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RESEARCH ARTICLE

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Fungal degradation of wood: initial proteomic analysis of extracellular proteins of *Phanerochaete chrysosporium* grown on oak substrate

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Abstract Two-dimensional (2-D) gel electrophoresis was used to separate the extracellular proteins produced by the white-rot fungus *Phanerochaete chrysosporium*. Solid-substrate cultures grown on red oak wood chips yielded extracellular protein preparations which were not suitable for 2-D gel analysis. However, pre-washing the wood chips with water helped decrease the amount of brown material which caused smearing on the acidic side of the isoelectric focusing gel. The 2-D gels from these wood-grown cultures revealed more than 45 protein spots. These spots were subjected to in-gel digestion with trypsin followed by either peptide fingerprint analysis by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) or by liquid chromatography (LC)/MS/MS sequencing. Data from both methods were analyzed by Protein Prospector and the local *P. chrysosporium* annotated database. MALDI-TOF/MS only identified two proteins out of 25 analyzed. This was most likely due to problems associated with glycosylation. Protein sequencing by LC/MS/MS of the same 25 proteins resulted in identification of 16 proteins. Most of the proteins identified act on either cellulose or hemicellulose or their hydrolysis products. Thus far no lignin peroxidase, Mn peroxidase or laccases have been detected.

Keywords White-rot fungi · 2-D gel · MALDI-TOF · Peptide mapping

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Introduction

Cellulose, hemicellulose and lignin accounts for the majority of the carbons fixed by photosynthesis (Kirk 1983). The degradation of these components plays a major role in carbon recycling on earth. While cellulose and hemicellulose are degraded by a large number of microbes, lignin is highly recalcitrant and is degraded predominantly by fungi (Crawford and Crawford 1980). Of these fungi, the most extensively characterized are the white-rot fungi (Kirk 1983). White-rot fungi degrade both the lignin and carbohydrate components of woody biomass (Kirk and Farrell 1987). This complicated process involves an ensemble of both oxidative enzymes and hydrolytic enzymes. Cellulose and hemicellulose are degraded by hydrolytic enzymes whereas lignin, a much more formidable substrate, is degraded by oxidative enzymes (Kirk and Cullen 1998).

In 1998, Kirk and Cullen (1998) wrote "The picture of how the hyphae bring about the decay of wood is becoming increasingly clear at the molecular level, even though much remains to be learned about the specific enzymes of the white-rot fungi per se." Although many of the hydrolytic and oxidative enzymes have been purified and their cDNAs isolated, little is known about their expression on woody substrates. The recent sequencing of the genome of the white-rot fungus *Phanerochaete chrysosporium* (Martinez et al. 2004) now allows for proteomic methods to identify all of the enzymes involved in the degradation of woody substrates. Furthermore, this approach can potentially lead to the discovery of new enzymes involved in wood decay and also provide information on the succession of enzymes involved in this process. Here, advances in instrumentation such as matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and capillary liquid chromatography–nanoelectrospray ionization–tandem MS (CapLC-ESI-MS/MS), coupled with the very old method of two-dimensional (2-D) gel electrophoresis, now allow proteins of small samples sizes to be identified

by peptide mass mapping and tandem mass spectra matching, respectively.

The present paper describes methodology for the preparation of extracellular proteins from solid substrate cultures for use in 2-D gels. The present study also compares peptide fingerprinting versus CapLC-ESI-MS/MS as a method for identifying these extracellular proteins. These initial studies are the first to initiate the identification of the extracellular proteome of *P. chrysosporium* when grown on red oak.

Materials and methods

Wood preparation for liquid culture

Red oak wood chips were extracted with water three times to remove brown-colored extractives. Approximately 5 vol. distilled deionized water were added to the chips and then autoclaved for 30 min. The brown-colored water was then decanted and this process was repeated two more times. The wood was then dried (40°C) and ground to a powder, using a coffee grinder.

Strains and culture conditions

P. chrysosporium BKM-1767 (ATCC 24725) was maintained on malt agar slants as described by Kirk et al. (1990). *P. chrysosporium* was grown on either solid-substrate cultures or liquid submerged cultures, using wood chips as the carbon source. For growth on solid substrates, *P. chrysosporium* was first cultured in 250-ml Erlenmeyer flasks containing 20 g millet, 10 g wheat bran and 30 ml water at 37°C for 10 days and then used to inoculate polypropylene growth bags (Unicorn, Commerce, Tex.) containing 255 g wood chips, 30 g millet, 15 g wheat bran and 300 ml distilled water. Proteins were extracted after 3 weeks at 37°C by mixing with 500 NaCl (0.5 M), followed by incubation for 2 h at 4°C with stirring. The mixture was subsequently squeezed through cheesecloth, centrifuged at 8,000 *g* for 30 min at 4°C. Ammonium sulfate was added to the supernatant to 100% saturation over 30 min under constant stirring. After overnight incubation at 4°C with stirring, the preparation was centrifuged at 8,000 *g* for 30 min at 4°C. The protein pellet was dissolved in 50 ml water. Trace ammonium sulfate was removed by repeated concentration (four times, 10-kDa cutoff; Amicon) and re-suspension in 50 ml water. The final concentrate (10 ml) was stored at −20°C.

For liquid submerged cultures, *P. chrysosporium* was grown using wood as a carbon source. Shallow-stationary cultures were prepared in 125-ml Erlenmeyer flasks containing 10 ml low-nitrogen medium containing 1% (w/v) pretreated wood powder (described above), Basal III (Kirk et al. 1990), 0.1 mg/l thiamine HCl and 0.2 mg/l ammonium tartrate in 10 mM dimethyl succinate, pH 4.5. Cultures were inoculated with homogenized mycelia (Kirk et al. 1990) grown on the same medium at 37°C without shaking and flushed with water-saturated O_2 on days 2 and 5 and then harvested on day 6. The extracellular fluid was separated from the mycelium and woody substrate by passage through cheese cloth and then centrifuged at 8,000 *g* for 30 min. The supernatant was concentrated 100-fold using a 10-kDa filter (Amicon). Distilled deionized water was added to one-third of the original volume and re-concentrated. This desalting was repeated three more times. The fluid was then lyophilized to further concentrate the sample.

2-D gel electrophoresis

Immobiline Drystrips (11 cm, pH 3–10; Amersham) were re-hydrated overnight, using re-hydration buffer containing 8 M urea, 2% Chaps, 2% immobilized pH gradient buffer (pH 3–10; Amersham) and a few crystals of bromophenol blue. The concentrated protein (100 μg) was added to the isoelectric focusing strips and subjected to electrophoresis at 300 V for 1 h and then 1,400 V for 18 h. The second dimension, SDS-PAGE, was performed with the Hoefer vertical slab gel unit (11×13 cm), using 12% polyacrylamide. The proteins were visualized by Coomassie brilliant R stain.

In-gel digestion

Proteins spots were excised and digested with trypsin (Koc et al. 2001). Gel pieces were washed three times with 100 μl ammonium bicarbonate (25 mM) and dehydrated with 100 μl acetonitrile (50%) after each wash. The gel pieces were then dried using a speed-vacuum. Trypsin (12.5 ng/μl in 25 mM ammonium bicarbonate) was added to cover the gel piece and then incubated overnight at 37°C. The peptides were extracted twice with 25 μl formic acid (5%), shaken for 15 min and then sonicated for 5 min. The extracts were dried using a speed-vacuum and resuspended in 10 μl trifluoroacetic acid (TFA, 0.1%). The samples were desalted using C18 Zip Tips (Millpore, Bedford, Mass.).

MALDI-TOF mass spectrometry

The molecular mass of the peptides from the in-gel digestions were determined using MALDI-TOF/MS. The pressure in the ion chamber was 4×10^{-7} Torr and the matrix solution contained 0.3 M α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% acetonitrile. The sample (1 μl sample-digest, 1 μl matrix solution) was spotted onto a sample plate and dried by hot air. The plate was loaded into a MALDI unit and the monoisotopic peptide molecular weights were determined in reflectron mode. The spectra were calibrated with angiotensin, neurotensin and bradykinin. Proteins were identified by searching these peptide molecular weight

values against the NCBI non-redundant protein (NR) database, using the MS-Fit utility of the Protein Prospector.

LC mass spectrometry

In-gel tryptic digests were analyzed by CapLC-ESI-MS/MS. A Micromass Q-TOF micro coupled with a Waters CapLC HPLC unit was used for the analyses. Samples (1–10 µl) were injected with solvent A (acetonitrile/water/formic acid, 5/95/0.2) which was delivered by the auxiliary pump of the CapLC and trapped in a Waters Symmetry 300 column (C-18, 5 µm film, 0.3×5.0 mm). The sample was then washed for 3 min with solvent A at 20 µl/min, after which the trapped peptides were back-flushed onto the analytical column, a Dionex PepMap fused silica capillary column (C-18, 5 µm film, 0.075×150.0 mm), using a 10-port valve. The sample was eluted from the analytical column with a gradient of 5–42% solvent B (acetonitrile/water/formic acid, 95/5/0.2) within 44 min. The flow rate from the HPLC pump was 10 µl/min and this flow was reduced to approximately 300 nl/min, using a Valco zero dead volume splitter. A nanolock spray source with dual sprays, one for analyte and one for reference compound, was utilized. The lock mass was Glu-Fib delivered at 0.5 µl/min, using the syringe pump of the Q-Tof micro. The spray voltage was set to 3,700 V and the source temperature was 100°C. No nebulizing gas was used. The Q-TOF mass spectrometer operated in data-dependent acquisition mode, using Mass Lynx ver. 4.0 SP1. To perform the MS/MS data acquisition, a survey scan over m/z from 400 to 2,500 was performed every second between 10 min and 50 min. From each survey scan, up to three of the most intense double- or triple-charged ions above an intensity threshold of 15 were selected for MS/MS to obtain the product ion spectra resulting from collision-induced dissociation in the presence of argon. The product ion spectra collected were processed using Protein Lynx Global Server ver. 2.0 prior to database-searching. The processed spectra were searched against both a locally generated protein database for *P. chrysosporium* genes (Martinez et al. 2004) and the NCBI NR database using Protein Lynx Global Server and/or the on-line version of Mascot (http://www.matrixscience.com).

Protein database generation

To identify the proteins, we obtained from Dr. Dan Cullen (USDA Forest Products Laboratory, Madison, Wis.), prior to publication (Martinez et al. 2004), the modeled annotated database for *P. chrysosporium* (all of the putative proteins encoded by *P. chrysosporium* in a FASTA format). The annotated sequence database was obtained by two methods. The first method entailed comparing the entire genome in translation against all known proteins in GenBank at low stringency, using ungapped BLASTX (Blosum62, score > 30). The second method was obtained from outputs of GeneWise and GrailEXP. Thus, with this set of protein sequences, we were then able to utilize Protein Prospector software (MS-Fit; http://prospector.ucsf.edu/) to identify proteins by peptide fingerprinting.

Miscellaneous methods

Protein content was measured by Bradford (1976). Where indicated, extracellular proteins were treated with either trichloroacetic acid (TCA)/acetone (Jiang et al. 2004) or phenol (Hernandez-Macedo et al. 2002).

Results and discussion

Extraction of extracellular proteins

Our initial attempts to isolate and characterize extracellular proteins from wood-grown cultures utilized *P. chrysosporium* grown on red oak medium for 2 weeks (Orth et al. 1993; Varela et al. 2003). In contrast to proteins isolated from brown-rot cultures, these preparations contained a brown-colored extractive, probably lignin-derived. A sample 2-D gel, shown in Fig. 1, reveals extensive streaking at the acidic end. The area of streaking corresponded to a brown-colored material. Attempts to identify the proteins from such samples were not successful.

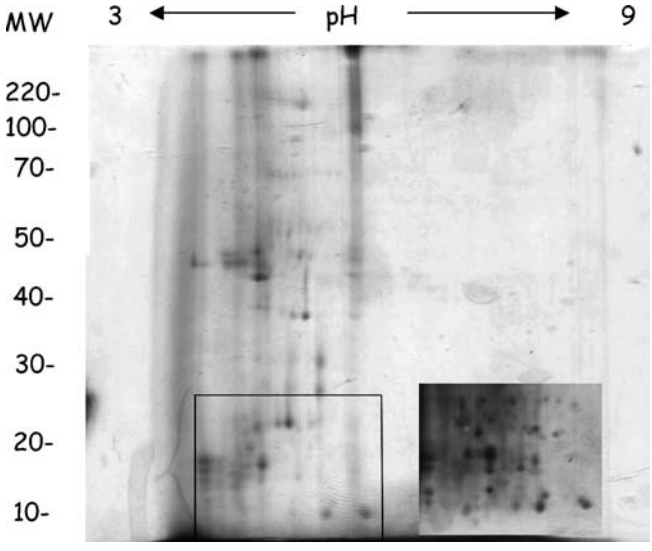

Fig. 1 2-D gel of extracellular proteins from *P. chrysosporium* grown on solid wood substrate for 3 weeks. The wood medium contained 85% (w/w) wood chips, 10% millet and 5% wheat bran. The gel was stained with Coomassie blue, except for the *inset* where after visualizing with Coomassie blue, the gel was destained and then visualized using silver stain. The inset shows the portion of the gel that is *boxed*

A number of different methods were used to remove the brown extractive from the extracellular proteins. Extraction of the proteins with 1% TCA in acetone (Jiang et al. 2004)decreased the brown color, although extensive streaking was still observed. Hernandex-Macedo et al. (2002) utilized phenol extraction to isolate proteins from *P. chrysosporium*. Again, this method was not effective in removing the brown-colored extractive.

Washing the wood substrate prior to fungal incubation help improve the protein preparations. The woody substrate was then used in shallow-liquid stationary cultures. Mycelial mats were visible after 2 days of growth. The extracellular fluid was isolated from 6-day cultures and concentrated. The concentrated proteins were subjected to 2-D gel electrophoresis. A 2-D gel of the extracellular proteins is shown in Fig. 2. Over 45 protein spots are visible, with the more prominent spots labeled.

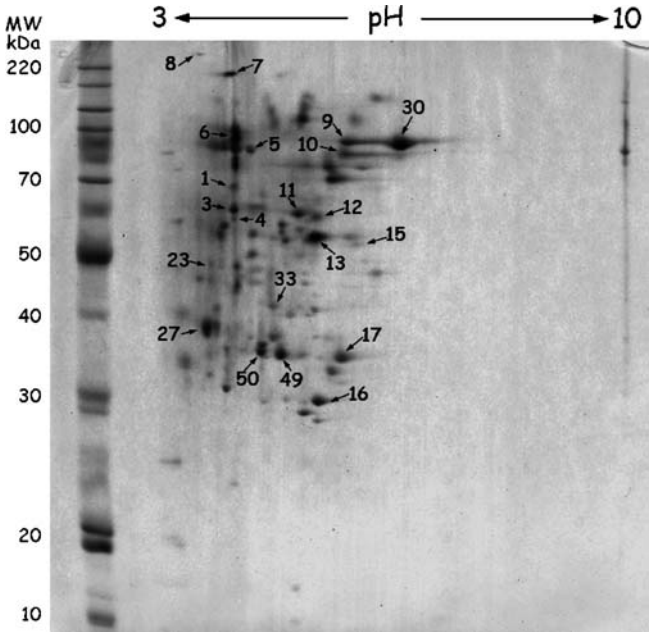

Fig. 2 Representative 2-D gel of extracellular proteins. The fungal cultures were grown on liquid cultures containing oak wood chips as the carbon source (see Materials and methods). The gel shows over 45 spots, with the prominent spots numbered. For clarity, not all of the proteins spots are labeled. However, all of the visible bands (over 45) were excised from the gel

Protein identification after 2-D gels and in-gel digestion

The protein spots from the 2-D gels were excised and subjected to in-gel digestion, using trypsin. The tryptic peptides were then analyzed by MALDI/TOF and from the 25 proteins analyzed, only two were identified: laminarinase (Table 1) and cellobiose dehydrogenase (Table 2). This was not surprising due to a prior difficulty reported by other investigators with extracellular fungal proteins (Fryksdale et al. 2002). These workers attributed the failure of MALDI-TOF protease fingerprinting to be due to glycoslyation, which could not only alter the peptide molecular weights but also potentially inhibit the action of the trypsin protease in the in-gel digestion. Chemical deglycosylation methods using trifluormethanesulfonic acid to remove the carbohydrates (Fryksdale et al. 2002) prior to 2-D electrophoresis were not successful (data not shown).

However, the CapLC-ESI MS/MS method was able to identify the proteins using the MS/MS of the non-glycosylated tryptic peptides. This enabled the identification of 16 protein spots, two of which were corroborated by MALDI-TOF/MS. We detected enzymes involved in both cellulose and hemicellulose hydrolysis, as described below.

We then utilized CapLC-ESI-MS/MS. After trypsin digestion, the resulting peptide mixtures were analyzed by nanoscale CapLC/MS/MS, using a quadrupole TOF/MS. A representative product ion spectrum for one of the peptides analyzed is shown in Fig. 3. The product ion spectra obtained by CapLC-MS/MS were searched against the annotated *P. chrysosporium* local and NCBI NR databases, using Protein Lynx Global Server ver. 2.0. As shown in Table 3, from the same 25 spots

Table 1 Peptide masses and predicted amino acid sequence of laminarinase. The peptide masses were analyzed by Protein Prospector, using the *P. chrysosporium* local database; and the best match was pc.78.37.1. A BLAST homology search of the fungal database indicated that the protein is laminarinase

m/z (minimum)	*m/z* (average)	Start	End	Missed cleavages	Database sequence
616.4146	616.7867	94	98	1	(R)IRSIK (T)
709.3885	709.8257	212	217	0	(R)TNSFIK (V)
744.448	744.8776	90	95	1	(R)NSVRIR (S)
851.4568	852.0333	218	223	0	(K)VWFFPR (N)
1292.685	1293.472	51	62	0	(R)VNYVDQATALAK (N)
1396.682	1397.497	76	89	0	(R)ADHTTTLSPSGPGR (N)
1408.723	1409.595	99	110	0	(K)TYTTHVAVFDVR (H)
1436.775	1437.646	63	75	0	(K)NLTYASGDTLILR (A)
1541.827	1542.836	212	223	1	(R)TNSFIKVWFFPR (N)
1736.934	1738.009	96	110	1	(R)SIKTYTTHVAVFDVR (H)
1852.927	1854.002	76	93	1	(R)ADHTTTLSPSGPGRNSVR (I)
2710.442	2712.095	51	75	1	(R)VNYVDQATALAKNLTYASGDTLILR (A)
2814.439	2816.12	63	89	1	(K)NLTYASGDTLILRADHTTTLSPSGPGR (N)

Table 2 Peptide masses and predicted amino acid sequence of cellobiose dehydrogenase. The peptide masses were analyzed by Protein Prospector, using the *P. chrysosporium* local database; and the best match was pc.5.157.1. A BLAST homology search of the fungal database indicated that the protein is cellobiose dehydrogenase

m/*z* (minimum)	*m*/*z* (average)	Start	End	Missed cleavages	Database sequence
607.4122	607.3356	135	138	0	(K)WVFR (C)
742.5745	742.4827	259	264	0	(K)VLLLER (G)
1060.607	1060.47	587	596	0	(R)AYSGSDGFTR (Y)
1178.799	1178.653	479	490	0	(R)VILSAGAFGTSR

that were analyzed by CapLC-MS/MS, 16 sequences could be identified from the annotated *P. chrysosporium* database (Table 3 also shows additional spots which were identified on subsequent analyses). Two of the original 16 were the same as the ones identified by MALDI-TOF, thus corroborating the two methods. The calculated pI and calculated molecular weights were compared with the values obtained from the gel. The sequences (accession numbers given in Table 3) were then used in a BLAST search of the fungal database at NCBI and identified by homology to sequences in the fungal database, as discussed below.

Cellulose hydrolysis

Enzymes required for cellulose hydrolysis were detected in our 2-D gels. Complete hydrolysis of cellulose, the least diverse of the three major wood components (Atalla 1993) requires an ensemble of three enzymes which, within themselves, contain numerous isozymes (for a review, see Kirk and Cullen 1998): (1) endoglu-

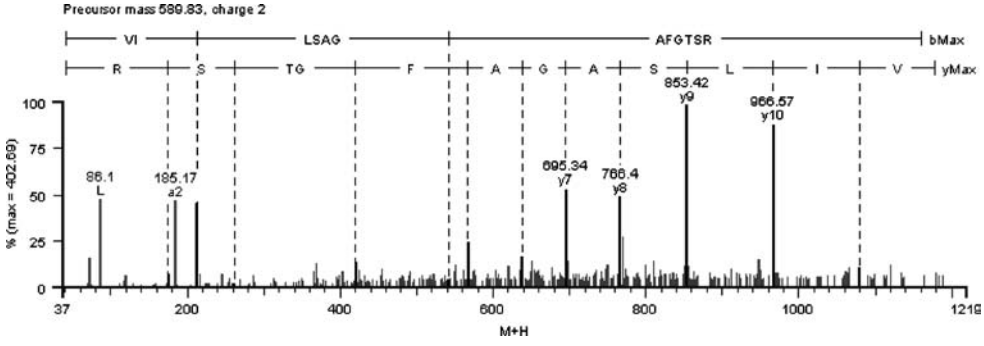

Fig. 3 Product ion spectrum of the doubly charged precursor ion (*m*/*z* 589.83) of a tryptic peptide (VILSAGAFGTSR) of chain A of cellobiose dehydrogenase

Table 3 Results of BLAST homology search of genes identified by Protein Prospector. Each protein spot corresponds to a number in the gel shown in Fig. 1. The sequences were obtained from CapLC-ESI-MS/MS and then used to search the local *P. chrysosporium* database by Protein Prospector. The identified enzyme is listed (*Putative enzyme*) and its identified gene (*Accession*) was used in a BLAST search of the fungal database (the *E* values of the BLAST search are shown). The calculated molecular weight (*MW*) and pI are shown, along with the experimentally determined values (in parentheses)

Protein spot	Sequence	Putative enzyme	Accession	*E* value	MW	pI
1	VVLDSNWR	Cellobiohydrolase	pc.33.51.1	0.0	58,142 (66,000)	4.9 (4.9)
3	TAFGDTNWFAQK	Cellobiohydrolase	pc.33.51.1	0.0	58,142 (59,000)	4.9 (4.9)
5	SAFDFLNGK	Cellobiose dehydrogenase	pc.5.157.1	0.0	82,007 (86,000)	5.2 (5.1)
6	IGIDAALR	Cellobiose dehydrogenase	pc.5.157.1	0.0	82,007 (95,000)	5.2 (5.0)
7	SEQLAVWTVK	β-Glucosidase	Genewise2nd.66.3.1	$2e^{-42}$	69,848 (160,000)	4.9 (4.8)
8	IIDFAAPVGQK	α-Galactosidase	pc.11.202.1	0.0	47,706 (200,000)	4.6(4.6)
13	ISGTFTNVR	Endopolygalacturonase	Genewise2nd.1.81.1	$8e^{-93}$	32,503 (52,000)	4.7 (5.7)
15	IPDLGTYLASASALGK TTYLACVNYALTNLAK	Exocellobiohydrolase II	pc.3.82.1	0.0	48,417 (50,000)	5.0 (6.0)
16	ADVSYDIWFGK	β-Endoglucanase	pc.163.7.1	$3e^{-41}$	25,418 (30,000)	4.7 (5.8)
17	TYTTHVAVFDVR	Laminarinase	pc.78.37.1	e^{-180}	33,961 (34,000)	5.0 (6.0)
23	LPSTDHPSTDGQR GQGYNQATINDNPNYK VILSAGFGTSR AYSGSDGFTR	Cellobiose dehydrogenase	pc.5.157.1	0.0	82,007 (45,000)	5.2 (4.8)
27	ISGTFTNVR	Endopolygalacturonase	Genewise2nd.1.81.1	$8e^{-93}$	32,503 (38,000)	4.7 (4.7)
30	GDGNTDDTAAIQAAIN-AGGR	Glucan 1,3-β glucosidase	pc.67.67.1	e^{-172}	82,632 (80,000)	5.6 (6.2)
33	GVFTFANADTIANLAR	Endo-1,4 β xylanase A	pc.143.21.1	e^{-127}	35,784 (42,000)	5.3 (5.3)
49	LQTDHLFAR TGIQGTVMSPLK MVFHDSIAISPK TGIQGTVMSPLKGEMR	Lignin peroxidase, isozyme H2, H1	pc.19.174.1	0	39,534 (39,000)	5.02 (5.2)
50	LQTDHLFAR TGIQGTVMSPLK	Lignin peroxidase, isozyme H2	pc.19.174.1	0	39,534 (39,000)	5.02 (5.3)

canases (endo-1, 4-β-glucanase) which hydrolyze internal bonds, (2) cellobiohydrolases (exo-1,4-β-glucanase) which act processively on the existing chain ends and on those created by the endoglucanases, releasing cellobiose and (3) β-glucosidase which cleaves cellobiose into two glucose molecules. The activity of all three of these enzymes have been detected in *P. chrysosporium* (Eriksson and Rzedowski 1969; Eriksson and Pettersson 1975; Streamer et al. 1975; Eriksson et al. 1990; Uzcategui et al. 1991; Kawai et al. 2003) and some have been cloned (Vanden Wymelenberg et al. 1993).

Spot 16 was the only endoglucanase detected on the 2-D gel. The experimental and the calculated pI and molecular weight are close in value. A BLAST homology search of the *P. chrysosporium* genome showed one other homologous sequence (Table 4).

There are two types of cellobiohydrolase and both have been detected in *P. chrysosporium* (Uzcategui et al. 1991) and cloned (Tempelaars et al. 1994; Van Rensburg et al. 1998). Cellobiohydrolase I (spots 1, 3) acts on the reducing end. Both spots are very close in molecular weight and pI. On occasion, we detected another spot (originally numbered spot 2) which migrated close to spot 1 and was identical to spot 1 in sequence. Differences in mobility of the same gene product may be due to heterogeneity in post-translational modification processes, such as glycosylation or phosphorylation. We observed such differences previously with lignin peroxidases from *P. chrysosporium*, where isozyme H1 and H2 had an identical amino acid sequence but a different pI (Kuan and Tien 1989a). Homology searches showed three additional cellobiohydrolase I-like sequences in the *P. chrysosporium* genome with a similar molecular weight and pI (Table 4). Cellobiohydrolase II (spot 15) acts on the non-reducing end. The homology search found no other cellobiohydrolase II-like sequences.

Only one β-glucosidase was identified on the gel (spot 7). Although the predicted pI is in close agreement with the experimental value, the experimental molecular weight of 160,000 from the gel is much higher than that predicted for a β-glucosidase. This again could be due to a high level of glycosylation. A BLAST homology search indicated the presence of seven additional genes, all of which encoded a protein with a molecular weight of approximately 80,000–90,000.

Table 4 BLAST homology search of the *P. chrysosporium* database

Putative enzyme	Spot	pI	MW
Cellobiohydrolase I (pc.33.51.1)[a]	1, 2, 3	4.96	58,142
pc.116.24.1	–	4.23	53,841
pc.95.47.1	–	4.55	53,900
pc.139.26.1	–	5.03	55,006
Cellobiose dehydrogenase (pc.5.157.1)[a]	5, 6, 23	5.19	82,007
β-Glucosidase (genewise2nd.66.3.1)[a]	7	4.96	69,848
pc.80.2.1	–	5.74	91,753
pc.83.26.1	–	5.93	93,594
pc.63.34.1	–	5.4	90,613
pc.47.98.1	–	5.71	94,448
pc.2.181.1	–	5.42	86,824
pc.11.220.1	–	6.45	86,576
pc.114.39.1		5.45	81,344
α-Galactosidase (pc.11.202.1)[a]	8	4.65	47,706
genewise2nd.55.13.1	–	4.72	41,173
genewise2nd.17.81.1	–	4.66	39,686
Endopolygalacturonase(genewise2nd.1.81.1)[a]	13, 27	4.69	32,503
Exocellobiohydrolase (pc.3.82.1)[a]	15	5.04	48,417
β-Endoglucanase (pc.163.7.1)[a]	16	4.73	25,418
Pc.5.247.1	–	4.74	26,984
Laminarinase (pc.78.37.1)[a]	17	5.05	33,961
pc.58.2.1	–	6.87	38,422
pc.116.7.1	–	5.39	36,118
genewise2nd.21.14.1	–	4.72	25,584
pc.19.47.1	–	5.01	31,656
genewise.78.46.1	–	4.38	29,856
pc.10.73.1	–	5.27	42,790
pc.22.125.1	–	5.29	33,860
pc.22.189.1	–	4.64	36,974
Glucan 1,3-β glucosidase (pc. 67.67.1)[a]	30	5.55	82,632
pc.3.254.1	–	7.23	85,043
Endo-1,4 β Xylanase A (pc.143.21.1)[a]	33	5.30	35,784
pc.5.251.1	–	6.51	39,268
genewise2nd.42.28.1	–	4.73	42,050
genewise2nd.74.12.1	–	5.21	43,570
Pc.47.69.1	–	5.35	37,553
genewise2nd.32.42.1	–	5.30	37,016
Lignin peroxidase (pc.19.174.1), see text	49, 50	5.02	39,534

[a]These putative genes (listed in Table 3) encoded the proteins identified from the 2-D gel and were used in the BLAST search of the *P. chrysosporium* genome. Homologous sequences are shown, along with the calculated pI and MW

Hemicellulose hydrolysis

Enzymic hydrolysis of xylans also requires multiple enzymes (Kirk and Cullen 1998). Datta et al. (1991) detected endoxylanase activity in extracts of pulp-grown cultures of *P. chrysosporium*. Endo-1,4-β-xylanases (endoxylanase) act on the backbone of xylans and produce xylo-oligomers of different lengths. Thus far, we have only detected endoxylanases in the 2-D gels (spot 33). The calculated and experimental pI and molecular weight values are in close agreement. Five other endoxylanase-like genes were found in the homology search (Table 3). Other enzymes involved in hemicellulose hydrolysis, such as α-glucuronidase (Castanares et al. 1995), α-arabinofuranosidase (Dobozi et al. 1992) and β-xylosidase (Dobozi et al. 1992), have all been detected in cultures of *P. chrysosporium*.

Multiple enzymes are also involved in the degradation of *O*-acetylgalactoglucomannans (see Johnson 1990). Endomannases, acetylmannan esterases, α-galactosidases, β-mannosidase and β-glucosidase are all involved in complete hydrolysis. From the 2-D gel, we detected α-galactosidase and β-glucosidase. Apparently, these enzymes are not well characterized in *P. chrysosporium*. Only α-galactosidase activity has been detected in *P. chrysosporium* cultures (Brumer et al. 1999). An enzyme first thought to be a β-glucosidase is actually a laminarinase, involved in cell wall metabolism rather than *O*-acetylgalactomannan degradation (Kawai et al. 2003).

Other enzymes

In addition to enzymes known to be involved in cellulose and hemicellulose hydrolysis, we also found lignin peroxidases (spots 49, 50). Lignin peroxidase (Tien and Kirk 1988) catalyzes the oxidation of phenolic and nonphenolic aromatic rings, to yield cation radicals (Hammel et al. 1985; Kersten et al. 1985). Both spot 49 and spot 50 have identical amino acid sequences and very similar molecular weights, but different pI values. Previous work from our laboratory showed that isozymes H2 and H1 were identical in amino acid sequence. However, isozyme H1 is phosphorylated (Kuan and Tien 1989b). Such may be the case with spots 49 and 50, where spot 49 is H1 and spot 50 is H2. We have yet to detect Mn peroxidase (Gold et al. 2000). However, our inability to detect an enzyme should not have any physiological relevance associated with it. We have yet to attempt identifying all spots; and expression patterns are influenced by growth conditions. We omitted the homology for lignin peroxidase since this gene family has been well characterized (Stewart et al. 1992).

Another oxidoreductase we detected is cellobiose dehydrogenase (spots 5, 6, 23). The homology search detected an unlikely candidate with a molecular weight of 23,000. Different roles have been proposed for this enzyme in lignocellulose degradation, but have yet to be clearly defined (Henriksson et al. 2000). This enzyme oxidizes celliobiose residues by two electrons and can reduce a variety of electron acceptors. Cellobiose dehydrogenase from *P. chrysosporium* has been extensively characterized, purified, cloned and expressed (Bao et al. 1993; Li et al. 1996; Igarashi et al. 1997). It has been proposed to provide hydrogen peroxide for the extracellular peroxidases and also to prevent phenoxy radical-dependent re-polymerization of lignin (Ander 1994; Duarte et al. 1999; Henriksson et al. 2000).

From the 2-D gels, we also identified endopolygalacturonases (spots 13, 27) and 1,3-β glucosidases (spots 17, 30). Endopolygalacturonase has been previously isolated and characterized from solid-state cultures of *P. chrysosporium* (Shanley et al. 1993). This enzyme is active with pectin, which accounts for up to 4% of the dry weight of wood. The 1,3-β glucosidases are most likely not involved in lignocellulose degradation. Laminarinases (spot 17) are endoglucosidases. A BLAST search detected a very large number of homologous sequences (Table 4). In contrast to laminarinase, 1,3-β-glucosidases (spot 30) are exo 1,3-β-glucosidases. The role of these enzymes may not be associated with lignocellulose degradation. The 1-3-β linkage is found in grass celluloses, but we are not aware of such linkages in woody biomass. It is, however, a common linkage in fungal cell walls and thus these enzymes are proposed to be involved in fungal cell wall metabolism associated with mycelial growth.

Conclusions

We detected over 40 different proteins spots on 2-D gels. Using in-gel digestion and CapLC-MS/MS, we identified 16 of these spots. With the exception of three proteins (spots 7, 8, 23), all of the calculated molecular weights were within 13,000 of the experimental molecular weight (determined from gel). We have no apparent explanation for the large discrepancies observed here. With the exception of spot 23, the experimental molecular weights were all greater than or equal to the calculated value, which is consistent with glycosylation or other post-translational modification which would decrease mobility during SDS-PAGE.

In this study, we detected enzymes that act on the three major components of wood: cellulose, hemicellulose and lignin (and their breakdown products). The process of identification is on-going as we continue to developed a methodology to identify the extracellular proteome of *P. chrysosporium* when grown on a solid substrate. We have provided conditions for the preparation of proteins with minimal contamination and a methodology for identifying the proteins by CapLC-MS/MS. The methods described here can now be used by other investigators interested in identifying extracellular fungal enzymes.

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