The Journal of Microbiology, December 2006, p. 617-621
Vol. 44, No. 6

Increase of Yeast Survival under Oxidative Stress by the Expression of the Laccase Gene from *Coprinellus congregatus*

Dongsik Kim, Eunjung Kwak, and Hyoung T. Choi*

Molecular Microbiology Lab, Department of Biochemistry, Division of Life Sciences, Kangwon National University, Chunchon 200-701, Republic of Korea

(Received August 1, 2006 / Accepted November 20, 2006)

Coprinellus congregatus **secreted a laccase isozyme when the culture was transferred to an acidic liquid medium (pH 4.1). The laccase cDNA gene (*clac2*) was used as a probe for cloning of the genomic laccase gene (*lac2*) including the promoter (*Plac2*). The open reading frame (ORF) of *lac2* had 526 deduced amino acids and four conserved copper binding domains as other fungal laccases. Recombinant plasmid (pRSlac2p-cDNA) of *lac2* cDNA with its own promoter was transformed in *Saccharomyces cerevisiae*. Expression of the transformed *lac2* gene was induced by oxidative stress (H_2O_2) in yeast and the survival rate of the transformed yeast strain was greatly increased when compared with that of the control strain transformed with pRS316 yeast vector.**

Keywords: *Coprinellus congregatus*, acidic laccase gene (*lac2*), gene expression, oxidative stress

Laccase (benzenediol:oxygen oxidoreductases, EC1.10.3.2) catalyzes one-electron transfer from a wide range of substrates. The most important sources of these enzymes are white-rot fungi, which degrade lignin in wood. Fungal laccases are responsible for many diverse biological phenomena, including fungal development in basidiomycetes (Wood, 1985; Choi *et al.*, 1987), pigment formation (Law and Timberlake, 1980), and fungal pathogenicity (Panepinto and Williamson, 2006). They are also involved in the degradation of recalcitrant compounds as well as lignin (Kirk and Farrell, 1987; Karlsson *et al.*, 2001; Han *et al.*, 2004). Many fungal laccase genes have been cloned and the expression of many laccase genes has been increased by metals (Baldrian and Gabriel, 2002; Galhaup *et al.*, 2002) and nitro-aromatic compounds (Cheong *et al.*, 2006). The promoters of laccase genes have many regulatory elements including a metal-responsive element, CreA-binding site, stress-responsive element, and a xenobiotic-responsive element (Galhaup *et al.*, 2002; Xiao *et al.*, 2006).

Coprinellus (= *Coprinus*) *congregatus* Fries is a mushroom forming fungus that is easily cultivated and induced to form mushrooms under laboratory conditions. This fungus produces several laccase isozymes during its development and produces sclerotial laccases in liquid shake cultures older than 8 days. Each of these laccase isozymes are membrane-associated and are never secreted into culture supernatant under normal growth conditions (Choi *et al.*, 1987; Choi and Ross, 1990). However, when a liquid culture (pH 7.0) of this fungus is transferred to an acidic liquid medium (pH 4.1), an acidic laccase is secreted into the culture supernatant (Kim *et al.*, 1997). The pH of the acidic medium is neutralized to higher than 5.0 after 24 h, at which time the fungal growth rate increases (Choi *et al.*, 1994). cDNA of the acidic laccase (*clac2*) gene has been cloned and its expression under acidic conditions has been confirmed (Kim *et al.*, 2001).

In this study, a laccase genomic DNA fragment which contained an ORF identical to the cloned cDNA of *lac2* (Kim *et al.*, 2001) and the promoter region (1.2 kb) has been cloned. Additionally, the laccase cDNA (*clac2*) gene was recombined to its own promoter to generate a *Plac2-lac2* construct, which was then inserted into a yeast vector (pRS316) to monitor external factors for induction of the laccase gene in yeast. Since the single cell yeast shows a more rapid response against environmental changes than filamentous fungi, we tried to examine the expression of the laccase gene in the yeast. Here we report a positive regulation of the *lac2* promoter by oxidative stress in *Saccharomyces cerevisiae*.

* To whom correspondence should be addressed.
(Tel) 82-33-250-8511; (Fax) 82-33-242-0459
(E-mail) htchoi@kangwon.ac.kr

Materials and Methods

Cloning of an acidic laccase genomic DNA fragment

C. congregatus monokaryon (a1) cells grown in a YpSs (Difco, USA) liquid medium for 1 day at 25°C were immediately frozen in liquid N_2 and chromosomal DNA was isolated by following the method described by Kim (Kim *et al.*, 2001). The DNA was partially digested with *Sau*3AI, then used to construct a genomic DNA library with a Lambda DASH II kit (Stratagene, USA). A DIG-labeled probe for the acidic laccase gene was synthesized using the laccase cDNA (*clac2*) as a template (Kim *et al.*, 2001). Five phage clones were selected through three consecutive confirmation screenings. Clone No. 1-5 was selected for further analysis since it generated a 4-10 kb DNA fragment when digested with *Bam*HI, which fell within the desired size range. When they were hybridized with the laccase probe, a 4 kb fragment (No. 7148) showed a strong band. This 4 kb *Bam*HI DNA fragment was subcloned into the pBluescript SK(+) vector and its DNA sequence was determined. However clone No. 7148 had only part of the structural gene with promoter, therefore another DNA fragment (*Bam*HI-*Bgl*II) was selected by Southern hybridization using the *clac2* probe. This DNA fragment was also cloned and sequenced and appeared to have the downstream portion of the structural gene and terminator. The genomic gene of the acidic laccase (*lac2*) and promoter sequence were reported to the EMBL Nucleotide Sequence Database and assigned accession numbers AJ542532 and AY217088, respectively.

Construction of acidic laccase (clac2) expression vector regulated by its own promoter

In order to combine the promoter region with *lac2* cDNA, two specific primers were synthesized as follows: forward primer; 5'-GCTCTAGACTCTAGCAGTTG-3' with a *Xba*I linker at the 5'-end, and reverse primer; 5'-AA GGGGGGAGGAATTCA-3' with *Eco*RI linker at the 3'-end. When these primers were used for amplification of the promoter using No. 7148 as the template, a 1.2 kb PCR product was generated. Laccase cDNA was also amplified using two specific primers as follows: a forward primer comprised of the complementary sequence of the reverse primer for the promoter, and reverse primer; 5'-GCTCTAGATCACAGTATATT-3' with *Xba*I linker at the 3'-end. Each amplified product was digested with two restriction enzymes and ligated with pRS316 yeast transformation vector which was previously digested with *Xba*I and dephosphorylized for safe recombination. This final construct was designated as pRSlac2p-cDNA.

Transformation of S. cerevisiae to uracil prototroph using pRS316 and pRSlac2p-cDNA

S. cerevisiae KM3 (*MATa, his3Δ200, trp1Δ101, ura3-52*) was grown on a YPD (1% yeast extract, 2% peptone, 2% glucose) plate at 30°C. Yeast transformation was carried out using pRS316 and pRSplac2p-cDNA through the electroporation method (0.2 mm gap; 450 V, 150 μF, 99R). Transformants were selected using a dextrose agar plate containing basal salts with amino acid and vitamin mixtures excluding uracil (C-ura plate). PCR was conducted to demonstrate the transformation of pRSplac2p-cDNA using primers for the *lac2* cDNA.

Determination of laccase promoter expression in transformed yeast under the various stress conditions

A transformant KM3 with pRSplac2-cDNA was grown on a C-ura plate then transferred to the same liquid medium. $CuSO_4$ 1 mM, $MnSO_4$ 1 mM, TNT 0.44 mM, or NaN_3 1 mM was added to the C-ura liquid medium and the laccase activities in both the culture supernatants and the cells were determined in each culture.

For adaptation against oxidative stress, a transformant was grown in C-ura liquid medium for 3 days, then transferred to the same fresh medium plus 100 μM H_2O_2 for 4 h. Adapted yeast cells were transferred to new C-ura medium (10% v/v) with H_2O_2 (0, 0.5 M, 1.0 M, and 1.5 M), then their growth was determined for 3 days at 12 h intervals by spectrophotometry at 600 nm. Total RNAs were isolated from the transformed strain (KM3) under adaptation conditions (at H_2O_2 100 μM for 4 h and 24 h), and stress conditions (at H_2O_2 1.5 M for 24 h and 48 h), then 15 μg of total RNA from each culture was transferred to a nylon membrane using a slot blot apparatus. Northern hybridization was performed with a probe generated by a Klenow reaction using laccase cDNA as the template.

Results and Discussion

Sequence analysis of the lac2 genomic DNA

The 2,362 bp *lac2* genomic DNA revealed the Lac2 ORF of 2,158 bp with 10 introns interrupted by 11 exons, encoding a protein of 526 amino acids (Fig. 1). The length of the predicted introns ranged from 50-68 bp. A putative TATA box was found 78 bp upstream of the start codon and 34 bp upstream of the mRNA starting site. The polyadenylation signal was located 87 bp downstream from the stop codon.

The deduced amino acid sequence of Lac2 showed 96%, 96%, and 91% amino acid similarities when compared with the laccases of *Agaricus bisporus* (*lcc2*: Smith *et al.*, 1998), *Pleurotus ostreatus* (*pox1*:

Fig. 1. Genomic DNA sequence and deduced amino acid sequence of *lac2* in *Coprinellus congregatus*. Capital letters represent mRNA transcripts, capital italics represent untranslated regions. Promoter, introns and flanking regions are written in lower case letters, and a poly-A+ tail is attached bp 2,364 after the capital letter followed by a slash. TATA box and polyadenylation signals are underlined. Triangle represents signal peptide processing site, and star represents the stop codon. Four copper binding sites are shaded.

Giardina *et al.*, 1995), and *Trametes versicolor* (*lcc1*: Jönsson *et al.*, 1995), respectively. Lac2 protein had four conserved copper binding domains that are similar to those found in other fungal laccases (Fig. 1).

Construction of expression vector for acidic laccase cDNA regulated by its own promoter

In order to construct an expression vector of the *lac2* gene regulated by its own promoter (pRSlac2p-cDNA) in *S. cerevisiae*, two DNA components were amplified using specific primers then introduced into the multiple cloning site of yeast vector pRS316 (see Materials and Methods for details). The final pRSlac2p-cDNA construct was confirmed by restriction enzyme digestion and agarose gel electrophoresis. *S. cerevisiae* transformed with the expression vector and transformants were selected using the C-ura plate. Yeast transformants carrying the *lac2* expression vector were confirmed by PCR using *lac2*-specific primers on the total DNA of each transformant to see the *lac2*-specific band (data not shown).

Induction of laccase under the various stress conditions in the transformed yeast

When the transformed yeast strain KM3 was grown in the C-ura liquid media containing heavy metals (Cu and Mn), TNT, and NaN_3, no induction of the *lac2* transcript was observed (data not shown). In addition, no laccase activity in the cell or the culture supernatant was observed under acid stress in a liquid medium (pH 4.1). Yeast cells usually show good growth at pH 4.1 while many filamentous fungi, including *C. congregatus*, showed almost no growth. This may explain why there was no promoter induction under acid stress in the transformed KM3. However, when the KM3 cell was challenged to an oxidative stress with H_2O_2, laccase activity in the culture supernatant was detected even though the specific activity of acid laccase was lower than that of in *C. congregatus*. The laccase activity from the induced cultures was much lower than expected during the early growth phase (12 h or earlier), and showed almost no activity thereafter (data not shown). However, the transformant showed a much increased survival rate in the presence of H_2O_2 (Fig. 2A). The growth rate of the transformant strain was much higher than that of the control strain transformed with pRS316 vector (Fig. 2B). The expression of *lac2* by H_2O_2 was confirmed through the Northern hybridization (Fig. 3). The expression level of 24 h culture with 1.5 M H_2O_2 was higher than that of 48 h culture, which was parallel to the growth curve. The growth of the transformant with 1.5 M H_2O_2 was fast during the first 36 h, then showed slower growth (Fig. 2B). Even though the precise mechanism of the protective action of the laccase in yeast against H_2O_2 stress is not yet clear, Lac2 was expressed resulting in a

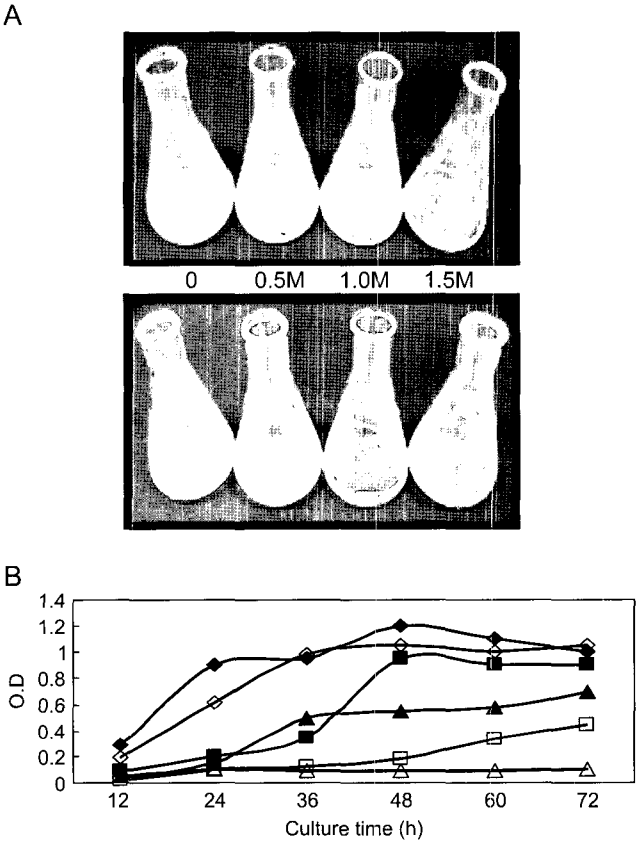

Fig. 2. Survival and growth in the selective liquid medium with H_2O_2 stress. A. Liquid cultures of transformant (KM3, upper panel) and the control strain transformed with pRS316 (lower panel). The concentrations of the H_2O_2 are shown. B. Growth curves of the liquid cultures of Fig. 2A. ◆, 0 M; ■, 0.5 M; ▲, 1.5 M for the transformant. ◇, 0 M; □, 0.5 M; △, 1.5 M for the control strain. All experiments were run in triplicate, and the representative results are shown. (Cultures of 1.0 M H_2O_2 are not shown to simplify the graph)

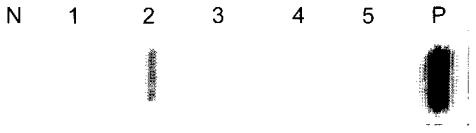

Fig. 3. Confirmation of lac2 expression by Northern hybridization. Total RNA (15 μg) was loaded in each lane. Lane N, negative control with pRS316 vector; 1, transformant KM3 (48 h culture) with 1.5 M H_2O_2; 2, KM3 (24 h culture) with 1.5 M H_2O_2; 3, KM3 (24 h culture) with 100 μM H_2O_2; 4, KM3 (4 h culture) with 100 μM H_2O_2; 5, KM3 (24 h culture) without H_2O_2 stress; P, positive control with pRSlac2p-cDNA.

positive effect on the survival against oxidative stress.

There are many reports of the laccase gene expression under various conditions such as in the presence of heavy metals, especially copper (Galhaup *et al.*, 2002; Xiao *et al.*, 2006) several aromatic compounds such as o-toluidine (Xiao *et al.*, 2006), TNT and its catabolic intermediates (Cheong *et al.*, 2006), and acid stress (Kim *et al.*, 2001). In the case of a fungal pathogen, *Cryptococcus neoformans*, laccases have been shown to synthesize melanin and contribute to the fungal virulence and survival in macrophages. Two laccase genes of this fungal pathogen showed higher expression in response to oxidative and nitrosative stresses, and deletions of the genes with thiol peroxidase resulted in peroxide sensitivity (Missall *et al.*, 2005). In this report, oxidative stress by H_2O_2 also induced the laccase expression in yeast cloned from a mushroom forming fungus while there was no induction by other stresses such as the presence of heavy metal or toxic compounds. As a next step, we will determine the regulation of the *lac2* promoter in *C. congregatus* using green fluorescent protein as the reporter gene.

Acknowledgment

This work was supported by Korea Research Foundation Grant (2004-015-C00492).

References

Baldrian, P. and J. Gabriel. 2002. Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. *FEMS Microbiol. Lett.* 206, 69-74.

Choi, H.T. and I.K. Ross. 1990. Localization of phenoloxidases in *Coprinus congregatus* grown on a low-temperature-liquifying medium. *Kor. J. Microbiol.* 28, 274-277.

Choi, H.T., R.L. Wilks, and I.K. Ross. 1987. Formation of sclerotia in liquid culture of *Coprinus congregatus* and their phenoloxidase isozymes. *Mycol.* 79, 166-172.

Choi, Y., E. Ha, S. Kim, H. Choi, and K. Yoon. 1994. Regulation of membrane-associated laccase synthesis in liquid culture of *Coprinus congregatus*. *Kor. J. Mycol.* 22, 46-49.

Cheong, S., S. Yeo, H. Song, and H.T. Choi. 2006. Determination of laccase gene expression during degradation of 2,4,6-trinitrotoluene and its catabolic intermediates in *Trametes versicolor*. *Microbiol. Res.* 161, 316-320.

Galhaup, C., S. Goller, C. Peterbauer, J. Strauss, and D. Haltrich. 2002. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiol.* 148, 2159-2169.

Giardina, P., R. Cannio, L. Martirani, L. Marzullo, G. Pamieri, and G. Sannia. 1995. Cloning and sequencing of a laccase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 61, 2408-2413.

Han, M., H. Choi, and H. Song. 2004. Degradation of phenanthrene by *Trametes versicolor* and its laccase. *J. Microbiol.* 42, 94-98.

Jönnson, L., K. Sjötröm, I. Häggström, and P.O. Nyman. 1995. Characterization of a laccase gene from the white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. *Biochim. Biophys. Acta* 1251, 210-215.

Karlsson, S., B. Holmbom, P. Spetz, A. Mustranta, and J. Buchert. 2001. Reactivity of *Trametes* laccases with fatty and resin acids. *Appl. Microbiol. Biotechnol.* 55, 317-320.

Kim, S., Y. Leem, and H.T. Choi. 1997. Role of laccase in a low pH liquid medium in *Coprinus congregatus*. *Kor. J. Microbiol.* 33, 27-30.

Kim S., Y. Leem, K. Kim, and H.T. Choi. 2001. Cloning of an acidic laccase gene (*clac2*) from *Coprinus congregatus* and its expression by external pH. *FEMS Microbiol. Lett.* 195, 151-156.

Kirk, T.K. and R. Farrell. 1987. Enzymatic combustion: the microbial degradation of lignin. *Ann. Rev. Microbiol.* 41, 467-505.

Law, D. and W. Timberlake. 1980. Developmental regulation of laccase level in *Aspergillus nidulans*. *J. Bacteriol.* 144, 507-517.

Missall, T.A., J. Moran, J. Corbett, and J.K. Lodge. 2005. Distinct stress responses of two functional laccases in *Cryptococcus neoformans* are revealed in the absence of the thiol-specific antioxidant Tsa1. *Eukaryot. Cell* 4, 202-208.

Panepinto, J.C. and P. Williamson. 2006. Intersection of fungal fitness and virulence in *Cryptococcus neoformans*. *FEMS Yeast Res.* 6, 489-498.

Smith, M., A. Shnyreva, D. Wood, and C. Thurston. 1998. Tandem organization and highly disparate expression of the two laccase genes *lcc1* and *lcc2* in the cultivated mushroom *Agaricus bisporus*. *Microbiol.* 144, 1063-1069.

Wood, D. 1985. Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. *J. Gen. Microbiol.* 176, 656-664.

Xiao, Y.Z., Y. Hong, J. Li, J. Hang, P. Tong, W. Fang, and C. Zhou. 2006. Cloning of novel laccase isozyme genes from *Trametes* sp. AH28-2 and analyses of their differential expression. *Appl. Microbiol. Biotechnol.* 71, 493-501.