Structure and Biochemistry of the laccases from the Lignin - Degrading Basidiomycete *Ganoderma lucidum*.

Kranthi Kumar, P., Zach Mathew and C. A. Reddy.

G. lucidum is one of the most important and widely distributed ligninolytic white rot fungi from habitats such as forest soils, agricultural soils, and tropical mangrove ecosystems and produce laccases as an important family of lignin modifying enzymes. Biochemically, laccases are blue multi copper oxidases that couple four electron reduction of molecular oxygen to water. There is a growing interest in the use of laccases for a variety of industrial applications such as bio-pulping and bio-bleaching as well as in their ability to detoxify a wide variety of toxic environmental pollutants. These key oxidative enzymes are found in all the three domains of life: *Eukaryota. Prokarya, and Archaea*. *Ganoderma lucidum* (strain no.103561) produces laccase with some of the highest activity (17,000 micro katals per mg of protein) reported for any laccases to date. Our results showed that this organism produces at least 11 different isoforms of laccase based on variation in mol. weight and/or PI.

Our Studies showed that the presence of copper in the medium yields 15- to 20-fold greater levels of enzyme by *G. lucidum*. Dialysation of extra cellular fluid of *G. lucidum* against 10mM sodium tartrate (pH5.5) gave an additional 15 to 17 fold stimulation of activity with an observed specific activity of 17,000 µkatals/mg protein. Dialysis against acetate buffer gave five fold increase in activity while dialysis against glycine showed inhibition of activity. Purification by FPLC and preparative gel electrophoresis gave purified fractions that resolved into eleven isoforms as separated by isoelectric focusing, and the PI,s were 4.7, 4.6, 4.5, 4.3, 4.2, 4.1, 3.8, 3.7, 3.5, 3.4 and 3.3.

Genomic clones of laccase were isolated using *G. lucidum* DNA as a template and using inverse PCR and forward/reverse primers corresponding to the sequences of the conserved copper binding region in the N-terminal domain of one of the laccases of this organism. Inverse PCR amplication of *Hin*dIII digested and ligated *G.lucidum* DNA was done using ABI Geneamp XL PCR kit in Ribocycler. The 5' conserved copper binding region of laccase was used for designing forward primer (5'TCGACAATTCTTTCCTGTACG3') and reverse primer (5' TGGAGATGGG ACACT GGCTTATC 3'). The PCR profile was 95^0C for 3min, 94^0C for 1min, 57^0C for 30 sec and 68^0 C for 5min. for 30 cycles, and the final extension was at 72^0C for 10min. The resulting ~2.7 Kb inverse PCR fragment was cloned into ZERO TOPOII blunt ligation vector (INVITROGEN) and screened on Kanamycin plates. Selected putative clones containing inserts were digested with a battery of restriction enzymes and analyzed on 1% agarose gels. Restriction digestion of these clones with *Bam*HI, *Pst*I, *Sal*I, *Pvu*II, *Eco*RI, and *Xho*I revealed 8 distinct patterns suggesting gene diversity. Two clones were sequenced using overlapping primers on ABI system. The sequences were aligned using Bioedit program. The aa sequences of the clones were deduced by Genewise2 program using *Aspergillus* as the reference organism. Eukaryotic gene regulatory sequences were identified using GeneWise2 Program. Laccase sequence alignments and similarity indexes were calculated using ClustalW and BioEdit programs.

Blast analysis of two distinct *Bam*HI clones, *lac*1 and *lac*4, showed that the proteins encoded by these clones are fungal laccase sequences. The coding sequence of *lac1*gene is interrupted by 6 introns ranging in size from 37-55 nt and encodes a mature protein consisting of 456 aa (*Mr*: 50,160), preceded by a putative 37-aa signal sequence. This predicted *Mr* is in agreement with the range of *Mr*s previously reported by us for the laccases of *G. lucidum*. The deduced aa sequence of LAC1 showed relatively high degree of homology with laccases of other basidiomycetes. It showed 96% homology to full-length LAC4 protein and 47-53% similarity to unpublished partial laccase sequences of other *G. lucidum* strains. Among the other basidiomycete laccases, LAC1 showed the highest similarity of 53-55% to *Trametes versicolor*LAC3 and LAC4. The consensus copper-binding domains found in other basidiomycete laccases are conserved in the LAC1 protein of *G.lucidum*.

Eight putative *N*-glycosylation sites as well as consensus eukaryotic promoter sequence and polyadenylation signal sequences are also found. Coding sequence of *lac4* is interrupted by 7 introns (similar in size to those of *lac1*), encodes a mature protein of 525aa (*Mr*: 57,750), and has 98% nt homology to *lac1*, but was otherwise identical. Molecular masses of GLAC1 and GLAC4 were 49.8 kDa (462aa) and 52.5 kDa (524aa) in comparison to *T. versicolr* laccase which was 56.3 kDa (524aa). Predicted PI values of GLAC1, GLAC4 and *T. versicolor* laccase are, respectively 4.5, 4.7, and 4.2. Eight other laccase clones, distinct from *lac1* and *lac4* have recently been isolated from *G. lucidum* Our results show the existence of a laccase multi-gene family in *G. lucidum* in agreement with our earlier results showing multiple isoforms of laccase in this organism.