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TRANSCRIPTION OF LIGNINASE H8 BY PHANEROCHAETE CHRYSOSPORIUM UNDER NUTRIENT NITROGEN SUFFICIENT CONDITIONS

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Dot blot analyses showed that more ligninase H8 mRNA was present in ammonium sufficient cultures of Phanerochaete chrysosporium than in ammonium limited cultures. Reverse transcription followed by polymerase chain reaction and DNA sequencing verified that H8 mRNA was present under both conditions. Fast protein liquid chromatography profiles indicated that H8 was present but lacked heme in ammonium sufficient cultures. These data indicated that H8 was transcribed and translated by day 5 under ammonium sufficient conditions, but was inactive due to lack of heme.

Lignin peroxidases, (ligninases) first isolated in 1984 (1,2), are responsible for lignin degradation by white rot fungi and can play a role in the biodegradation of a wide range of environmental pollutants (3). It is important to understand the regulation of these enzymes to optimize bioremediation efforts. Phanerochaete chrysosporium produces extracellular ligninases in cultures limited in nutrient nitrogen (4-6), carbon (7), or sulfur (8). Under nutrient sufficient conditions, enzyme activity was not detected and it was concluded that transcription did not take place (7,9). We report that under these conditions, transcription does take place but no ligninase enzyme activity is detected. This is controversial since it is commonly thought that regulation is at the level of transcription (9-11).

MATERIALS AND METHODS

Culture Conditions, Enzyme Purification and Assay

P. chrysosporium strain BKM-F-1767 (ATCC 24725) was grown from spores at 39°C under nitrogen sufficient and limiting

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conditions (24 and 2.4 mM ammonium as ammonium tartrate) with trace elements as described previously (12). Fast protein liquid chromatography (FPLC) of the extracellular, proteins and enzyme assays were conducted as previously described (13).

RNA Isolation

Mycelium was taken from nitrogen sufficient and limiting cultures after 5 days of growth. The mycelium was washed and ground with mortar and pestal at -70°C using liquid nitrogen. Triplicate poly(A) mRNA samples were extracted from separate flasks using Micro-Fast Track mRNA isolation materials (Invitrogen, San Diego, CA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription was done with 0.2 μg of poly(A)RNA and 50 U of Muloney murine leukemia virus reverse transcriptase with 2.5 μM of random hexamers and the other components from a GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) according to manufacturer's protocol. The PCR reaction was done according to manufacturer's protocol except final primer concentrations were 0.2 μM each in the 100 μl reactions. A 402 bp coding region spanning 4 introns of the P. chrysosporium ligninase H8 gene was targeted using primers H8I and H8II (TGA GGC GCA CGA GTC GAT TCG TCT and GAC AAG CTC GAG CTC ATC GAA CTC) corresponding to base positions starting at 252 and 868 in the sequence reported by Smith et al. (14).

Dot Blots

Dot blots were done using poly(A) RNA (0.5 μg) extracted from day 5 cultures of P. chrysosporium. Alkaline RNA denaturation and fixation on a Zeta-Probe membrane (BioRad, Hercules, CA) was done according to the manufacturer's protocol. The probe was a 402 bp segment of ligninase H8 gene generated by PCR amplification with primers H8I and H8II using H8 cDNA (ML-1 described in Tien and Tu (9)) within a pBluescript plasmid (Stratagene, LaJolla, CA) as a target. The PCR amplified probe was randomly labeled with alpha ^{32}P-dATP using T7 DNA polymerase from Prime-it Random Primer kit (Stratagene) to a specific activity of 10^9 dpm/μg. Nuctrap Push Columns (Stratagene) were used to purify the probe according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Ligninase H8 mRNA was detected by RT-PCR (Fig. 1) as well as by dot blot analysis (Fig. 2) in day 5 cultures of P. chrysosporium grown under both nutrient nitrogen (ammonia) limiting and sufficient conditions. Dot blot analysis is more quantitative than RT-PCR amplification and indicated that higher levels of ligninase H8 mRNA were present under nitrogen sufficient conditions. All reagent controls showed no evidence of contamination. Specificity of RT-PCR was confirmed using asymmetric PCR of the double stranded cDNA template to prepare single stranded DNA for DNA sequencing (15) with components from a sequence 2.0 DNA sequencing kit (USB, Cleveland, OH) according

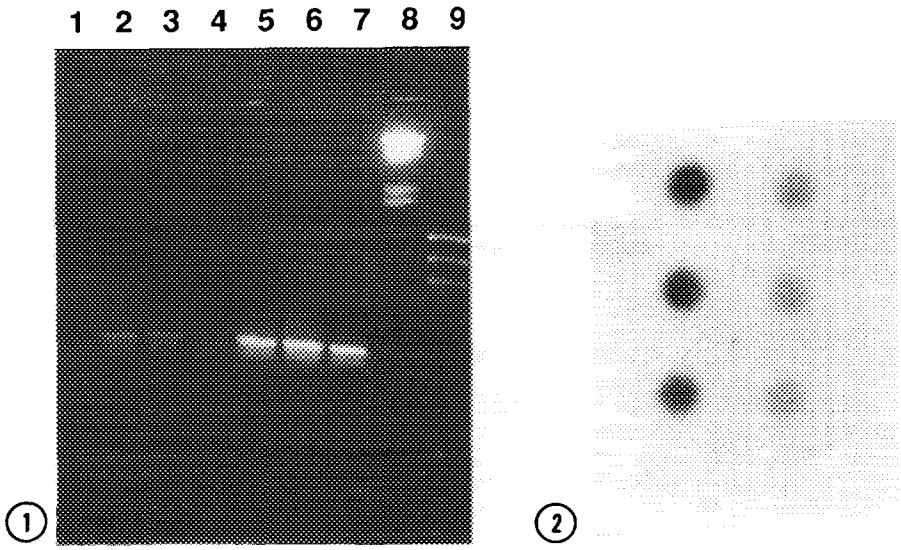

Figure 1. Detection of ligninase H8 mRNA by reverse transcription and polymerase chain reaction in day 5 cultures of P. chrysosporium.
Lane 1: Reagent blank.
Lane 2-4: RNA (0.2 µg) extracted from triplicate ammonium sufficient cultures.
Lane 5-7: RNA (0.2 µg) extracted from triplicate ammonium deficient cultures.
Lane 8: DNA size marker, λ(Hind III digest).
Lane 9: DNA size marker, ϕ x 174 (HaeIII digest).

Figure 2. Detection of ligninase H8 mRNA by dot blots of mRNA extracted from day 5 cultures of ammonium sufficient (left dots) and deficient (right dots) cultures of P. chrysosporium. Control (rat liver mRNA) is below right. The probe used was ^{32}P labeled ligninase H8 cDNA.

to the manufacturer's instructions. PCR amplification of any contaminating P. chrysosporium ligninase H8 DNA would show up as a 616 bp fragment.

Although ligninase RNA was present in day 5 cultures of nitrogen sufficient cultures, ligninase activity was undetectable. However, FPLC elution profiles of protein from these cultures resembled the protein profiles obtained from nitrogen limited cultures (Fig. 3) but did not contain heme, as evidenced by the lack of any absorbance at 409 nm.

Heme bleaching reactions (16,17) or protease activity (18,19) may be responsible for the absence of ligninase activity. Protease has previously been measured in nitrogen limited cultures but not nitrogen sufficient cultures of P. chrysosporium (18,19) and was linked to the decay of the lignolytic system. Protease activity was measured using azocol as described by Dorsetz et al. (18). Protease activity was 23 times higher in

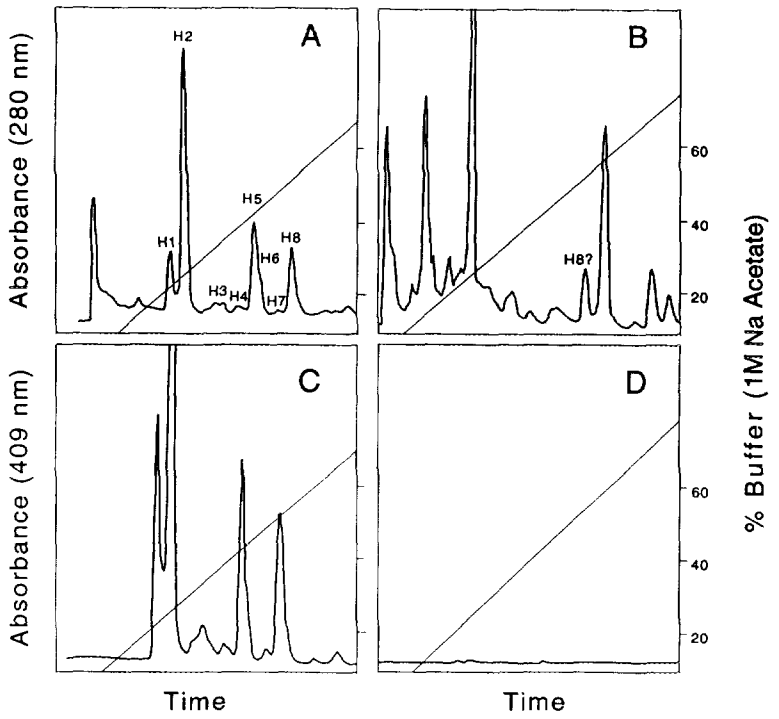

Figure 3. FPLC profile of extracellular protein taken from day 5 ammonium limited (A, C) and sufficient (B, D) cultures of P. chrysosporium. Figures 3A and 3B show protein absorbance at 280 nm. Figures 3C and 3D show heme absorbance at 409 nm. The sloping line represents the acetate gradient.

day 5 nitrogen sufficient cultures (21.7 U/ml) than in nitrogen limited cultures. However, protease activity responsible for the loss of heme would have to be very limited proteolysis as the FPLC elution profiles of inactive preparations obtained from nitrogen sufficient cultures were almost identical to the FPLC profile of active enzymes obtained from nitrogen limited cultures.

Other researchers that have reported ligninase production by P. chrysosporium under nitrogen sufficient conditions have either used mutant strains or did not eliminate the possibility of carbon limitation. Over production of ligninase by P. chrysosporium was reported under non-limiting nutrient nitrogen conditions in a solid matrix (20). However, the glucose concentration was zero before enzyme activity was detected. Ligninase activity was also found under conditions of nitrogen sufficiency with glycerol as a carbon source (21). There is a nitrogen deregulated mutant strain of P. chrysosporium that

produces active enzyme under nutrient nitrogen sufficient conditions (22). However, the reason for the lack of heme in the proteins observed in this study are unknown but are being investigated.

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