell walls will have direct contact with the substrate, as will hyphae growing within or “sunken” into the secondary wood cell wall (Fig. 8B). We have previously shown the association of another H$_2$O$_2$-producing high-molecular-weight (~300 kDa) oxireductase, pyranose 2-oxidase (EC 1.1.3.10), with extracellular slime during the decay of wood by the white rot fungi P. chrysosporium, Trametes versicolor, and Oudemansiella mucida (7). This enzyme is also synthesized by G. trabeum (our unpublished observations) and may function in the production of active oxygen species synergistically with AOX. Extracellular glyoxal oxidase suggested as a major possible source of H$_2$O$_2$ for fungal (P. chrysosporium) lignocellulolonic activities (33) has not yet been reported with G. trabeum.

As an enzyme-protein “supporting” matrix, extracellular slime in its various forms, such as sheaths, mycofibrils, and tripartite membranes, has been shown associated with most types of fungal and bacterial wood decay (6, 17, 22, 26, 46), including basidiomycete fungi. It was shown to be associated in the white rot decay of lignocellulose with ligninolytic peroxidases (6, 10, 46) and laccase (9, 42), as well as aryl-alcohol oxidase (2). Thus, a similar role of a functional matrix for the slime in G. trabeum, a fungus known to produce extracellular sheaths during degradation of cellulose (26), would seem reasonable. From TEM-immunogold labeling it was, however, not possible to determine whether AOX was located directly on the tripartite membranes or associated with the more electron-dense slime materials bound to its structure. Previous studies on the chemical composition of tripartite membranes from Sporotrichum pulverulentum have shown the presence of several proteins of different molecular weights (17). Other studies have shown a monokaryotic strain of the brown rot fungus Postia placenta that lost production of extracellular slime unable to degrade wood (40).

A better understanding of AOX’s molecular and genetic properties and its involvement in oxidative wood degradation by G. trabeum increases our knowledge of the principles underlying brown rot decay. Through finding the enzyme’s specific inhibitors, an aspect currently under study, it may further help in the development of targeted wood preservatives.

Conclusions. These new findings in support of the role of AOX in brown rot decay of wood by G. trabeum include (i) the extracellular nature of G. trabeum AOX, as shown by the enzyme’s periplasmic distribution and localization on secreted tripartite membranes and slime materials; (ii) a unique AOX C-terminal sequence that does not contain a signal for peroxisomal targeting, in accord with finding i; and (iii) the high activities of AOX in liquid cultures, which are consistent with a major metabolic role for the enzyme. Finally, methanol, the preferred AOX substrate, is available through its production during concomitant lignin demethylation (thus, a coupling between cellulose and lignin biotransformation is possible).

Acknowledgments

This study was supported by the Swedish Council for Environment, Agriculture, and Spatial Planning (FORMAS) (2004-1850) and Institutional Research Concept No. AVIZ200510 (Czech Republic).

References


2532.


