J. Microbiol. Biotechnol. (2002), **12**(6), 972–978

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of 2,4-Dichlorophenol Oxidizing Peroxidase from *Streptomyces* sp. AD001

JEON, JEONG-HO[1], YUN-JON HAN[1], TAE-GU KANG[1], EUNG-SOO KIM[2], SOON-KWANG HONG[1], AND BYEONG-CHUL JEONG[1]*

[1]*Department of Biological Sciences/BK21 Graduate Program in Environmental and Biological Engineering, MyongJi University, Nam-dong, Yongin-si, Kyunggi-do 449-728, Korea*
[2]*Department of Biotechnology, Inha University, Yonghyeon-dong, Nam-gu, Inchon 402-751, Korea*

Received: November 5, 2002
Accepted: November 19, 2002

Abstract *Streptomyces* sp. AD001 is a Gram-positive soil actinomycetes secreting an uncharacterized 2,4-dichlorophenol (DCP) oxidizing enzyme, whose activity is similar to the previously known Actinomycetes lignin-peroxidase (ALiP). This extracellular peroxidase was purified from *Streptomyces* sp. AD001 as a single protein band on an SDS-PAGE by ammonium sulfate fractionation, Q-sepharose, concanavalin A, and Bio-Gel HTP column chromatographies. The molecular mass of the purified peroxidase was determined by SDS-PAGE to be 45.2 kDa, and 49.7 kDa with MALDI-TOF-MS, respectively. The highest level of peroxidase activity was observed at pH 7.5 and 30°C. The amino terminal sequence of the purified peroxidase (G-E-P-E-E-G-N-V-D-G-T-L) showed no significant homologies to any known proteins, suggesting that *Streptomyces* sp. AD001 may secrete a novel kind of bacterial peroxidase. Initial rate kinetic data of the 2,4-DCP oxidation were best modeled with a random-binding bireactant system.

Key words: Peroxidase, purification, *Streptomyces* sp. AD001, 2,4-dichlorophenol

Streptomyces are Gram-positive, filamentous soil bacteria, which undergo a unique morphological differentiation. *Streptomyces* are important industrial microorganisms owing to their ability to produce a large number of secondary metabolites, such as antibiotics, and several classes of enzymes having various industrial applications [4, 14, 19, 24]. In addition, some *Streptomyces* species have also been recognized as being ecologically and environmentally valuable microorganisms due to their superior capabilities of degrading many recalcitrant substances with diverse structures [1, 7, 10, 22].

*Corresponding author
Phone: 82-31-330-6196; Fax: 82-31-335-8249;
E-mail: bcjeong@mju.ac.kr

Among the many *Streptomyces* industrial enzymes, *Streptomyces* peroxidases have been intensively studied due to their potential biotechnological application for the production of animal feedstock and raw materials for the chemical, agricultural, and paper industries. And, their capability of oxidizing a large number of aromatic substances [7, 10, 25] also implies the potential for treatment of industrial effluents containing recalcitrant compounds.

S. viridosporus T7A, one of the best characterized xenobiotic-degrading *Streptomyces*, was previously reported to secrete four isoforms (P1 to P4) of extracellular Actinomycetes lignin-peroxidase (ALiP) [22]. Among the four ALiP isoforms found in *S. viridosporus* T7A, only P3 isoform exhibited 2,4-dichlorophenol oxidizing activity in the presence of hydrogen peroxide, indicating its usefulness as the most promising bacterial enzyme for xenobiotic-biodegradation, due to its broad substrate specificity [7].

In general, enzyme reaction mechanisms involving two substrates are described by one of three models: the random-binding bireactant (Bi Bi), the ordered Bi Bi, or the ping-pong Bi Bi kinetic model. The kinetic mechanism of the ALiP-P3 from *S. viridosporus* T7A, which catalyze the oxidation of 2,4-DCP, was proposed to be a random-binding bireactant system. [7].

Recently, a soil actinomycetes, named *Streptomyces* sp. AD001, was isolated from Korean soil, based on superior dye-decoloring activity on the plate containing congo-red as well as new-fuchin dyes, and was found to secrete an ALiP-like enzyme which oxidized 2,4-DCP in the presence of hydrogen peroxide [10]. This strain produced ALiP-P3-like enzyme to an extracelluar culture broth, and this enzyme can oxidize 2,4-DCP [10]. We describe in the present study the purification and characterization of the extracellular 2,4-DCP oxidizing peroxidase from *Streptomyces* sp. AD001 strain. The enzyme kinetic data indicated

that *Streptomyces* AD001 peroxidase followed a random-binding bireactant mechanism.

MATERIALS AND METHODS

Bacteria and Culture Conditions

The microorganism used in this study was *Streptomyces* sp. AD001, isolated from Korean soil, and was known to secrete an extracellular ALiP-like enzyme [10]. *Streptomyces* sp. AD001 spore suspensions (10 ml) were prepared from R1R2 slants, which had been incubated at 30°C for 7 days. The spore suspensions from each slant were divided and stored as either long-term stock cultures (stored at −72°C) or working spore suspensions (stored at −20°C). A modified DJMM (40 g corn starch/l, 20 g casein/l, 7 g $CaCO_3$/l, 3 g yeast extract/l, 1 g NH_4Cl/l, 1 ml R2YE trace metal stock solution/l, 1 g xylan/l, pH 7.0) was used for enzyme production [8]. Five ml of *Streptomyces* sp. AD001 spore suspension was inoculated in 1 l medium. A 2-l Erlenmeyer flask containing 500 ml of the medium was incubated at 30°C and 200 rpm. Peak enzyme production was observed at 6 days of cultivation.

Purification of Peroxidase

All purification steps were performed at 4°C. The culture supernatant of *Streptomyces* sp. AD001 was first treated with 70% ammonium sulfate, and the precipitate was then recovered by centrifugation at 7,000 rpm for 30 min. After suspending in 50 mM MES buffer (pH 6.0), the suspension was dialyzed against the same buffer. Next, the enzyme suspension was loaded on a Q-sepharose fast-flow anion exchange column (26/32, Pharmacia, Sweden), which had been pre-equilibrated with 50 mM MES buffer (pH 6.0). The protein was eluted with 0, 100, 150, 200, and 400 mM NaCl step gradient in the same buffer at a flow rate of 10 ml/min. The active fractions were pooled and then applied to a concanavalin A sepharose affinity column (16/5, Pharmacia, Sweden). The column was eluted batchwise with 0, 50, 100, 150, and 200 mM methyl-α-D-glucopyranoside at a flow rate of 1 ml/min. The active fractions were pooled and dialyzed against 20 mM phosphate buffer (pH 6.8), and the dialysate was applied to a Bio-Gel-hydroxylapatite column (16/10, Bio-Rad, U.S.A.), which had been pre-equilibrated with the same buffer. The column was washed with the same buffer followed by elution with a 50, 100, 150, 200, and 500 mM phosphate buffer (pH 6.8) stepwise gradient. The active fractions, which were eluted with 150 mM phosphate buffer, were concentrated using an Amicon YM 10 membrane (Millipore, U.S.A.) and then used for characterization.

SDS-PAGE and Native PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the methods of Laemmli [15]. The protein preparation was boiled for 10 min in the presence of 1.6% (wt/vol) SDS, 4% (wt/vol) β-mercaptoethanol, and 0.1% bromophenol blue in the sample buffer. The molecular weight standards (Invitrogen, The Netherlands) used were Myosin (188 kDa), Phosphorylase B (98 kDa), BSA (62 kDa), GDH (49 kDa), ADH (38 kDa), CAH (28 kDa), Myoglobin Red (19 kDa), Lysozyme (14 kDa), Aprotinin (6 kDa), and Insulin B chain (3 kDa). The electrophoresis was performed with 12% polyacrylamide gel containing 0.1% SDS, at a constant voltage, using a vertical system (Mini Gel system, Bio-Rad U.S.A.). Native PAGE was carried out at a temperature of 4°C under the same conditions as SDS-PAGE, without the addition of SDS. After electrophoresis, protein bands were visualized by Coomassie brilliant blue R250 staining. Peroxidase bands on nondenaturing PAGE gels were developed by activity staining with 2,4-DCP as the substrate.

Effect of pH and Temperature on Activity and Stability of Enzyme

The optimum pH for the purified peroxidase was determined over a pH range of 4.0 to 9.5 using the following buffers (100 mM): acetic acid (4.0–5.5), MES (5.5–6.5), phosphate (6.5–8.0), and boric acid (8.0–9.5). For the pH stability test, the enzyme was incubated at different pHs at 30°C for up to 40 h, and then the residual activity was measured after readjustment of the pH to 7.0.

To determine the effect of temperature on the stability of the enzyme, the enzyme solution was incubated in 100 mM MOPS (pH 7.0) buffer for up to 40 h at various temperatures (4, 20, 40, and 60°C), then the residual enzyme activity was assayed. To examine the optimum temperature, the enzyme activity was measured at various temperatures (20, 30, 35, 40, 45, 50, and 60°C).

Table 1. Purification of extracellular peroxidase from *Streptomyces* sp. AD001.

	Volume (ml)	Total protein (mg)	Total activity (Uint)	Specific activity (Uint/)	Yield (%)	Purification (fold)
Crude extracts	1,700	2,407.2	5,304.0	2.2	100	1
Ammonium sulfate	110	1,556.4	2,326.5	1.5	44.2	0.7
Q-sepharose	30	60.0	788.1	13.1	14.9	6.0
Concanavalin A	3	4.9	383.91	80.0	7.2	35.9
Bio-Gel HTP	3	1.1	116.4	102.1	2.2	46.3

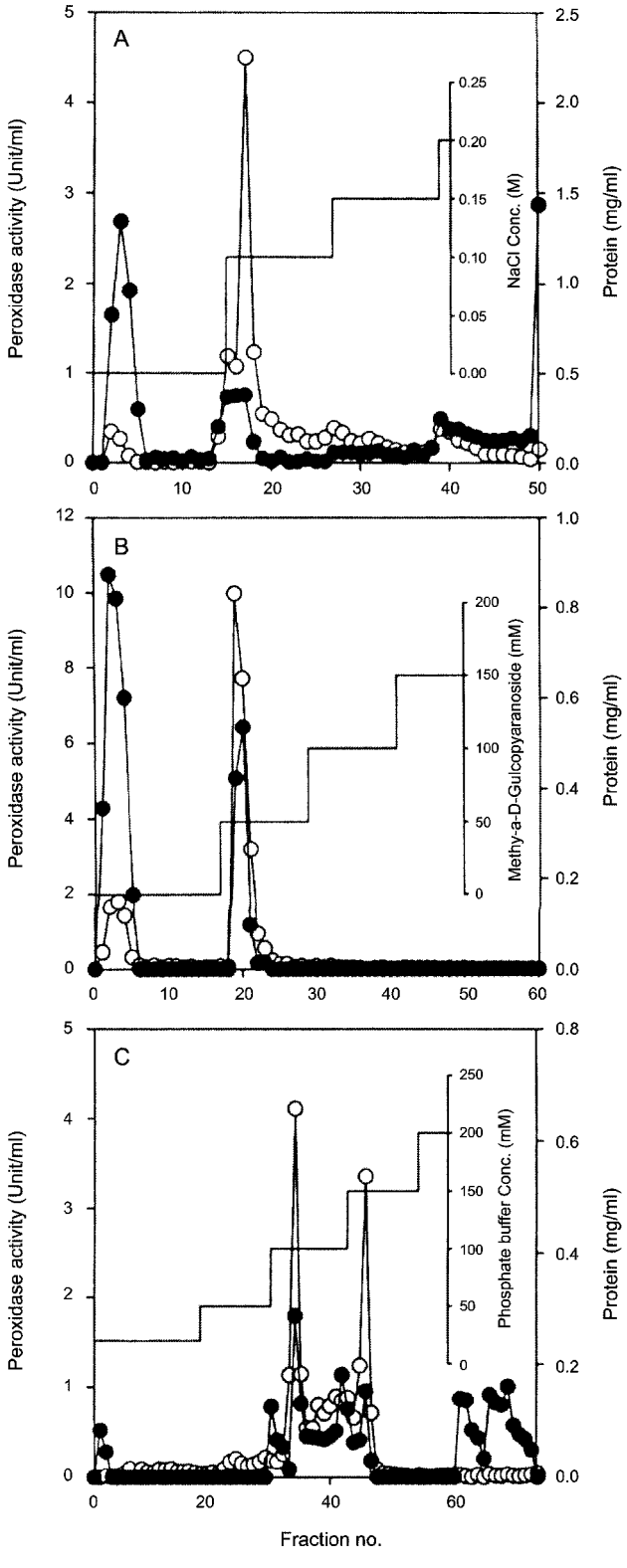

Fig. 1. Elution profiles of protein and peroxidase activity in the column chromatography.
(A) Q-sepharose FF anion exchange column (26/32) chromatography. (B) Concanavalin A affinity column (16/5) chromatography. (C) BIO-Gel-hydroxylapatite column (16/10) chromatography. ●: Protein; ○: Peroxidase activity.

Peroxidase Assay and Protein Concentration Determination

Peroxidase activity was assayed using a modified 2,4-dichlorophenol assay [22], which is based on the reaction of ALiP-P3-oxidized 2,4-dichlorophenol with 4-aminoantipyrene to form colored antipyrylquinonimine that strongly absorbs at 510 nm. The final concentrations of the 1.0 ml aqueous assay mixture consisted of 100 mM MOPS buffer (pH 7.0), 9.0 mM 2,4-dichlorophenol, 8.2 mM 4-aminoantipyrene, and 40 mM H_2O_2. After the addition of H_2O_2, absorbance change at 510 nm was monitored for 2 min at 30°C. One unit of enzyme activity corresponded to an increase of 1.0 absorbance/min. Protein concentration was determined by the method of Bradford *et al*. [2] using bovine serum albumin as the standard.

Molecular Mass Determination and Amino-Terminal Amino Acid Sequence Analysis

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Voager™ DE-STR (PerSeptive Biosystems, Inc.). The N-terminal amino acid sequence of peroxidase in the PVDF membrane was determined by using a protein sequencing system (Milligen, Applied Biosystems).

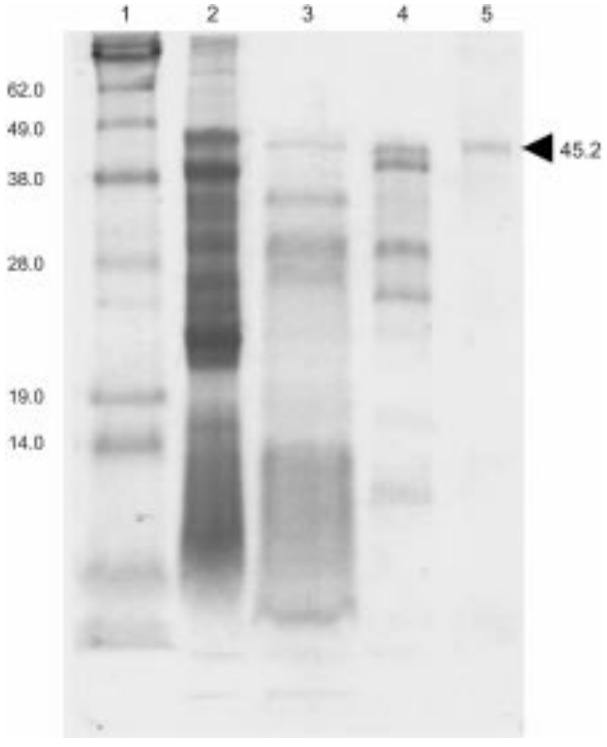

Fig. 2. SDS-PAGE of proteins during purification steps.
Lane 1: Molecular size markers (in kDa); Lane 2: 0–70% ammonium sulfate precipitate; Lane 3: Q-sepharose anion exchange column fraction; Lane 4: Concanavalin A column fraction; Lane 5: Bio-Gel-hydroxylapatite column fraction.

Table 2. Effect of inhibitors on peroxidase activity.

Inhibitor (1 mM)	Inhibition rate (%)±STD*
Potassium cyanide	88.9±0.6
Sodium azide	89.1±0.7
EDTA	63.7±5.5
Mercuric sulfate	100±0.0
Magnesium sulfate	43.5±5.5
Zinc sulfate	53.6±13.7
Copper sulfate	68.3±5.3
Cobalt chloride	73.3±8.2

*STD was from 3 separate experiments.

RESULTS AND DISCUSSION

Purification of Peroxidase

Peroxidase was purified from 2 l of *Streptomyces* sp. AD001 culture broth by ammonium sulfate fractionation, chromatographies on Q-sepharose FF, concanavalin A, and Bio-Gel-hydroxylapatite as described in Materials and Methods. As shown in Table 1, the final purification step resulted in 46.3-fold purification with an overall yield of 2.2%. The enzyme had a specific activity of 102.1 U/mg protein. The concanavalin A affinity chromatography (Fig. 1B) was particularly useful for the peroxidase, which was the main subject of this research. Bio-Gel HTP chromatogram and native PAGE activity staining showed that more than three 2,4-DCP oxidizing peroxidases existed in the *Streptomyces* sp. AD001 culture broth (Figs. 1B, 2C, and 3B). Finally, purified peroxidase was eluted with 150 mM phosphate buffer on the Bio-Gel HTP column. The purified enzyme showed as a single protein band on an SDS-PAGE after staining with Coomassie Brilliant Blue R250. The apparent molecular mass of the purified peroxidase was estimated to be 45.2 kDa by SDS-PAGE (Fig. 2) and 49.7 kDa by MALDI-TOF-MS. These results indicate that the purified peroxidase was in monomeric form. The N-terminal amino acid sequence of the purified peroxidase was identified as G-E-P-E-E-G-N-V-D-G-T-L. No other protein with the same N-terminal amino acid sequence was found in the database analyzed by NCBI's BLAST (Basic Local Alignment Search Tool) program.

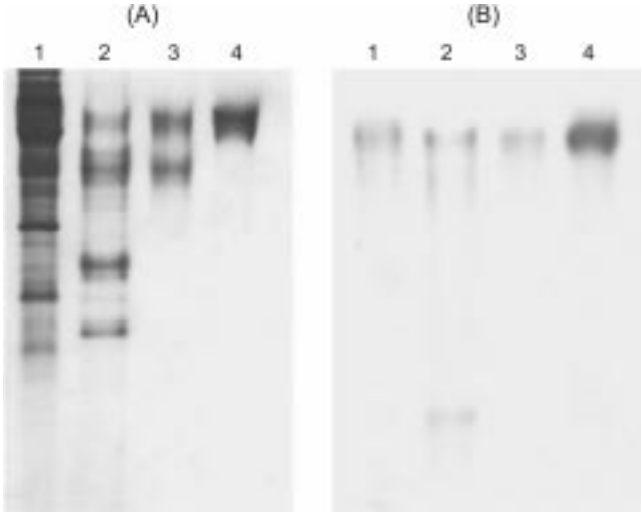

Fig. 3. Native PAGE (A) and activity staining (B).
The activity staining was performed in peroxidase assay mixture (pH 7.0, 100 mM MOPS) for 5 min at 30°C after native PAGE. Lane 1: 0–70% ammonium sulfate precipitate; Lane 2: Q-sepharose anion exchange column fraction; Lane 3: Concanavalin A column fraction; Lane 4: Bio-Gel-hydroxylapatite column fraction.

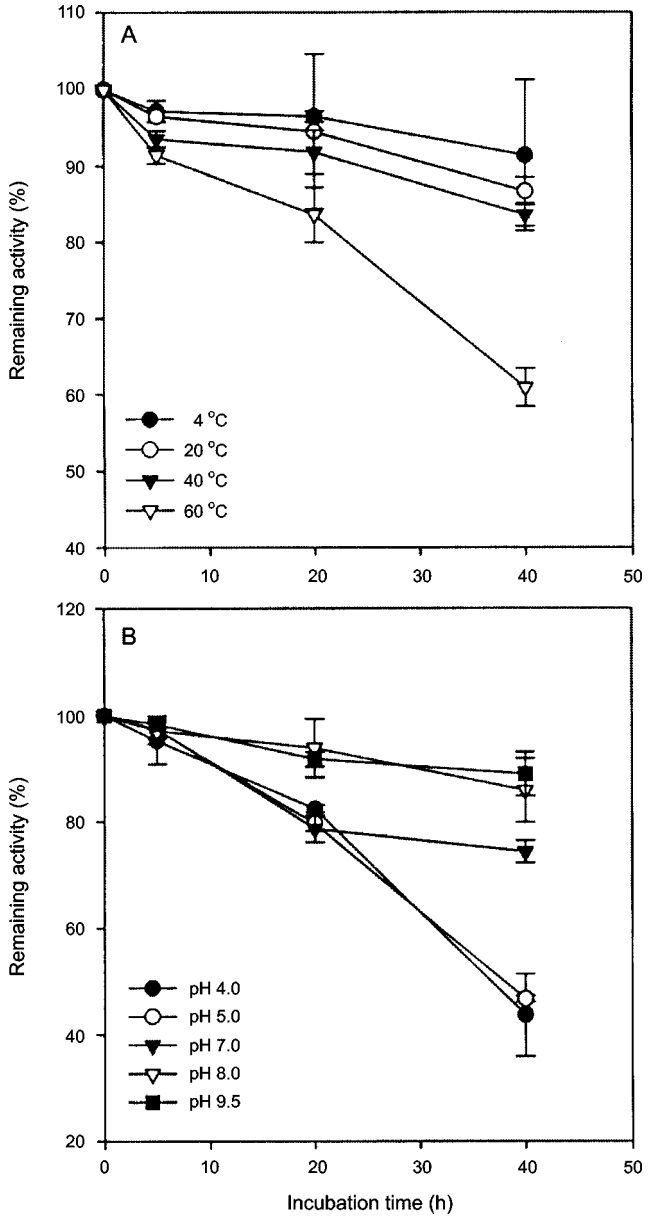

Fig. 4. Effects of temperature (A) and pH (B) on the peroxidase stability.

Effects of pH and Temperature on the Peroxidase Activity

The optimal pH and temperatures for the peroxidase activity were 7.5 and 30°C, respectively (data not shown). The purified peroxidase was very stable below 40°C, remaining over 80% of its initial activity after 40 h of incubation (Fig. 4A). Also, this peroxidase was more stable at alkaline pH (pH 8, 9.5) and lost about 60% of its initial activity after storage at acidic pH (Fig. 4B). The purified peroxidase was a thermostable and alkalinestable enzyme. Thus, the peroxidase can be stored at pH 9.5 and 4°C.

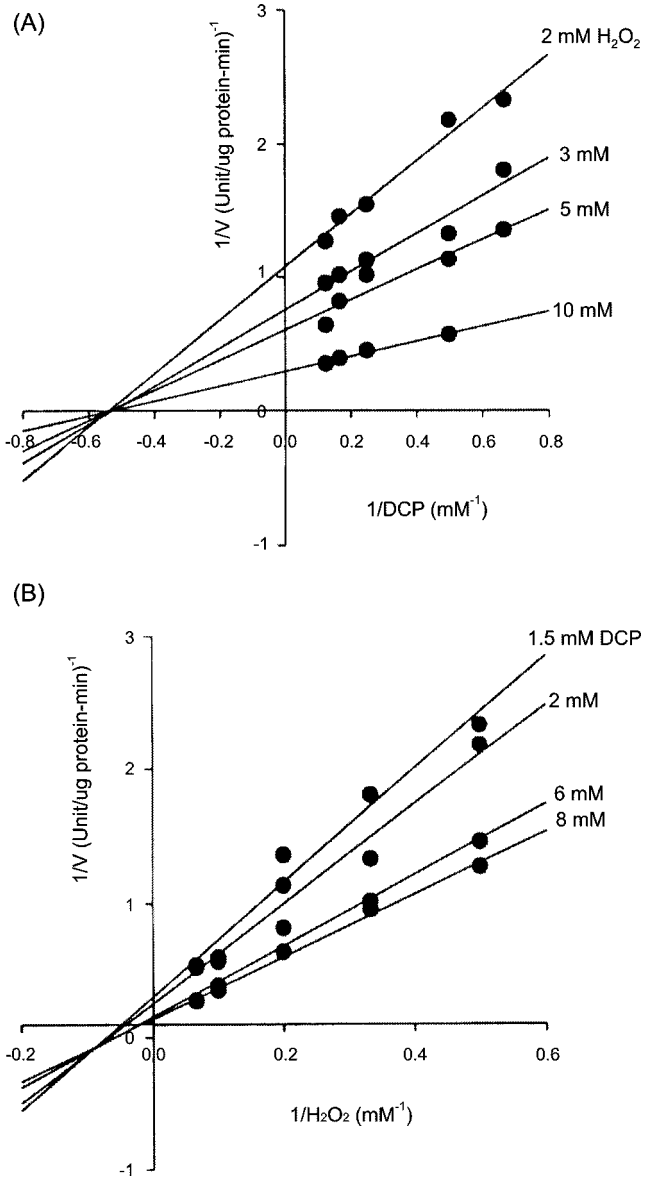

Fig. 5. Initial plot of 1/rate vs 1/[DCP] and 1/[H_2O_2] at fixed H_2O_2 and DCP concentrations.
Data are the average of triplicate initial rate measurements.

Effect of Metal Ions and Metabolism Inhibitors on the Peroxidase Activity

The purified peroxidase was pre-incubated in the presence of various metal ions and chemical reagents at 4°C for 30 min at 1 mM concentration, and its residual activity was assayed. As shown in Table 2, the peroxidase activity was inhibited by about 90% with known heme protein inhibitors such as potassium cyanide and sodium azide, and the chelating agent, EDTA, showed less effect (64%). Additionally, the purified peroxidase exhibited a maximum absorption at 400 nm (data not shown). Thus, these observations indicated that this peroxidase is a heme protein, similar to ALiP-P3 [22]. The peroxidase activity was completely abolished by 1 mM mercuric sulfate, whereas Mg^{2+}, Zn^{2+}, Cu^{2+}, and Co^{+} showed less inhibition.

Kinetic Study of Purified Peroxidase

Initial rate data with varying concentrations of 2,4-DCP or H_2O_2 did not match with both ping-pong bireactant system (which would result in parallel curves that do not intersect) and ordered Bi Bi system (in which the curves do intersect on 1/[DCP]=0) (Fig. 5). Therefore, the random binding bireactant kinetic model is the only possible model system. The parameters of the random binding bireactant kinetic model are summarized in Table 3. Figure 6 shows the actual rates and predicted rates by the random Bi Bi model with the calculated kinetic constants. This result suggests that the purified peroxidase catalyzed the oxidation of 2,4-DCP by a random Bi Bi system. This reaction mechanism was similar to that of ALiP-P3 from *Streptomyces viridosporus* T7A [7]. However, the presently purified peroxidase and ALiP-P3 showed different kinetic constants (Table 3). The kinetic parameter, α, of the random bireactant system is a measure of the change in the enzyme's binding affinity to one substrate, after the other has formed an enzyme-substrate complex. The binding factor determined for the purified peroxidase (α=1.8) in the present study indicates that, once 2,4-DCP or H_2O_2 is bound to the enzyme, the affinity of the resulting complex for the remaining substrate is higher than that of the native enzyme. This binding factor shows that the purified peroxidase has substrate

Table 3. Kinetic constants for the purified peroxidase and ALiP-P3-catalyzed 2,4-DCP oxidation.

Kinetic constant	ALiP-P3*	Purified peroxidase
α	0.7	1.8
V_{max} [nmol/mg protein.min]	465.8	529.7
K_{DCP} (mM)	0.372	1.7
$K_{H^+O^2}$ (mM)	38	11.0

α: Change in enzyme's binding affinity to one substrate after the other has formed an enzyme-substrate complex.
*This data was obtained from reference 7.

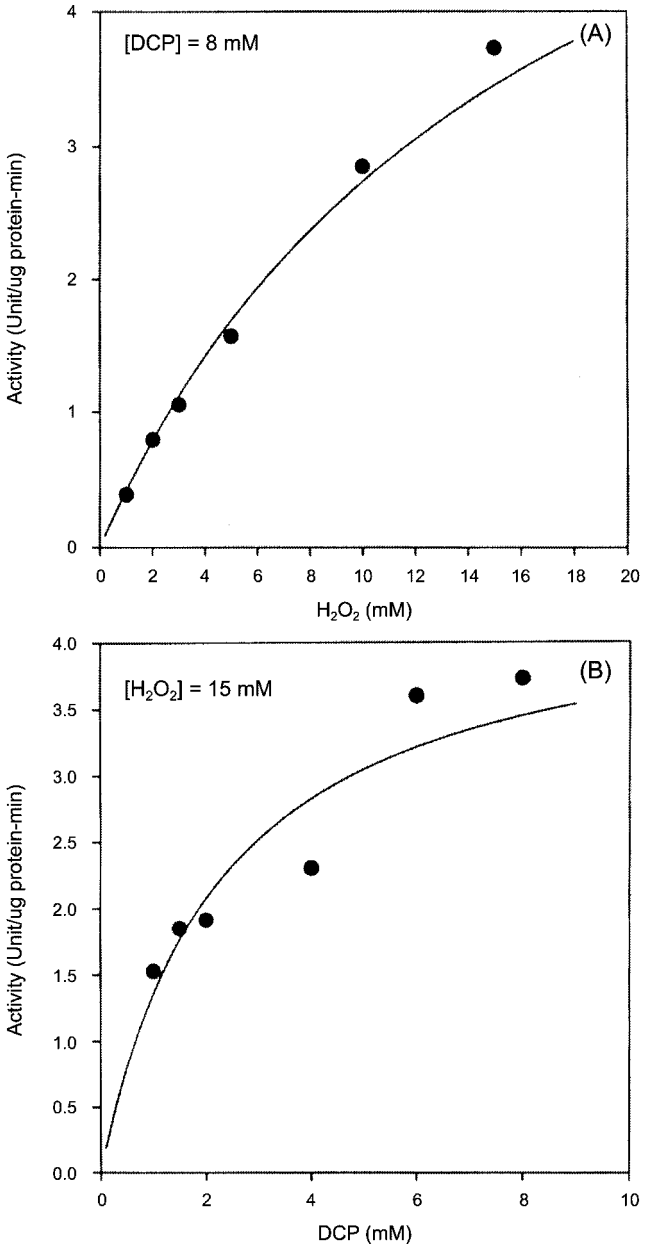

Fig. 6. Test of kinetic parameters determined from initial rate data.
The predicted initial rates are compared to the measured rates at fixed DCP concentration of 8 mM (A) and H_2O_2 concentration of 15 mM (B). Experimental data, model prediction.

specificity different from ALiP-P3: The K_{DCP} and $K_{H_2O_2}$ of the purified peroxidase were 1.7 mM and 11.0 mM, respectively (Table 3), and these results indicate that the purified peroxidase has higher affinity for H_2O_2, and a lower affinity for 2,4-DCP than ALiP-P3. The V_{max} of the purified peroxidase was similar to ALiP-P3. These characteristics indicate that the purified peroxidase was an ALiP-P3-like enzyme.

Acknowledgment

This work was supported by a grant from the MOST and KOSEF through Regional Research Center at MyongJi University.

REFERENCES

1. An, H. R., H. J. Park, and E. S. Kim. 2000. Characterization of benzoate degradation via *ortho*-cleavage by *Streptomyces setonii*. *J. Microbiol. Biotechnol*. **10:** 111–114.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Burke, N. S. and D. L. Crawford. 1998. Use of azo dye ligand chromatography for the partial purification of a novel extracellular peroxidase from *Streptomyces viridosporus* T7A. *Appl. Microbiol. Biotechnol.* **49:** 523–530.
4. Chun, H. S., H. B. Chang, Y. I. Kwon, and H. C. Yang. 2001. Characterization of an α-glucosidase inhibitor produced by *Streptomyces* sp. CK-4416. *J. Microbiol. Biotechnol.* **11:** 389–393.
5. Chung, N. H., G. Kang, G. H. Kim, I. S. Lee, and W. G. Bang. 2001. Effect of nutrient nitrogen on the degradation of pentachlorophenol by white rot fungus, *Phanerochaete chrysosporium. J. Microbiol. Biotechnol.* **11:** 704–708.
6. Chung, N. H., I. S. Lee, H. S. Song, and W. G. Bang. 2000. Mechanisms used by white rot fungus to degrade lignin and toxic chemicals. *J. Microbiol. Biotechnol*. **10:** 737–752.
7. Dennis, C. Y. and T. K. Wood. 1997. 2,4-Dichlorophenol degradation using *Streptomyces viridosporus* T7A lignin peroxidase. *Biotechnol. Prog.* **13:** 53–59.
8. Dennis, C. Y., D. J. Jahng, and T. K. Wood. 1996. Enhanced expression and hydrogen peroxide dependence of lignin peroxidase from *Streptomyces viridosporus* T7A. *Biotechnol. Prog*. **12:** 40–46.
9. Elba, P. S. B., H. J. Nascimento, J. M. B. Macedo, and J. G. Silva Jr. 1999. Lignin peroxidase isoform *Streptomyces viridosporus* T7A: Are they a monomer based structure? *Biotechnology Techniques* **13:** 289–293.
10. Kang, M. J., J. K. Kang, and E. S. Kim. 1999. Isolation and characterization of soil *Streptomyces* involved in 2,4-dichlorophenol oxidation. *J. Microbiol. Biotechnol.* **9:** 877–880.
11. Kim, J. S. and J. K. Lee. 2000. Cloning, DNA sequence determination, and analysis of growth-associated expression of *sod*F gene coding for Fe- and Zn-containing superoxide dismutase of *Streptomyces griseus*. *J. Microbiol. Biotechnol*. **10:** 700–706.
12. Kim, T. K., J. H. Choi, and I. K. Rhee. 2002. Purification and characterization of a cyclohexanol dehydrogenase from *Rhodococcus* sp. TK6. *J. Microbiol. Biotechnol*. **12:** 39–45.
13. Kintz, P., A. Tracqui, and P. Mangin. 1992. Accidental death caused by the absorption of 2,4-dichlorophenol through the skin. *Arch.Toxicol.* **66:** 298–299.

14. Kwon, H. J., S. E. Hwang, J. T. Han, C. J. Kim, J. R. Rho, and J. H. Shin. 2001. Production of oleamide, a functional lipid, by *Streptomyces* sp. KK90378. *J. Microbiol. Biotechnol*. **11:** 1018–1023.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
16. Leda, M. F. G., M. B. M. Jacyara, and P. S. B. Elba. 1999. Lignin peroxidase production by *Streptomyces viridosporus* T7A. *Appl. Biochem. Biotechnol.* **77–79:** 771–778.
17. Lee, J. S., E. J. Kang, M. O. Kim, D. H. Lee, K. S. Bae, and C. K. Kim. 2001. Identification of *Yarrowia lipolytica* Y103 and its degradability of phenol and 4-chlorophenol. *J. Microbiol. Biotechnol*. **11:** 112–117.
18. Maria, G. M., S. B. Andrew, J. R. Brandon, S. Gary, N. Peter, and T. W. Michael. 2001. Extracellular heme peroxidases in Actinomycetes: A case of mistaken identity. *Appl. Environ. Microbiol*. **67:** 4512–4519.
19. Park, J. W., J. K. Lee, T. J. Kwon, D. H. Yi, Y. I. Park, and S. M. Kang. 2001. Purification and characterization of a cytochrome P-450 from pravastatin-producing *Streptomyces* sp. Y-110. *J. Microbiol. Biotechnol*. **11:** 1011–1017.
20. Park, S. S. and S. M. Hwang. 1999. Purification and characterization of iron-containing superoxide dismutase from *Lentinus edodes*. *J. Microbiol. Biotechnol*. **9:** 854–860.
21. Ramachandran, M., D. L. Crawford, and A. L. Pometto. 1987. Extracellular enzyme activities during lignocellulose degradation by *Streptomyces* spp.: A comparative study of wild-type and genetically manipulated strains. *Appl. Environ. Microbiol.* **53:** 2754–2760.
22. Ramachandran, M., D. L. Crawford, and G. Hertel. 1988. Characterization of an extracellular lignin peroxidase of the lignocellulolytic Actinomycete *Streptomyces viridosporus*. *Appl. Environ. Microbiol.* **54:** 3057–3063.
23. Ramachandran, S., T. S. Magnuson, and D. L. Crawford. 2000. Isolation and analysis of three peroxide sensor regulatory gene homologs ahpC, ahpX and oxyR in *Streptomyces viridosporus* T7A - a lignocellulose degrading actinomycete. *DNA Seq*. **11:** 51–60.
24. Seo, Y. W., K. W. Cho, H. S. Lee, T. M. Yoon, and J. H. Shin. 2000. New polyene macrolide antibiotics from *Streptomyces* sp. M90025. *J. Microbiol. Biotechnol*. **10:** 176–180.
25. Spiker, J. K., D. L. Crawford, and E. C. Thiel. 1992. Oxidation of phenolics and non-phenolic substrates by the lignin peroxidase of *Streptomyces viridosporus* T7A. *Appl. Microbiol. Biotechnol.* **37:** 518–523.
26. Syracuse Research Corp. 1992. Toxicological profile for 2,4-dichlorophenol; U.S. Public Health Service: Washington, DC, U.S.A.
27. Thomas, L. and D. L. Crawford. 1998. Cloning of clustered *Streptomyces viridosporus* T7A lignocellulose catabolism genes encoding peroxidase and endoglucanase and their extracellular expression in *Pichia pastoris*. *Can. J. Microbiol.* **44:** 364–372.
28. Timothy, S. M., M. A. Roberts, D. L. Crawford, and G. Hertel. 1991. Immunologic relatedness of extracellular ligninases from the Actinomycetes *Streptomyces viridosporus* T7A and *Streptomyces badius* 252. *Appl. Biochem. Biotech.* **28/29:** 433–443.
29. Valli, K. and M. H. Gold. 1991. Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. *J. Bacteriol.* **173:** 345–352.
30. Varela, E., A. T. Martinez, and M. J. Martinez. 2000. Southern blot screening for lignin peroxidase and aryl-alcohol oxidase genes in 30 fungal species. *J. Biotechnol.* **83:** 245–251.