Heterologous Expression of *Phanerochaete chrysoporium* Glyoxal Oxidase and its Application for the Coupled Reaction with Manganese Peroxidase to Decolorize Malachite Green

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cDNA of the *glx1* gene encoding glyoxal oxidase (GLX) from *Phanerochaete chrysoporium* was isolated and expressed in *Pichia pastoris*. The recombinant GLX (rGLX) produces H₂O₂ over 7.0 nmol/min/mL using methyl glyoxal as a substrate. Use of rGLX as a generator of H₂O₂ improved the coupled reaction with recombinant manganese peroxidase resulting in decolorization of malachite green up to 150 µM within 90 min.

**KEYWORDS**: Dye decolorization, Glyoxal oxidase, Manganese peroxidase, *Phanerochaete chrysoporium*, *Pichia pastoris*

Due to their ability to degrade lignin and lignin model compounds, the importance of white rot fungi in lignin degradation has become increasingly apparent [1, 2]. Among them, *Phanerochaete chrysoporium*, which is used as a model organism for understanding the lignin-degrading mechanism and for degradation of various organopollutants, has been studied extensively during the last two decades [1-5]. Under ligninolytic conditions, *P. chrysosporium* produces extracellular lignin peroxidase (LiP) isozymes and manganese peroxidase (MnP) isozymes, which are required for extracellular H₂O₂ to degrade lignin or xenobiotic compounds [6, 7]. Production of extracellular H₂O₂ involves either intracellular enzymes, such as glucose oxidase 1 [8], glucose oxidase 2 [9], fatty acetyl-coenzyme [10], or extracellular gloxal oxidase, which reduces O₂ to H₂O₂ using aldehyde and α-hydroxyl carbonyl compounds, such as glyoxal and methyl glyoxal, as an electron source [11].

Compared with intact cells of *P. chrysosporium*, degradation of lignin or organopollutants using lignin degrading enzymes, such as LiP, MnP, and laccase (phenol oxidase), has some advantages, including no nutrient supply and easy control of temperature and pH [12], however, this enzymatic degradation is influenced by the H₂O₂ supply strategy. At the beginning of the reaction, activity of LiP or MnP is inhibited by excess H₂O₂. Use of glucose oxidase for degradation of lignins or dyes by lignin-degrading enzymes has recently been reported [6, 13]. The rate of generation of H₂O₂ by glucose oxidase could be easily controlled so that activity of Lip or MnP is constantly sustained; thus, inactivation of these enzymes by excess H₂O₂ was avoided. Studies on glucose oxidase and lignin degrading enzymes that catalyze degradation of recalcitrant compounds have been reported [6, 13-16]. However, even though gloxal oxidases are useful enzymes for H₂O₂ generation, studies on the use of gloxal oxidase as a lignin-degrading enzyme to catalyze degradation of lignins or dyes are very limited [17, 18]. In this study, we report on cloning and expression of the *glx1* gene encoding glyoxal oxidase (GLX) from *Phanerochaete chrysoporium* in *Pichia pastoris*. In addition, results of this study demonstrated the usefulness of recombinant GLX (rGLX) as an H₂O₂-supplying enzyme for decolorization of malachite green in combination with the recombinant manganese peroxidase (rMnP).

*P. chrysosporium* BKM-F-1767 (also ATCC 24725) was obtained from the Korean Collection for Type Culture and maintained in medium described by Tien and Kirk [19]. Transformants with a *glx1* gene expression vector were selected in low-salt Luria Bertani (LB) medium, containing 50 µg/mL Zeocin. The host strain used for heterologous expression was *Pichia pastoris* GS115 (his4), which was grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar). The pPICZC vector (Invitrogen, *Corresponding author* <E-mail : smpark@chonbuk.ac.kr>

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Carlsbad, CA, USA) was used for expression of the glx1 gene in *P. pastoris*. Expression of the insert in this vector is controlled by the methanol-inducible AOX1 promoter. To induce expression of GLX genes, *P. chrysosporium* was cultured under nitrogen limiting conditions in stationary flasks at 39°C for five days. Total mRNA was extracted using the Oligotex mRNA Mini kit (Qiagen, Hilden, Germany) and total cDNA was synthesized using the SMARTer PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA).

The glx1 cDNA of *P. chrysosporium* BKM-F-1767, including the signal peptide sequence, was amplified by PCR using the forward primer 5'-GCCAATTCTATTTGTGGCACGTCTAGCGCTAGT-3' and the reverse primer 5'-CGTACGTAACTCCAGGTCGCGAGGGTG-3'. The purified PCR products were cloned into the pGEM-T vector, excised using EcoRI and SmaBI, purified from an agarose gel, and inserted into pPICZC, resulting in the pPICZC/ss-glx1 construct. The glx1 gene expression construct, pPICZC:ss-glx1, was confirmed by PCR and restriction enzyme digestion, followed by sequencing of the insert in the expression vector.

For transformation of the yeast strain, plasmid (10 µg) was linearized using Pmel and transformed into *P. pastoris* using electroporation methods as recommended by manufacturer (Bio-Rad, Hercules, CA, USA). Transformed cells were selected on YPD agar plates containing 100 µg/mL Zeocin, at 30°C until colony formation was observed (2–3 days). Recombinant *P. pastoris* was confirmed by PCR using glx1-specific primers. Five *P. pastoris* transformants were cultured in YPD medium at 28°C to confirm whether the glx1 gene was highly expressed in the host. After an overnight incubation, 5 mL of the cultures were transferred into fresh YPD medium in a shaking incubator at 28°C and 220 rpm for one day. The cells were washed with sterile distilled water and resuspended in 10 µL BMKY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin, 1% methanol). The suspension was slowly added to 90 mL of fresh BMKY medium and cultured for three days. One mL of pure methanol was added to the 24 hr-culture at a final concentration of 1% to induce expression of the glx1 gene. One mL of culture was collected every 24 hr for measurement of enzyme activity. The clone exhibiting the highest level of rGLX release was selected and stored at −80°C. Enzyme activity was assayed using a peroxidase-coupled assay with phenol red as the peroxidase substrate, as described by Kersten and Krik [11]. Culture supernatant (100 µL) was mixed with distilled water to reach a volume of 300 µL and was then added to a reaction mixture containing 50 mM 2, 2-dimethylsuccinate (pH 6.0), 10 mM methylglyoxal, 0.01% phenol red, and 10 µg of horseradish peroxidase (type II; Sigma Chemical Co., St. Louis, MO, USA) in a total reaction volume of 1 mL. The reaction was stopped by addition of 50 µL of 2 N NaOH; the preparation was then assayed at 610 nm. *P. pastoris* recombinants were induced for expression of the glx1 gene in three days. Enzyme activity reached a maximum at two days of incubation and decreased thereafter. The GLX activity in *P. chrysosporium* culture was 6 µM H₂O₂/min, which was a similar order of magnitude as that of the glucose oxidase activity (4.5 µM H₂O₂/min) reported by Kelly and Reddy [8]. Enzyme activity in the culture filtrate of recombinant yeast was 1,200 µM H₂O₂/min, which was 200-fold higher than that of *P. chrysosporium* and the glucose oxidase activity of *P. chrysosporium*. However, this comparison was a rough index of the relative levels of activity, as many factors, such as differences in culture conditions, enzyme recovery, and culture age were not considered.

To purify rGLX from the two-day-culture, 100 mL of cell-free supernatant obtained by centrifugation and filtration through a 0.45 µm filter was mixed with 10 mL of 10× binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole pH 7.4) and applied to a Ni²⁺-nitrilotriacetic acid agarose column (Histrap; GE Healthcare, München, Germany) using the AKTA fast protein liquid chromatography purification system. Protein was eluted with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole and collected in a 15 mL conical tube. All fractions containing the purified enzyme were dialyzed in distilled water for removal of salt and imidazole. Protein concentration was measured by the Bradford method using the Thermo Scientific Protein Assay kit (Thermo Scientific, Rockford, IL, USA) with serum albumin as the standard. SDS-PAGE was performed as described by Sambrook and Russell [20]. The optimum temperature of rGLX was determined by measuring the activity at 20–80°C. To determine the optimal pH and temperature of

**Fig. 1.** SDS-PAGE analysis of recombinant glyoxal oxidase (GLX) purified from *Pichia pastoris*. M, molecular weight markers; lane 1, recombinant GLX; lane 2, recombinant GLX treated with PNGase F.
rGLX, sodium citrate, sodium phosphate, and sodium carbonate buffers were used to generate a pH range from 3 to 10. The molecular mass of rGLX was approximately 70 kDa (Fig. 1), which was larger than the predicted 57 kDa mature polypeptide [17]. This difference in molecular size between rGLX and the predicted protein appeared to be due to post-translational modification, such as glycosylation [11, 17]. The amino acid sequence of the predicted mature polypeptide showed five potential N-glycosylation sites (Asn-Xaa-Ser/Thr), indicated at Asn11, Asn24, Asn78, Asn209, and Asn399 [18]. After rGLX treatment with PNGase F, the molecular mass of this enzyme was reduced to 60 kDa, but was still larger than the expected size of 57 kDa, which appeared to be due to the 2.5 kDa of fused myc epitope and His-tag amino acid residues.

The optimal temperature for rGLX was 30°C, and the optimal pH was 5.0 (Fig. 2A and 2B), which is comparable with pH 6.0 of GLX1 from P. chrysosporium [18], although the pH range of rGLX was narrower than that of native GLX1. The enzyme activity showed a rapid decrease at pHs < 3.0 or > 7.0, which may have been due to differences in the protein folding form.

The protein in the cell-free supernatant obtained above for purification of rGLX was concentrated by ultrafiltration (Centricon Plus-70; Amicon Corp., Lexington, MA, USA) for use of GLX as a H2O2 source for decolorization of malachite green using recombinant MnPH4. In our previous study [21], rMnPH4 from P. chrysosporium expressed in P. pastoris easily decolorized methyl orange, malachite green, and remazol brilliant blue R. To determine the optimal pH of this coupling enzymatic reaction, experiments with 1 mL of reaction volume were performed under the following conditions: pH range of 3.75–4.25, rGLX to produce 4.78 μM H2O2/min, 500 U/L rMnPH4, 0.5 mM Mn2+, 50 mM methylglyoxal, and 35 μM malachite green in 50 mM malonate buffer at 30°C. Controls were run in parallel without methylglyoxal. In addition, rGLX was added to the reaction mixture to reach a final H2O2 concentration of 0–12.5 μM/min for determination of the effect of GLX concentration on decolorization of dyes by rMnPH4. In addition, malachite green was added to the reaction mixture at a final concentration of 0–150 μM for determination of the maximum treatment capacity of the dye in this coupling reaction. All experiments were performed in triplicate. The decolorization of malachite green was measured photometrically at 600 nm using a Schimadzu UV spectrophotometer. The decolorization degree of the dye was calculated as the extent of decrease from the initial optical density value of the dye.

One of the important parameters affecting the enzymatic coupling reaction for degradation of lignin or dyes is the pH range. In the LiP-catalyzing oxidation of veratryl alcohol (VA) together with glucose oxidase used as a H2O2 supplying enzyme, the initial velocity of VA oxidation varies according to the pH value due to the difference in the optimal pH of LiP (pH 3.5) and glucose oxidase (pH

Fig. 2. The optimal pH (A) and temperature (B) for recombinant glyoxal oxidase (GLX) and effect of pH (C) on the decolorization of malachite green with recombinant MnPH4 coupled with recombinant GLX (4.78 μM H2O2/min). In malachite green decolorization, the pH range used was 4–5.25 at 0.25 intervals. After 45 min of incubation, the remaining color of the malachite green was measured at 600 nm. The reaction mixture contained 35 μM malachite green, 50 mM methylglyoxal, 500 U/L MnP, and 0.5 mM MnSO4. All experiments were performed in triplicate.

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As the glucose oxidase concentration was increased, the optimal pH of this coupling reaction shifted from 4.0 to 3.5. The optimal pH of rMnPH4 was 4.5 but that of rGLX was 5.0. To optimize pH of the coupling reaction, the experiment was performed in the pH range of 3.75–4.25, so that malachite green was well decolorized by rMnPH4. The effect of pH on the decolorization of malachite green by rMnPH4 coupled with rGLX is shown in Fig. 2C. The rate of decolorization of malachite green varied according to pH. The optimal pH of this coupling reaction was 4.7. The pH and temperature used in further experiments were fixed at 4.7 and 30°C, respectively, for decolorization of malachite green using this enzyme system.

The amount of H$_2$O$_2$ generated in the coupled enzymatic reaction could be easily controlled by changing the concentration of added rGLX. To demonstrate the usefulness of this enzyme for generation of H$_2$O$_2$, malachite green was degraded using rMnPH4 coupled with rGLX. In addition, for comparison of the decolorization efficiency between the enzymatic supply of H$_2$O$_2$ by rGLX and external addition of H$_2$O$_2$, H$_2$O$_2$ was added to the reaction mixture to initiate the rMnPH4-catalyzing decolorization of malachite green (Fig. 3A). In the presence of rGLX production of 4.78, 7.00, 9.55, or 12.5 μM H$_2$O$_2$/min, the incubation time required for complete decolorization of malachite green was 90, 65, 50, or 30 min, respectively, indicating that enzymatic reaction time decreased, as the concentration of added rGLX increased. It took 60 min to reach 100% decolorization with 100 μM externally added H$_2$O$_2$. Below the rate of 9.55 μM H$_2$O$_2$/min production, the decolorization rate was retarded in the initial enzymatic coupling reaction. This lag phase is a common phenomenon in enzymatic coupling reactions [17]. The mechanism of this lag remains unclear. However, this lag phase did not appear with a high concentration of rGLX (over 12.5 μM H$_2$O$_2$/min).

Experiments with different initial dye concentrations were performed in order to determine the maximal decolorization capacity of the enzymatic coupling reaction (Fig. 3B). In the presence of 25, 50, 75, 100, 125, or 150 μM malachite green, the decolorization rate was 100, 100, 94.0, 88.9, 90.4, and 83.1%, respectively. High decolorization efficiency was achieved at dye concentrations from 25 to 150 μM within 90 min. When working at higher loads, longer periods are required for achievement of acceptable decolorization.

In a previous study on the enzymatic coupling reaction for degradation of lignin model compounds or dyes, glucose oxidase received considerable attention as a H$_2$O$_2$ producing enzyme [6]. This study reported that the glx1 gene encoding GLX1 from P. chrysosporium is well expressed in P. pastrois and suggested that this rGLX would be useful as an H$_2$O$_2$-generating enzyme in the enzymatic coupling reaction. Until now, no attempt to degrade lignins or dyes using both recombinant enzymes has been reported. Most importantly, one of the two enzymes used in the coupling reaction for degradation of lignins or dyes was purified from a fungal culture [17] or purchased from a company [10]. We have established a system for to decolorization of a dye using with rMnP and rGLX. The results of this study demonstrate the potential for application of this system to degradation of dyes or recalcitrant compounds.

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References

1. Bumpus J, Tien M, Wright D, Aust SD. Oxidation of persistent environmental pollutants by a white rot fungus. Science 1985;228:1434-6.

2. Seo YS, Ryu WR, Kim CJ, Chang YK, Cho MH. Biodegradation of PAHs (polycyclic aromatic hydrocarbons) using immobilized cells of *Phanerochaete chrysosporium*. Korean J Biotechnol Bioeng 2000;15:247-53.

3. Huang X, Wang D, Liu C, Hu M, Qu Y, Gao P. The roles of veratryl alcohol and nonionic surfactant in the oxidation of phenolic compounds by lignin peroxidase. Biochem Biophys Res Commun 2003;311:491-4.

4. Martins MA, Ferreira IC, Santos IM, Queiroz MJ, Lima N. Biodegradation of bioaccessible textile azo dyes by *Phanerochaete chrysosporium*. J Biotechnol 2006;118:437-43.

5. Kersten PJ, Cullen D. Cloning and characterization of a cDNA encoding glyoxal oxidase, a H2O2-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Proc Natl Acad Sci U S A 1993;90:7411-3.

6. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Vol. 1. 3rd ed. New York: Cold Spring Harbor; 2003.

7. Thiyagarajan S, Kim HY, Park SM. Heterologous expression and characterization of *Phanerochaete chrysosporium* manganese peroxidase isoenzyme H4. In: Proceeding of 2011 International Symposium and Annual Meeting of the Korean Society for Microbiology and Biotechnology; 2011 Oct 14-15; Seoul, Korea. Seoul: Korean Society for Microbiology and Biotechnology; 2011. p. 52.