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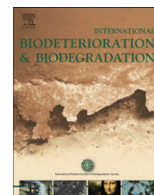

Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-D PAGE and MALDI-TOF/TOF/MS

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Proteins expressed by the brown-rot fungus *Gloeophyllum trabeum* were characterized from inoculated southern yellow pine sapwood undergoing decay, from pure cultures of the fungus and from uninoculated pinewood. Analysis was carried out by two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF/TOF/MS. No proteins were detected from the clean uncontaminated wood. The inoculated wood undergoing active brown-rot decay produced 76 proteins, including the Fenton-chemistry related enzymes, alcohol oxidase, lipoxygenase, and catalase. One hundred and eleven proteins were detected from the pure culture and most were common metabolic proteins. A majority of proteins in both samples were identified as hypothetical proteins. A surprising result is that there was very little overlap between proteins found in both sets of samples, indicating a very different mechanism in action when the fungus is growing on a cellulose-based nutrient source (wood) versus glucose media. This study also highlights a current limitation of this approach, which is the limited protein and genomic sequence information annotated on the public databases. Of the 187 proteins characterized, only 36 were identified with confidence. To our knowledge, this is the first reported proteomic analysis of pinewood decayed by a brown-rot fungus and provides the initial characterization of proteins involved in this type of wood biodeterioration. Although significant limitations still exist in identifying the proteins, this limitation will diminish as functional proteins are identified and added to the databases.

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1. Introduction

Wood is an important renewable and natural resource with a multitude of uses (Goodell, 2003). Wood decay results in the loss of billions of U.S. dollars annually (Preston, 2000). The traditional method for protecting wood from decay is treatment with broad-spectrum chemical preservatives, usually without knowing the specific identity of the fungi causing decay in many different environments. Identification of the microorganisms involved in decay is necessary in order to develop a complete understanding of the roles the different microorganisms play in the decay process. However, the presence of a microorganism does not mean it is metabolically active in decay. Wood decay is caused by enzymes and low-molecular-weight mediators secreted by fungi in their efforts to obtain a carbon source for their survival (Cease et al., 1989; Flournoy et al., 1991; Pointing et al., 2003). The resulting wood decay is classified into three types: soft rot, brown rot, and white rot. Basidiomycota members typically cause the more active and destructive brown- or white-rot decay (Nilsson et al., 1989; Bruce et al., 1991).

Brown-rot fungi and brown-rot decay predominate on pines and pine products such as lumber in the USA (Goodell, 2003). During brown-rot decay the carbohydrate fraction of the holocellulose is actively metabolized, leaving behind a chemically modified lignin residue, which is typically brown and crumbly. The depolymerization of the cellulosic fraction of the S2 cell wall dramatically and rapidly reduces the strength of the wood. The S2 layer is degraded in advance of the actual hyphae, and since most enzymes are too large to penetrate intact cell wall layers, it is hypothesized that non-enzymatic low-molecular mediators or chealators diffuse into the cell wall, producing localized hydroxyl free radicals via modified Fenton reactions (Green and Highley, 1997; Goodell, 2003; Daniel et al., 2007). However, the exact mechanism of brown-rot decay is still hypothetical and controversial. Recently, the genome of the brown-rot fungus *Postia placenta* was sequenced and annotated (Martinez et al., 2009). In relation to the decay of wood, these authors detected none of the common lignin decay enzyme genes, such as lignin peroxidase, manganese peroxidase, or versatile peroxidase; however, there were two possible laccase genes present. There were high transcript levels of numerous hemicellulases and a single putative β 1-4 endoglucanase from cultures grown on cellulose media compared to those grown on glucose media. No genes coding for cellobiohydrolases or cellulose-binding

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domains were found. On cellulose media there were also high transcript levels of a methanol oxidase similar to one in *Gloeophyllum trabeum*, as well as genes tentatively identified as glucose-1-oxidases, polyphenol oxidases, iron reductases, and quinone reductases, all supporting Fenton chemistry and hydroxyl radicals as the brown-rot mechanism of decay. The annotated genome is available at http://genome.jgi-psf.org/Postia.

During the process of wood decay, fungi express different proteins involved in both the decay of wood and the metabolism of the fungus. Proteomic profiles or protein fingerprints can be used to compare decaying wood to non-decayed wood or cultured fungi to determine which proteins are being uniquely expressed during the decay process. The proteome itself is defined as the complete set of proteins generated by the genome (Gromov and Celis, 2000; Phillips and Bogyo, 2005). The genome is the complete set of an organism's DNA. The proteome of a given organism is constantly changing in response to internal and external conditions including its environment and carbon sources. Proteomics is much more complex than either genomics or transcriptomics because each protein can be chemically modified in different ways post synthesis (Keller and Hettich, 2009). Strong gene expression results in abundant mRNA and includes all transcripts in the cell. The transcriptome reflects the genes that are being actively expressed at any given time, but it does not necessarily mean that the corresponding protein is also abundant or active in the cell (Prabakaran et al., 2001; Zhou et al., 2004). Expressional proteomics can provide both a qualitative and quantitative snapshot of all proteins expressed by an organism at the time of extraction (Keller and Hettich, 2009).

In the wood-decay field, a proteomics approach has been used by numerous authors toward understanding the white-rot decay process using *Phanerochaete chrysosporium* as the model (Abbas et al., 2005; Vanden Wymelenberg et al., 2005; Sato et al., 2007; Matsuzaki et al., 2008; Shary et al., 2008). The genome of this white-rot fungus has been sequenced (Martinez et al., 2004). To our knowledge, this is the first reported proteomic analysis of pine-wood deteriorated by a brown-rot fungus and provides a useful tool for characterization of proteins involved in wood biodeterioration. Our objective was to identify those proteins expressed during the decay of pine. This research will lay the foundation to begin to understand the suites of decay genes and their proteins which are expressed during brown-rot biodeterioration. A related current study is looking at the hypothesis that gene expression of wood-decay enzymes is influenced by the decay resistance of the wood species being degraded.

2. Material and methods

2.1. Wood samples and fungal culture

The wood wafer samples for these studies were from loblolly pine (*Pinus taeda* L.) sapwood lumber. This wood had been kiln-dried at high pressure and temperature to 15% moisture content before being cut into wafers ($18 \times 18 \times 5$ mm). Samples consisted of uncontaminated wood (a: undecayed pine stake), inoculated decayed wood (b: *G. trabeum* inoculated pine stake) and pure culture of *G. trabeum*. The uncontaminated wood (UW) was clean and no microorganisms were detected; its moisture content was less than 15%. The inoculated wood (IW) was incubated with the brown-rot fungus, *G. trabeum*, for approximately 2 months at room temperature at a moisture content $\sim$60%. The uncontaminated wood (Fig. 1a) and inoculated wood after incubation (Fig. 1b) were ground in a Wiley mill. The proteins were extracted from each of the ground samples (listed as c and d in Fig. 1). Furthermore, pure cultures of *G. trabeum* (GT) were grown on a malt extract medium, and the proteins were extracted by the same procedure.

2.2. Extraction and quantification of proteins from wood and fungi

The proteins were extracted and quantified from all three samples by published methods (Bradford, 1976; Shimizu and Wariishi, 2005). In general, 1.0 g of each sample (ground samples and fungal tissue) was homogenized using a clean pestle with protein extraction buffer (5.0 ml for 1.0 g wet weight of biomass). The protein extraction buffer was composed of 0.7 M sucrose, 0.5 M Tris–HCl (pH 8.5), 0.05 M Na_2EDTA, 0.1 M KCl, and 2% (v/v) 2-mercaptoethanol. Water-saturated phenol was then used to extract total proteins from the buffer. Proteins in the phenol phase were precipitated with five volumes of ammonium acetate in methanol (0.1 M NH_4OAc and 1% 2-mercaptoethanol in methanol) at -70 °C for 2 h. The resultant pellets were sequentially washed with ammonium acetate in methanol and 80% acetone. The protein samples were then air-dried and stored at -70 °C until needed. The concentration of the fungal proteins extracted from the four samples was quantified by the 2-D-Quant Kit (Bio-Rad Hercules, CA) using bovine serum albumin (BSA) as the standard.

2.3. Analysis of the protein patterns using two-dimensional PAGE (2-D PAGE)

Immobilized pH gradient (IPG) strips (Dry Strips, 7 cm, pH 3–10 non-linear, Bio-Rad, Hercules, CA) were passively rehydrated overnight with the protein sample buffer (9.5 M urea, 4% CHAPS, 1% DTT, 0.2% ampholites). The isoelectric focusing (IEF) of sample proteins (300 μg) was performed using the following step gradient: 500 V for 1 h, 1000 V for 1 h, and 8000 V until a total of 67,500 V h was reached. After IEF, strips were equilibrated in a buffer containing 7 M urea, 2% SDS, 375 mM Tris (pH 8.8), and 10% glycerol plus either 50 mM DTT for reduction or 100 mM iodoacetamide for alkylation. Equilibrated IPG strips were loaded onto a 12% acrylamide gradient sodium dodecyl sulfate (SDS) – PAGE gel and sealed with 1% agarose.

Two-dimensional electrophoresis was carried out using a Protean IIxi system (Bio-Rad, Hercules, CA) until the dye front reached the bottom (Fryksdale et al., 2002). Protein molecular markers were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). Isoelectric focusing and 2-D PAGE were conducted three times on each of the three protein extractions. The proteins in the gels were stained with Commassie Brilliant Blue R-250, and images were acquired with a digital camera. Protein spots were detected and numbered with PD Quest software (Bio-Rad, Hercules, CA). The proteins on each gel were cut using a robotic digester and spot cutter (Investigator Proprep 4 block system, Genomics Solutions, Ann Arbor, MI; Robotic Bio-Rad proteome work station) in the Life Sciences and Biotechnology Institute (LSBI) at Mississippi State University following the procedure of Pechanova et al. (2008). All proteins were digested by trypsin following the in-gel digestion method of Pechanova et al. (2008) using the ProPrep Robotic Digester (Genomics Solution). The digested peptides were desalted with C18 ZipTips and subjected to MALDI-TOF/TOF/MS (matrix-assisted laser desorption ionization – time of flight/mass spectrometry) analysis (ABI 4700, Applied Biosystems, Foster City, CA).

2.4. Identification of proteins and functional grouping

From the mass spectral data, protein identification was automatically performed with ABI GPS Explorer software using the Result Dependent Analysis Mode. This system ran a search for each protein against the NCBInr (National Center for Biotechnology non-redundant database) using the MASCOT (version 1.8.0, Matrix Science Ltd., London, UK) search engine (Pappin et al., 1993).

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