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Enhanced Expression of Glucose 2-Oxidase in *Phlebia tremellosa* by Addition of Phthalates

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Most fungi possess several hydrogen peroxide-generating enzymes, glucose oxidase and pyranose oxidase. Pyranose oxidase can use glucose as its substrate to generate hydrogen peroxide. White rot fungi, which degrade diverse recalcitrant compounds, contain lignin-degrading enzymes, and lignin peroxidase and manganese peroxidase require hydrogen peroxide for their enzymatic reactions. In this study, we isolated a cDNA fragment of pyranose oxidase from *Phlebia tremellosa* using PCR and examined its expression under the degradation conditions of diethylphthalate (DEP). Pyranose oxidase expression was enhanced up to 30% by the addition of DEP, and this result supports the possible involvement of pyranose oxidase in the degradation of recalcitrant compounds.

KEYWORDS : Hydrogen peroxide generation, *Phlebia tremellosa*, Pyranose oxidase

White rot fungi, which degrade lignins, possess related enzymes such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP). Since these enzymes show relatively broad substrate specificities, white rot fungi can degrade polycyclic aromatic hydrocarbons, explosives, and endocrine-disrupting chemicals. We have previously reported the degradation of explosives [1] and endocrine-disrupting chemicals [2, 3]. We have also cloned the genes for laccase and MnP from *Phlebia tremellosa* and *Trametes versicolor* as well as constructed expression vectors. Genetic transformants using the expression vectors have been generated, and they show enhanced degrading activities against recalcitrant compounds [4-6].

Even though the original compounds of endocrine-disrupting chemicals in culture supernatants were removed up to 90% during the first 2~3 days, there were 30~40% residual estrogenic activities in the culture supernatants. This unbalanced degradation of endocrine-disrupting chemicals was observed in the wild type as well as in the genetic transformants of laccase or MnP [4-6]. This result implies that both of the original compounds and the degradation intermediates possessed estrogenic activities. Gartshore *et al*. [7] reported that the degradation intermediates of five different plasticizers have toxic effects, and phthalates can be changed to have strong estrogenic activities by UV irradiation [8]. These results also suggest that even though the original compounds were almost completely removed, the degradation intermediates still showed estrogenic activities in our previous experiments.

LiP and MnP require hydrogen peroxide as a co-substrate in their enzymatic reactions. Glucose oxidase (EC 1.1.3.4) catalyzes the oxidation of glucose to hydrogen peroxide and glucono-δ-lactone. Pyranose oxidase (EC 1.1.3.10; POx) is another enzyme that generates hydrogen peroxide; it produces hydrogen peroxide and 2-dehydro-glucose and therefore has another name, glucose 2-oxidase. Since genetic transformants containing extra copies of laccase or MnP cDNA are not fully satisfactory as mentioned earlier, we tried to examine other enzymes that would efficiently remove the estrogenic activity of an endocrine-disrupting chemical. In the first step, we determined whether or not POx is involved in the degradation of endocrine-disrupting chemicals. Since we used a white rot basidiomycete, *P. tremellosa*, for the degradation of endocrine-disrupting chemicals, isolation of a cDNA fragment of pyranose oxidase from this fungus was performed.

POx cDNA (D73369) was found in a white rot fungus, *T. versicolor*, and the DNA sequence was used for construction of PCR primers as follows: forward primer 5'-ATGTCTACTAGCTCGAGCGA-3' and reverse primer 5'-GGGTGGTAACAGCCATACGC-3'. Total RNAs were isolated from the *P. tremellosa* liquid culture, and the cDNAs were synthesized by following a previous report [3]. PCR with the primers resulted in isolation of a cDNA fragment consisting of 1,872 bp, including the open reading frame and terminator. The nucleotide sequence showed 97% identity with that of POx of *T. versicolor*, and the deduced amino acid sequence showed 99% identity with the same enzyme of *T. versicolor* (Fig. 1). This was reported to the EMBL nucleotide sequence database with the accession number FR671428.

The degradation of phthalates by *P. tremellosa* has been reported [3], and genetic transformants containing extra-

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P. tremellosa    MSTSSSDPFFNFTKSSFRSAAAQKASATSLPPLPGPDKKVPGMDIKYDVVIVGSGPIGCT  60
                 MSTSSSDPFFNFTKSSFRSAAAQKASATSLPPLPGPDKKVPGMDIKYDVVIVGSGPIG-T
T. versicolor    MSTSSSDPFFNFTKSSFRSAAAQKASATSLPPLPGPDKKVPGMDIKYDVVIVGSGPIGST  60

P. tremellosa    YARELVEAGYKVAMFDIGEIDSGLKIGAHKKNTVEYQKNIDKFVNVIQGQLMSVSVPVNT  120
                 YARELVEAGYKVAMFDIGEIDSGLKIGAHKKNTVEYQKNIDKFVNVIQGQLMSVSVPVNT
T. versicolor    YARELVEAGYKVAMFDIGEIDSGLKIGAHKKNTVEYQKNIDKFVNVIQGQLMSVSVPVNT  120

P. tremellosa    LVIDTLSPTSWQASSFFVRNGSNPEQDPLRNLSGQAVTRVVGGMSTHWTCATPRFDREQR  180
                 LVIDTLSPTSWQASSFFVRNGSNPEQDPLRNLSGQAVTRVVGGMSTHWTCATPRFDREQR
T. versicolor    LVIDTLSPTSWQASSFFVRNGSNPEQDPLRNLSGQAVTRVVGGMSTHWTCATPRFDREQR  180

P. tremellosa    PLLVKDDQDADDAEWDRLYTKAESYFKTGTDQFKESIRHNLVLNKLAEEYKGQRDFQQIP  240
                 PLLVKDDQDADDAEWDRLYTKAESYFKTGTDQFKESIRHNLVLNKLAEEYKGQRDFQQIP
T. versicolor    PLLVKDDQDADDAEWDRLYTKAESYFKTGTDQFKESIRHNLVLNKLAEEYKGQRDFQQIP  240

P. tremellosa    LAATRRSPTFVEWSSANTVFDLQNRPNTDAPNERFNLFPAVACERVVRNTSNSEIESLHI  300
                 LAATRRSPTFVEWSSANTVFDLQNRPNTDAPNERFNLFPAVACERVVRNTSNSEIESLHI
T. versicolor    LAATRRSPTFVEWSSANTVFDLQNRPNTDAPNERFNLFPAVACERVVRNTSNSEIESLHI  300

P. tremellosa    HDLISGDRFEIKADVFVLTAGAVHNAQLLVNSGFGQLGRPDPANPPQLLPSLGSYITEQS  360
                 HDLISGDRFEIKADVFVLTAGAVHNAQLLVNSGFGQLGRPDPANPPQLLPSLGSYITEQS
T. versicolor    HDLISGDRFEIKADVFVLTAGAVHNAQLLVNSGFGQLGRPDPANPPQLLPSLGSYITEQS  360

P. tremellosa    LVFCQTVMSTELIDSVKSDMIIRGNPGDLGYSVTYTPGAETNKHPDWWNEKVKNHMMQHQ  420
                 LVFCQTVMSTELIDSVKSDMIIRGNPGDLGYSVTY-PGAETNKHPDWWNEKVKNHMMQHQ
T. versicolor    LVFCQTVMSTELIDSVKSDMIIRGNPGDLGYSVTYKPGAETNKHPDWWNEKVKNHMMQHQ  420

P. tremellosa    EDPLPIPFEDPEPQVTTLFQPSHPWHTQIHRDAFSYGAVQQSIDSRLIVDWRFFGRTEPK  480
                 EDPLPIPFEDPEPQVTTLFQPSHPWHTQIHRDAFSYGAVQQSIDSRLIVDWRFFGRTEPK
T. versicolor    EDPLPIPFEDPEPQVTTLFQPSHPWHTQIHRDAFSYGAVQQSIDSRLIVDWRFFGRTEPK  480

P. tremellosa    EENKLWFSDKITDTYNMPQPTFDFRFPAGRTSKEAEDMMTDMCVMSAKIGGFLPGSLPQF  540
                 EENKLWFSDKITDTYNMPQPTFDFRFPAGRTS-EAEDMMTDMCVMSAKIGGFLPGSLPQF
T. versicolor    EENKLWFSDKITDTYNMPQPTFDFRFPAGRTSQEAEDMMTDMCVMSAKIGGFLPGSLPQF  540

P. tremellosa    MEPGLVLHLGGTHRMGFDEQEDKCCVNTDSRVFGFKNLFLGGCGNIPTAYGANPTLTAMS  600
                 MEPGLVLHLGGTHRMGFDEQEDKCCVNTDSRVFGFKNLFLGGCGNIPTAYGANPTLTAMS
T. versicolor    MEPGLVLHLGGTHRMGFDEQEDKCCVNTDSRVFGFKNLFLGGCGNIPTAYGANPTLTAMS  600

P. tremellosa    LAIKSCEYIKNNFTPSPFTDQAE  623
                 LAIKSCEYIKNNFTPSPFTDQA-
T. versicolor    LAIKSCEYIKNNFTPSPFTDQAQ  623
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Fig. 1. Comparison of amino acid sequences of pyranose oxidase between *Phlebia tremellosa* and *Trametes versicolor*. Hyphens indicate where different amino acids from the two fungal species are encoded.

copies of the laccase gene show increased degrading activity against phthalates [6]. In order to determine whether or not isolated POx is involved in the degradation of phthalates, analysis of diethylphthalate (DEP) degradation was performed as follows. *P. tremellosa* was grown in liquid minimal medium (100 mL in 250 mL flask) at 30°C for 5 days with shaking, and the culture was homogenized five times for 1 sec each using a Waring blender [3]. The homogenized cells were transferred to 200 mL of the same medium containing DEP (200 mg/L) (inoculums size; 10% v/v) and incubated for 3 days. Quantitative determination of DEP from culture supernatant and culture medium with EDC was carried out using a Waters HPLC (HP 1,525 series, Gemini 5 μm C6-phenyl 110A 150 × 4.6 mm column; Waters, Milford, MA, USA) as reported previously [5]. The residual concentration of DEP was decreased to 65~70% level in the culture supernatant when compared to that of control (Table 1). The gene expression of POx was determined by reverse transcriptase-PCR using the total RNAs from the 3-day grown cultures and the POx-specific primers (forward primer 5'-GCACCCGGACTGGTGGAACG-3'; reverse primer 5'-CGCAGCCACCGAGGAACAGG-3'), with actin used as the control. The

Table 1. Removal of diethylphthalate by *Phlebia tremellosa* on day 3

Strain	Removal of diethylphthalate (%)
Control	0 ± 1.5
P. tremellosa	33 ± 2.5

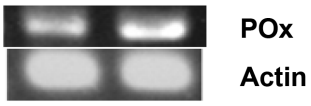

Fig. 2. Determination of pyranose oxidase (POx) expression by reverse transcriptase-PCR using POx-specific primers. Left lane, MM culture; Right lane, MM + DEP culture. MM, minimal medium; DEP, diethylphthalate.

expected length (542 bp) of the PCR product was amplified in both the control and DEP-degrading cultures, and POx gene expression was enhanced about 30% by the addition of DEP (Fig. 2). It has been reported that POx from white rot fungi can supply H_2O_2 during the degradation of lignin and recalcitrant compounds [9, 10], and the enhanced expression of POx gene under the degradation conditions of DEP shows that POx is possibly involved during the degradation of various recalcitrant compounds in *P. tremellosa*.

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