Biosci. Biotechnol. Biochem., **73** (8), 1793–1798, 2009

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Cloning and Homologous Expression of Novel Lignin Peroxidase Genes in the White-Rot Fungus *Phanerochaete sordida* YK-624

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Received February 23, 2009; Accepted April 24, 2009; Online Publication, August 7, 2009
[doi:10.1271/bbb.90152]

Two genes, encoding YK-LiP1 and YK-LiP2, were cloned from the white-rot fungus *Phanerochaete sordida* YK-624, and a homologous expression system for the gene was constructed. Two full-length cDNAs (*ylpA* and *ylpB*) were isolated by degenerate RT-PCR and RACE-PCR. The results of *N*-terminal amino acid sequence analysis of native YK-LiP1 and YK-LiP2 showed that *ylpA* and *ylpB* coded for YK-LiP2 and YK-LiP1 respectively. The promoter of glyceraldehyde-3-phosphate dehydrogenase cloned from *P. sordida* YK-624 (*PsGPD*) was used to drive the expression of *ylpA*. Expression vector p*GPD-g-ylpA* was transformed into a *P. sordida* YK-624 uracil auxotrophic mutant, UV-64. The YlpA protein was secreted in active form by the transformants after 4 d of growth in a medium containing an excessive nitrogen source, whereas endogenous YK-LiP1 and YK-LiP2 were not produced. The physical and catalytic properties of the purified YlpA protein were very similar to those of YK-LiP2. These results suggest that homologous expression of recombinant YK-LiP2 was successful.

Key words: *Phanerochaete sordida* YK-624; novel lignin peroxidase; cloning; homologous expression

Lignin is a heterogeneous, random, phenylpropanoid polymer that constitutes 20–30% of woody plant cell walls. White-rot basidiomycetous fungi are the only known microorganisms that are capable of degrading lignin extensively to CO_2 and H_2O.[1)] Many white-rot fungi produce multiple extracellular enzymes to degrade lignin polymers. These are separated into three major families: laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP).[2)] Moreover, a hybrid-type peroxidase of MnP and LiP, versatile peroxidase (VP), was discovered in several fungi.[3,4)] Among these, it is known that LiP and VP can directly oxidize nonphenolic compounds, including cytochrome *c* and lignin polymer.[4–8)] Several species of white-rot fungi have been studied intensively in recent years because of their ability to degrade and remove lignin from wood or pulp, *i.e.*, biobleaching.[9–16)] Furthermore, they were also studied due to their ability to biodegrade a wide spectrum of persistent environmental pollutants such as polychlorinated dibenzodioxin, DDT, lindene,[17)] chlorophenols,[18)] and polycyclic aromatic carbons.[19,20)] Recently, we found that ligninolytic enzymes such as MnP and laccase were effective in degrading methoxychlor[21)] and Irgarol 1051,[22)] and in eliminating the estrogenic activities of bisphenol A, nonylphenol,[23)] 4-*tert*-octylphenol,[24)] genistein,[25)] and steroidal hormones.[26,27)]

The white-rot fungus *P. sordida* YK-624, isolated from rotted wood, showed much higher ligninolytic activity and selectivity than *P. chrysosporium* or *Trametes versicolor*.[11)] The major extracellular ligninolytic enzymes of this strain are MnP[11,12)] and LiP.[14)] In particular, this strain produces two novel LiPs (YK-LiP1 and YK-LiP2),[28,29)] and these enzymes degrade lignin model compounds more effectively than the LiP from *P. chrysosporium* (Pc-LiP H8), and show high tolerance for hydrogen peroxide. Moreover, VA oxidation by YK-LiP1 and YK-LiP2 obey a bi-reactant sequential mechanism and an ordered bi-bi ping-pong mechanism respectively.[28,29)] These catalytic mechanisms of YK-LiPs are different from that of Pc-LiP (a peroxidase ping-pong mechanism).[8)] These interesting enzymatic differences are predicted to be one of the reasons for the lignin-degrading efficiency of this fungus.

In recent years, gene transformation systems for several white-rot fungi have been developed for overproduction of ligninolytic enzymes[30)] and for performing structure-function studies of these enzymes by site-directed mutagenesis.[31–33)] In our previous report, a gene transformation system for *P. sordida* YK-624 was constructed, and expression of the enhanced green fluorescent protein (EGFP) was successful.[34)] Development of a homologous gene expression system for this fungus is important to elucidate the reason it shows high lignin-degrading activity and selectivity. Moreover, a homologous gene expression system is the optimal method for ligninolytic enzyme production, because

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heterogeneous expression with other hosts has problems. In *Escherichia coli*, expression of recombinant LiP is needed to perform *in vitro* refolding procedure with hemin to produce the active form.[35,36] Expression of recombinant LiP by yeasts has been reported, but the molecular mass of the recombinant LiP was not consistent with native LiP[37] or expressed as cell-surface proteins,[38,39] whereas the ligninolytic enzymes produced by the homologous expression system gained active form without an *in vitro* refolding procedure and maintained the enzymic profiles.[31,32,40,41]

In the present study, cloning and homologous expression of YK-LiPs genes in *P. sordida* YK-624 were carried out and the recombinant protein was characterized.

Materials and Methods

Strains. P. sordida YK-624 (ATCC 90872) and uracil auxotrophic strain UV-64[34] were used. These strains and transformants were maintained on potato dextrose ager (PDA) slants at 4 °C.

Analysis of the N-terminal amino acid sequences of YK-LiP1 and YK-LiP2. YK-LiP1 and YK-LiP2 were obtained from extraceller culture fluid of Mn-deficient liquid media and purified by anion-exchange and size exclusion chromatography.[28,29] The N-terminal amino acid sequences of purified proteins were analyzed on a PPSQ-21A Protein Peptide Sequencer (Shimadzu, Kyoto, Japan).

Procedure for cloning of full-length cDNAs and genomic DNAs fragment encoding YK-LiP1 and YK-LiP2. The purification of genomic DNA and RNA and synthesis of cDNA were performed following the protocol established by Yamagishi *et al.*[34] cDNAs and genomic DNAs encoding YK-LiP1 and YK-LiP2 were cloned by a series of PCR procedures. Table 1 lists the primers used. Degenerate primers dFA, dFK, and dR were designed based on the results of N-terminal amino acid sequence analysis of YK-LiP1. cDNA of *P. sordida* YK-624 was used as the template HotStarTaq DNA polymerase was used (Qiagen, Valencia, CA). Each 60-bp product was amplified with primer sets dFA-dR and dFK-dR. The 3′ coding regions of these genes were cloned by 3′-rapid amplification of cDNA ends (3′-RACE) using reverse transcription primer RT, and four nested primers corresponding to cDNA fragments, FA1-R1, FA2-R1, FK1-R1, and FK2-R1. Moreover, the 5′ coding regions of these genes were cloned by 5′-rapid amplification of cDNA ends (5′-RACE) using four nested primers corresponding to cDNA fragments, Fadp-RA1, Fadp-RA2, Fadp-RK1, and Fadp-RK2. cDNA using 5′-RACE was synthesized by GeneRacer Kit (Invitrogen, La Jolla, CA). Primer sets FA3-RA3 and FK3-RK3, designed on results of 3′-RACE and 5′-RACE, was used to clone two full-length cDNAs and two genomic DNAs encoding YK-LiP1 and YK-LiP2. All of these PCR products were ligated in cloning vector pT7Blue T-Vector (Novagen, Madison, WI) using DNA Ligation kit ver2.1 (TaKaRa Bio, Shiga, Japan) and were introduced into *Escherichia coli* DH5α for sequencing.

Table 1. Oligonucleotides Used as Primers in This Study

Primer name	Nucleotide sequence (5′-sequence-3′)
dFA	GCNRCNTGYWSNAAYGGNGC
dFK	GCNRCNTGYWSNAAYGGNAA
dR	ACNKYRAACCANGCRCARCA
FA1	GCGACCGTCAGCGACGCGTCC
FA2	ACCGTCAGCGACGCGTCCTGCTG
RT	CTGATCTAGACCTGCAGGCTCGAG-TTTTTTTTTTTTTTTTT
R1	CTGATCTAGACCTGCAGGCTC
RA1	CGCCTCAGCACCGCACTGGGCACCCTG
RA2	CTGGAAGAGGTTCTGCTGGATGTCGTC
Fadp	CGACTGGAGCACGAGGAGCACACTGA
FA3	GAAAAGACACTCTCAGTCTCTACCAGC
RA3	AACTGTACAGATCGTATGCATCATTCA
FK1	AAGACGGTCAGCGCGTCGTCG
FK2	ACGGTCAGCGCGTCGTCGTGCTG
RK1	GGCCTCAGCGCCGCACTGGCCGCCGTT
RK2	GTTGAAGAGGTTCTCCTGGATGTCCGA
FK3	GAAAAGGTCACTCTCAGCCTCTCAGAC
RK3	GGGCCAGTAATACACGTGTATTTGCTT
GPD-F1	GTTTGTTTGAGCCTCCGGTGCGAA
GPD-*g-ylpA*-R1	CTGCTTGAAGGCCATGTTGAGTAGAGG
g-ylpA-F1	CCTCTACTCAACATGGCCTTCAAGCAG
g-ylpA-R1	GGGCGCGCCTTAGGCACCCGGGGGCGG
GPD-F2	CGGGGCGCGATTGATTTGAGGCGA
g-ylpA-R2	GCCTCAGCACCGCACTGGGCACCCTG

H(A/T/C), N(A/T/G/C), R(A/G), S(C/G), Y(C/T), W(A/T)

Construction of ylpA *expression plasmid.* Figure 1 shows the construction procedure for the *ylpA* expression plasmid. p*PsGPD-EGFP*, used in our previous study,[34] was used in the construction of the expression plasmid. Primers GPD-F1 and GPD-*g-ylpA*-R1 were designed to locate to the upstream region of the *Nde*I site in the *PsGPD* promoter and to join the downstream region of the first methionine codon of *ylpA* just outside the 3′-terminal sequence of the *PsGPD* promoter. These primers were used to amplify the *PsGPD* promoter fragment using PrimeStar HS DNA polymerase (step 1). Primers *g-ylpA*-F1 and *g-ylpA*-R1 were designed to join the 3′-terminal sequence of the *PsGPD* promoter just before the first methionine codon of *ylpA*, and to add an *Asc*I site just after the stop codon of *ylpA*. These primers were used to clone the genomic DNA of *ylpA* (step 2). A mixture of step 1 and step 2 PCR products was used as template in 2nd PCR, and the primers used were GPD-F1 and *g-ylpA*-R1 (step 3). The amplified DNA fragment and p*PsGPD-EGFP* were digested with *Nde*I and *Asc*I, and the DNA fragment was inserted between the *Nde*I and *Asc*I sites of p*PsGPD-EGFP*, yielding plasmid p*GPD-g-ylpA* (step 4). p*GPD-g-ylpA* was purified using a Quantum Prep Plasmid Midi Kit, and was sequenced to verify PCR errors.

Co-transformation of UV-64 with pPsURA5 *and* pGPD-g-ylpA *gene expression plasmids.* Preparation of UV-64 protoprasts and transformation were performed following the protocol established by Yamagishi *et al.*[34] Protoplasts of UV-64 (2×10^7 cells, 200 µl) were mixed with 120 µl of DNA solution (20 µg the p*GPD-g-ylpA*, 5 µg the p*PsURA5*,[34] 1.0 M SorbOsm, 40 mM $CaCl_2$) and incubated on ice for 10 min. The protoplast/DNA mixture was underlaid with an equivalent 50% w/v polyethylene glycol solution (PEG; PEG4000, in 1.0 mM SorbOsm, 10 mM MES, pH 6.2), incubated on ice for 10 min, mixed gently, and incubated for an additional 10 min. Two hundred milliliters of the regeneration medium (CYM medium with 1% SeaPlaque Agarose, Takara Bio) without uracil was then added, and this was poured into 15 petri dishes (diameter, 10 cm), and incubated at 30 °C. After 7 d, all regenerated clones were picked out and inoculated onto CYM semisolid without uracil. The clones growing on uracil-free medium were counted 5 d later, and were counted as uracil prototrophic transformants.

Screening of the ylpA*-overexpression recombinant.* Twenty-four regeneration transformants were subcultured on cellophane membranes overlaying uracil-free CYM semisolid for 3 d. Pieces of the mycelium were subsequently placed in a 96-well plate with Triton-X (0.05%) in TE and freeze-thawed 3 times. The supernatants were used as templates for PCR amplification to screen for *ylpA*-positive clones. Primers GPD-F2 and *g-ylpA*-R2 were designed to amplify the transformed genomic DNA of *ylpA*, which was joined to the *PsGPD* promoter. Eleven transformants screened by genomic PCR was grown on PDA plates (diameter, 9 cm) for 3 d at 30 °C. Two mycelial disks (diameter, 1 cm) punched from the growing edge of the mycelium were added to a 100-ml Erlenmeyer flask with 10 ml of Kirk medium (1.2 mM ammonium tartrate, pH 4.5). The culture was statically incubated at 30 °C for 7 d. After cultivation, LiP activity in the culture was determined by monitoring the oxidation of veratryl alcohol (VA) at 310 nm. The reaction mixture (1 ml) contained 1 mM VA, 20 mM succinate buffer (pH 3.0), and 0.2 mM hydrogen peroxide. One kat of LiP activity was defined as the amount of enzyme producing 1 mol of veratralaldehyde ($9.3\,mM^{-1}\,cm^{-1}$) from VA per s.

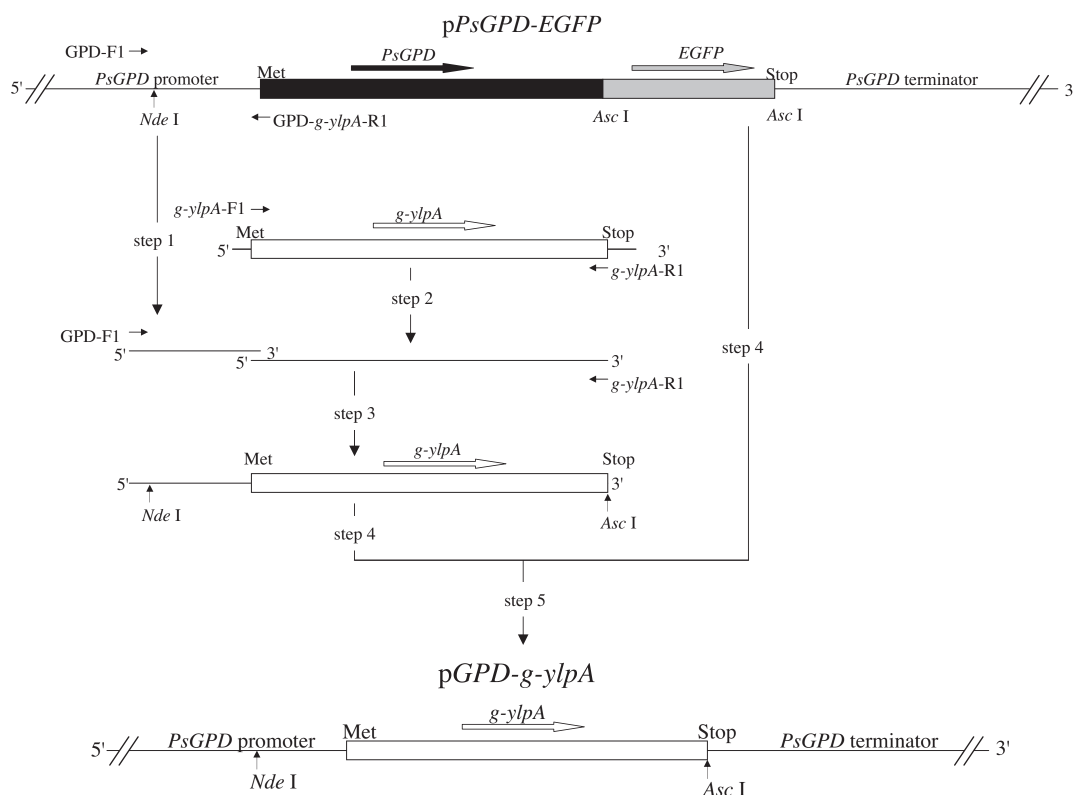

Fig. 1. Construction of *g-ylpA* Expression Plasmid p*GPD-g-ylpA*.

The procedure used to construct p*GPD-g-ylpA* is described under "Materials and Methods." The horizontal arrows indicate the locations and directions of primers mentioned in "Materials and Methods." Boxes indicate genes.

Cultivation in recombinant LiP suppression medium. Two mycelial disks of the A-11 strain selected by screening, the control strain transformed only by p*PsURA5*, and wild-type, were homogenized with 1 ml the sterile water. One ml of homogenate was added to petri dishes (diameter, 10 cm) with 20 ml the LiP suppression medium (modified manganese deficient-Kirk medium containing 40 mM ammonium tartrate, pH 6.5) and statically incubated at 30 °C. After incubation, the extracellular culture fluid was desalted, and LiP activity was measured as described above.

Enzyme preparation. The A-11 strain was grown under the conditions described above, for 6 d. Extracellular culture fluid was separated from the mycelia by filtration with glass fiber filter paper GA-100 and GB-140 (Advantec, Tokyo, Japan), and membrane filters (pore sizes 0.65, 0.45, and 0.2 µm, Advantec), and concentrated by ultrafiltration (10 kDa, Advantec). The concentrate was adjusted to pH 6.0 and loaded onto a Hitrap Q HP column (GE Healthcare, Little Chalfont, UK) equilibrated with 50 mM sodium phosphate buffer (pH 6.0). Proteins were eluted with a liner gradient from 0 to 0.25 M NaCl in the same buffer. The active fractions were collected, desalted by passage through a PD-10 column (GE Healthcare) equilibrated with distilled water, and concentrated by ultrafiltration. The concentrate was applied to a HiLoad 16/60 Superdex 75 pg column (GE healthcare), which was equilibrated with 20 mM succinate buffer (pH 4.5) containing 0.1 M NaCl, at a flow rate of 0.5 ml min^{-1}. The active fractions from the Superdex 75 column were collected, desalted, and concentrated by ultrafiltration. The concentrate was loaded onto a Mono Q HR 5/5 column (GE Healthcare) equilibrated with 20 mM succinate buffer (pH 4.5). Proteins were eluted with a linear gradient from 0 to 0.25 M NaCl in the same buffer. The active fractions were collected, concentrated by ultrafiltration, and desalted. The final product, a LiP-like peroxidase, was designed YlpA.

Enzyme characterization. The activities of YK-LiP2 and YlpA were determined by monitoring the oxidation of VA at 310 nm, as described above. The molecular mass was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5% polyacrylamide gel.[42] $1/v$ *versus* $1/[\mathrm{VA}]$ was plotted for calculated K_m and V_{max}. The reaction mixtures (1 ml) contained peroxidase (0.07 nkat), VA (20–200 µmol) and hydrogen peroxide (20 µmol) in 20 mM succinate buffer (pH 3.0). The reactions were initiated by the addition of hydrogen peroxide at 30 °C.

Nucleotide sequence accession numbers. The nucleotide sequences of genes *ylpA* and *ylpB* of *P. sordida* YK-624 have been deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/) under accession nos. AB455006 and AB455007 respectively.

Results

PCR-based isolation of cDNAs and genomic DNAs encoding YK-LiP1 and YK-LiP2

Based on the results of N-terminal sequence analysis of purified YK-LiP1, two forward degenerate primers and one reverse degenerate primer (dFA and dFK, and dR) were designed to perform degenerate PCR using *P. sordida* YK-624 cDNA as the template. Two differential PCR products were obtained by degenerate PCR, and the DNA fragments from the dFA-dR pair and the dFK-dR pair were named the *ylpA* fragment and the *ylpB* fragment respectively. The full-length cDNA fragments encoding *ylpA* (1,310 bp) and *ylpB* (1,303 bp) were cloned by a combination of the 3′-RACE and 5′-RACE procedures, and the genomic DNA fragments encoding the full-lengths of *ylpA* and *ylpB* (1,707 bp and 1,727 bp respectively) were cloned based on the results of full-length cDNA sequences (gene accession nos.: *ylpA*, AB455006; *ylpB*, AB455007). The N-terminal amino acid sequences of YK-LiP1 and YK-LiP2 were analyzed. *ylpB* and *ylpA* coded YK-LiP1 and YK-LiP2 respectively (Table 2) by the results of comparison with the predicted amino acid sequences of these cDNAs.

These cDNAs were compared with CDS coding LiP produced from several white-rot fungi (Table 3). *ylpA* showed highest identity with *LiP H8* from *P. chrysosporium* (cDNA sequence, 85.8%; predicted amino acid sequence, 92.2%). On the other hand, *ylpB* exhibited highest identity with *LiP6 (H10)* from *P. chrysosporium* (cDNA sequence, 85.2%; the predicted amino acid sequence, 89.2%). These two cDNA sequences and these predicted amino acid sequences showed 85.3% and 86.5% identity respectively. The genomic DNA of *ylpA* and *ylpB* was lacking Intron IV as compared with the genomic DNA of *LiP H8* and *LiP6* from *P. chrysosporium* respectively (data not shown).

Construction, co-transformation, and screening of the ylpA*-overexpression transformant*

An expression plasmid was constructed from p*PsGPD-EGFP* following our previous study[34] in order to use the *PsGPD* expression cassette. Genomic DNA of *ylpA* was inserted between the *PsGPD* promoter and *PsGPD* terminator by restriction enzyme digestion. This expression plasmid was named p*GPD-g-ylpA*. This plasmid was introduced into UV-64 using p*PsURA5* as the marker plasmid. The presence of the *PsGPD* promoter-*ylpA* fusion gene in each uracil prototrophic clone was confirmed by PCR using genomic DNA as the template (Fig. 2A). Eleven of 24 regenerated clones were co-transformed with p*GPD-g-ylpA*.

Each co-transformant recombinant *ylpA* expression cassette obtained was grown in Kirk medium (1.2 mM ammonium tartrate, pH 4.5) and the LiP activity in extracellular culture fluid was measured at 7 d. The results indicated that several transformants (A11, A17, and A24) showed higher LiP activity than the wild type (Fig. 2B). In particular, approximately 5 times as much LiP activity was detected in A11 and A24.

Production of recombinant LiP

For the purpose of suppressing endogenous LiP production, an excessive nitrogen source (ammonium

Table 2. Comparison of N-Terminal Amino Acid Sequences of Native YK-LiPs with the Predicted Proteins from cDNAs

	Amino acid number of N-terminal sequence											
	1	2	3	4	5	6	7	8	9	10	11	12
YK-LiP1	A	T	C	S	N	G	K	T	V	S	A	—
YK-LiP2	A	A	C	S	N	G	A	T	V	S	D	A
YlpA	A	A	C	S	N	G	A	T	V	S	D	A
YlpB	A	T	C	S	N	G	K	T	V	S	A	S

YK-LiP1 and YK-LiP2 were analyzed as described in the text. The mature N-terminal amino acid sequences of *ylpA* and *ylpB* were predicted based on the signal sequence regions and the mature N-terminal amino acid sequences of Pc-LiPs.

Table 3. Percentages of cDNA and Amino Acid Sequence Identities among YlpA, YlpB, and Several Other LiPs

Strain	Isozyme name	Gene (cDNA) Identities (%)		Protein Identities (%)	
		YlpA	YlpB	YlpA	YlpB
Phanerochaete sordida YK-624	YlpA	—	85.3	—	86.5
	YlpB	85.3	—	86.5	—
Phaerochaete chrysosporium	LiP H2	75.3	74.6	72.2	69.6
	LiP H8	85.8	82.1	92.2	84.4
	LiP 6(H10)	82.6	85.2	83.6	89.2
	LiP GLG6	83.0	80.7	89.2	83.3
Trametes versicolor	LiP 7	69.6	69.2	59.0	56.0
	LiP 12	72.5	71.6	62.6	60.7
	LiP GII	70.5	70.8	60.3	58.6
Phlebia radiata	LiP	68.3	66.1	60.1	58.1
Bjerkadera adusta	LiP	71.8	70.4	60.5	57.8

GenBank accession numbers. *Phanerochaete chrysosporium*: LiP H2:X15599, LiP H8:M27401, LiP 6:M63496, LiP GLG6:M77508. *Trametes versicolor*: LiP 7:Z30667, LiP 12:M64993, LiP GII:X75655. *Phlebia radiata*: LiP:126290. *Bjerkadera adusta*: LiP:444058

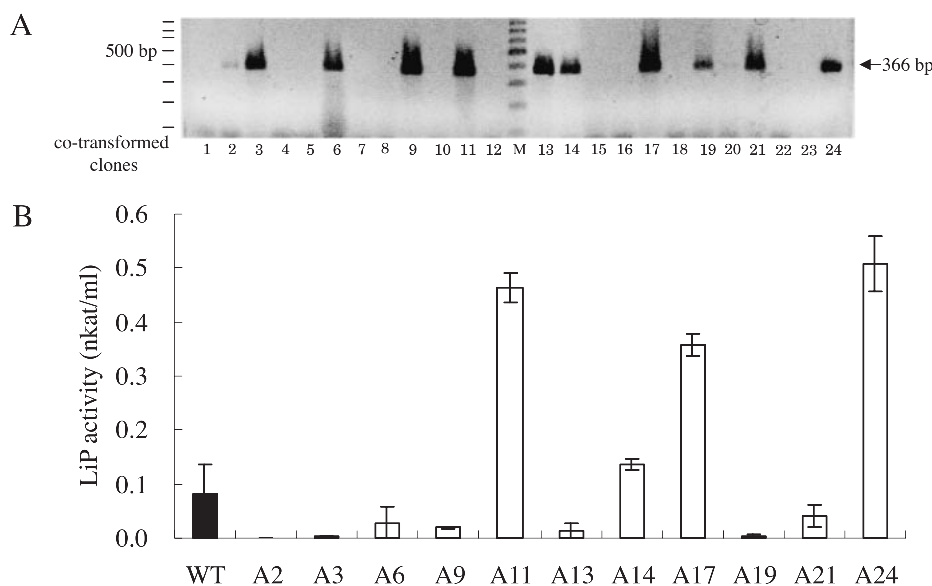

Fig. 2. Co-Transformation with p*GPD-g-ylpA*.

A, Detection of the *g-ylpA* gene linked with the *PsGPD* promoter from 24 regenerated clones co-transformed with p*PsURA5* and p*GPD-g-ylpA* by PCR amplification using primers GPD-F2 and *g-ylpA*-R2. *M* indicates a 100-bp ladder size marker. B, LiP activity in the extracellular culture fluid of the Kirk medium at 7 d. Black bar and White bar indicate the wild type and g-*ylpA* transformants respectively.

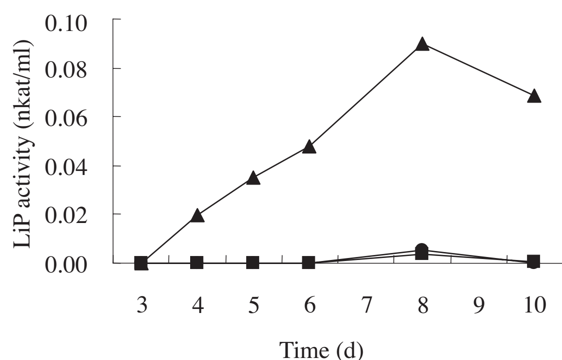

Fig. 3. LiP Activities in the Extracellular Culture Fluids of *P. sordida* YK-624 Transformants.

A11 (triangle) was the YlpA-expressing transformant, control (circle) was a p*PsURA5* transformant, and YK-624 (square) was the wild-type strain. LiP activities were calculated from VA oxidation rates.

tartrate) was added to the manganese-deficient Kirk medium (pH 6.5). Endogenous LiP production of the wild type was almost entirely repressed under culture conditions containing 40 mM ammonium tartrate (data not shown). Hence manganese deficient-Kirk medium containing 40 mM ammonium tartrate (pH 6.5) was used in the production of YlpA protein. *ylpA* transformant A11, the control transformant, and the wild type were cultured in this medium at 30 °C, and the LiP activity in each extracellular culture fluid was determined (Fig. 3). LiP activity in the culture inoculated with A11 was detected at 4 d of cultivation, and reached a maximum level at 8 d. In contrast, the wild type and the control transformant exhibited LiP activity at only 8 d of cultivation.

Characterization of recombinant YlpA

YlpA was purified from large-scale cultivation of transformant A11. The extracellular culture fluid was concentrated by ultrafiltration, and anion-exchange and size exclusion chromatographies were performed. The purified YlpA and the native YK-LiP2 were subjected to SDS–PAGE (Fig. 4). An intense band was observed in each lane, and the molecular mass of YlpA was very similar to that of native YK-LiP2. The absorption spectrum of purified YlpA (Fig. 5) exhibited a soret maximum at 407 nm and visible bands at 500 and 635 nm. The shapes and intensities of the absorption bands of YlpA were similar to those of YK-LiP2.

The apparent K_m and V_{max} of YlpA for VA at 20 µmol of hydrogen peroxide were determined to be 27.1 µM and 2.44 µM/min respectively. These values for native YK-LiP2 were determined to be 33.7 µM and 2.25 µM/min respectively.

Discussion

P. sordida YK-624 exhibits greater ligninolytic selectivity among beech woods than either *P. chrysosporium* or *Trametes versicolor*.[11] It produced two novel LiPs (YK-LiP1 and YK-LiP2) under manganase-deficient conditions,[28,29] and these enzymes effectively degraded lignin model compounds and showed high tolerance for hydrogen peroxide.[28,29] The catalytic mechanisms of YK-LiP1 and YK-LiP2 were different from that of

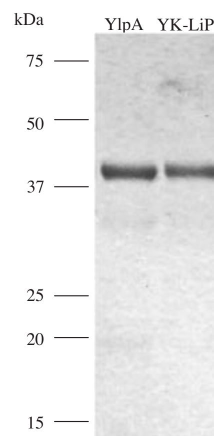

Fig. 4. SDS–PAGE of YlpA and YK-LiP2.

The proteins were loaded onto 12.5% Tris-glycine-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. The positions of the precision plus protein standard (Bio-Rad, Hercules, CA) are indicated.

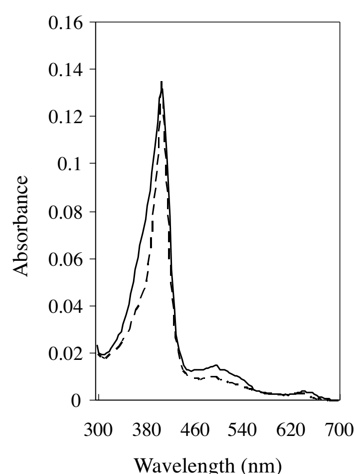

Fig. 5. Comparison of the Absorption Spectra of YlpA (straight line) and YL-LiP2 (dotted line).

Pc-LiP H8.[8,28,29] Cloning of the genes coding YK-LiP1 and YK-LiP2 and construction of a homologous expression system is necessary to investigate the structure-function of these enzymes by site-directed mutagenesis. In the present study, cloning and homologous expression of the YK-LiP1 and YK-LiP2 genes in *P. sordida* YK-624 were carried out and the recombinant protein was characterized. We obtained two full-length cDNAs and two genomic DNAs encoding YK-LiP1 and YK-LiP2. A comparison of the N-terminal amino acid sequences of YK-LiP1 and YK-LiP2 with the predicted amino acid sequences of the cloned genes suggested that *ylpA* and *ylpB* coded YK-LiP2 and YK-LiP1 respectively (Table 2). In *P. chrysosporium*, two major LiP isozimes (H2, H8) exist.[43] *ylpA* and *ylpB* had high identities with *Pc-LiP H8* and *Pc-LiP6* respectively. On the other hand, the enzymatic properties of native YK-LiP1 and YK-LiP2 were different from that of Pc-LiP H8. In Pc-LiP H8, several nearly heme amino acids involved in catalytic activity and substrate interaction have been found in previous research,[35,44] and all of them were conserved in the *ylpA* and *ylpB* coding proteins, whereas His 239, the electron transfer amino acid enzyme surface,[5] replaced Phe 239 in the *ylpB* coding protein

and Pc-LiP6. These results suggest that the replacement affects the degradation of lignin model compounds by YK-LiP1.

Here we describe the homologous expression of YlpA under the control of the *PsGPD* promoter.[34] This system allows production of recombinant YK-LiP under primary metabolic conditions when the endogenous YK-LiP genes are not expressed, facilitating purification of the recombinant protein. As a result of co-transformation introducing the expression vector and the selectable marker gene vector, 24 transformants were regenerated, and 11 of these carried the *ylpA* construct (Fig. 2A). Transformant A11 showed approximately 5 times as much LiP activity as the wild type. This transformant was chosen as the recombinant *ylpA*-overexpression strain. As shown in Fig. 4, only the introduced *ylpA* gene was expressed in manganese-deficient Kirk medium containing an excessive nitrogen source (40 mM ammonium tartrate).

Purification of YlpA was achieved by anion-exchange and size-exclusion chromatographies after ultrafiltation. SDS–PAGE analysis indicated that YlpA and YK-LiP2 are nearly identical in molecular mass (Fig. 4). This result suggests that the two proteins undergo very similar post-translational processing, including clevage of signal and propeptide sequences, folding, and glycosylation. YK-LiP2 and YlpA also have identical UV-visible spectral features (Fig. 5), indicating that the insertion, environment, and orientation of the heme are similar. Furthermore, the K_m and V_{max} values for YlpA for VA are similar to the values obtained for YK-LiP2. These results suggest that the substrate binding and catalytic efficiency of YlpA and YK-LiP2 are the same. This suggests that homologous expression of YK-LiP by *P. sordida* YK-624 is possible. Homologous expression of several lignin degrading peroxidases has been reported in some fungi, especially *P. chrysosporium*[31,32] and *Pleurotus ostreatus*.[33,41] This expression system can be used to investigate the structure and function these enzymes, especially high oxidation activities against lignin model compounds, by site-directed mutagenesis. Moreover, molecular breeding of the superior lignin degrading fungi applied in the pretreatment of wood in the production of bio-ethanol is possible using this homologous expression system, since *P. sordida* YK-624 degrades lignin more effectively and selectively than *P. chrysosporium* or *T. versicolor*.[11] Our next target is molecular breeding of the superior lignin degrading fungi from *P. sordida* YK-624 with this homologous expression system.

References

1) Kirk TK and Farrell RL, *Annu. Rev. Microbiol.*, **41**, 465–505 (1987).
2) Gold MH and Alic M, *Microbiol. Rev.*, **57**, 605–622 (1993).
3) Ruiz-Dueñas FJ , Camarero S, Pérez-Boada M, Martíne MJ, and Martínez AT, *Biochem. Soc. Trans.*, **29**, 116–122 (2001).
4) Kamitsuji H, Watanabe T, Honda Y, and Kuwahara M, *Biochem. J.*, **386**, 387–393 (2005).
5) Johjima T, Itoh N, Kabuto M, Tokimura F, Nakagawa T, Wariishi H, and Tanaka H, *Proc. Natl. Acad. Sci. USA*, **96**, 1989–1994 (1999).
6) Schoemaker HE, Lundell TK, Hatakka AI, and Piontek K, *FEMS Microbiol. Rev.*, **13**, 321–331 (1994).
7) Valli K, Wariishi H, and Gold MH, *J. Bacteriol.*, **174**, 2131–2137 (1992).
8) Wariishi H, Sheng D, and Gold MH, *Biochemistry*, **33**, 5545–5552 (1994).
9) Fujita K, Kondo R, Sakai K, Kashino Y, Nishida T, and Takahara Y, *Tappi J.*, **74**, 123–127 (1991).
10) Fujita K, Kondo R, Sakai K, Kashino Y, Nishida T, and Takahara Y, *Tappi J.*, **76**, 81–84 (1993).
11) Hirai H, Kondo R, and Sakai K, *Mokuzai Gakkaishi*, **40**, 980–986 (1994).
12) Hirai H, Kondo R, and Sakai K, *Mokuzai Gakkaishi*, **41**, 69–75 (1995).
13) Katagiri N, Tsutsumi Y, and Nishida T, *Appl. Environ. Microbiol.*, **61**, 617–622 (1995).
14) Machii Y, Hirai H, and Nishida T, *FEMS Microbiol. Lett.*, **233**, 283–287 (2004).
15) Paice MG, Jurasek L, Ho C, Bourbonnais R, and Archibald FS, *Tappi J.*, **72**, 217–221 (1989).
16) Reid ID, Paice MG, Ho C, and Jurasek L, *Tappi J.*, **73**, 149–153 (1990).
17) Bumpus JA, Tien M, Wright D, and Aust SD, *Science*, **228**, 1434–1436 (1985).
18) Joshi DK and Gold MH, *Appl. Environ. Microbiol.*, **59**, 1779–1785 (1993).
19) Bezalel L, Hadar Y, and Cerniglia CE, *Appl. Environ. Microbiol.*, **62**, 292–295 (1996).
20) Collins PJ, Kotterman M, Field JA, and Dobson A, *Appl. Environ. Microbiol.*, **62**, 4563–4567 (1996).
21) Hirai H, Nakanishi S, and Nishida T, *Chemosphere*, **55**, 641–645 (2004).
22) Ogawa N, Okamura H, Hirai H, and Nishida T, *Chemosphere*, **55**, 487–491 (2004).
23) Tsutsumi Y, Haneda T, and Nishida T, *Chemosphere*, **42**, 271–276 (2001).
24) Tamagawa Y, Hirai H, Kawai S, and Nishida T, *Environ. Toxicol.*, **22**, 281–286 (2007).
25) Tamagawa Y, Hirai H, Kawai S, and Nishida T, *FEMS Microbiol. Lett.*, **244**, 93–98 (2005).
26) Suzuki K, Hirai H, Murata H, and Nishida T, *Water Res.*, **37**, 1972–1975 (2003).
27) Tamagawa Y, Yamaki R, Hirai H, Kawai S, and Nishida T, *Chemosphere*, **65**, 97–101 (2006).
28) Sugiura M, Hirai H, and Nishida T, *FEMS Microbiol. Lett.*, **224**, 285–290 (2003).
29) Hirai H, Sugiura M, Kawai S, and Nishida T, *FEMS Microbiol. Lett.*, **246**, 19–24 (2005).
30) Tsukamoto A, Kojima Y, Kita Y, and Sugiura J, *Biosci. Biotechnol. Biochem.*, **67**, 2075–2082 (2003).
31) Mayfield MB, Kishi K, Alic M, and Gold MH, *Appl. Environ. Microbiol.*, **60**, 4303–4309 (1994).
32) Sollewijn GMD, Mayfield-Gambill M, Lin CGP, and Gold MH, *Appl. Environ. Microbiol.*, **65**, 1670–1674 (1999).
33) Tsukihara T, Honda Y, Sakai R, Watanabe T, and Watanabe T, *Appl. Environ. Microbiol.*, **74**, 2873–2881 (2008).
34) Yamagishi K, Kimura T, Oita S, Sugiura T, and Hirai H, *Appl. Microbiol. Biotechnol.*, **76**, 1079–1091 (2007).
35) Doyle WA, Blodig W, Veitch NC, Piontek K, and Smith AT, *Biochemistry*, **37**, 15097–15105 (1998).
36) Nie G, Reading NS, and Aust SD, *Biochem. Biophys. Res. Commun.*, **249**, 146–150 (1998).
37) Wang H, Lu F, Sun Y, and Du L, *Biotechnol. Lett.*, **26**, 1569–1573 (2004).
38) Ryu K and Lee EK, *J. Chem. Eng. Jpn.*, **35**, 527–532 (2002).
39) Ryu K, Kang JH, Wang L, and Lee EK, *J. Biotechnol.*, **135**, 241–246 (2008).
40) Yamazaki T, Okajima Y, Kawashima H, Tsukamoto A, Sugiura J, and Shishido K, *Biosci. Biotechnol. Biochem.*, **70**, 1293–1299 (2006).
41) Tsukihara T, Honda Y, Watanabe T, and Watanabe T, *Appl. Microbiol. Biotechnol.*, **71**, 114–120 (2006).
42) Laemmli UK, *Nature*, **227**, 680–685 (1970).
43) Farrell RL, Murtagh KE, Tien M, Mozuch MD, and Kirk TK, *Enzyme Microb. Technol.*, **11**, 322–328 (1989).
44) Edwards SL, Raag R, Wariishi H, Gold MH, and Poulos TL, *Proc. Natl. Acad. Sci. USA*, **90**, 750–754 (1993).