JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Glutathione-Dependent Biotransformation of the Fungicide Chlorothalonil

YOUNG-MOG KIM,† KUNBAWUI PARK,§ GIL-JAE JOO,† EUN-MO JEONG,#
JANG-EOK KIM,§ AND IN-KOO RHEE*,§

Institute of Agricultural Science and Technology and Department of Agricultural Chemistry, Kyungpook National University, Daegu 702-701, South Korea, and National Agricultural Products Quality Management Service, Daegu 702-010, South Korea

A gene responsible for the chlorothalonil biotransformation was cloned from the chromosomal DNA of *Ochrobactrum anthropi* SH35B, capable of efficiently dissipating the chlorothalonil. The gene encoding glutathione *S*-transferase (GST) of *O. anthropi* SH35B was expressed in *Escherichia coli*, and the GST was subsequently purified by affinity chromatography. The fungicide chlorothalonil was rapidly transformed by the GST in the presence of glutathione. LC-MS analysis supported the formation of mono-, di-, and triglutathione conjugates of chlorothalonil by the GST. The monoglutathione conjugate was observed as an intermediate in the enzymatic reaction. The triglutathione conjugate has not been previously reported and seems to be the final metabolite in the biotransformation of chlorothalonil. The glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST is reported.

KEYWORDS: Chlorothalonil; glutathione *S*-transferase; biotransformation

INTRODUCTION

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum chlorinated fungicide, which is highly efficient against the pathogens that infect mainly vegetables, fruits, and other crops (*1*). Although chlorothalonil is not highly toxic to mammals, it is extremely toxic to fish (*2*) and is classified in the B2 group, which is considered as a probable human carcinogen, by the U.S. Environmental Protection Agency (U.S. EPA) due to the carcinogen hexachlorobenzene that is produced as a byproduct during synthesis of the active ingredient (*3*). To remove toxic organic compounds such as pesticides, both biological and chemical treatments have been suggested. A biological treatment of the toxic organic compounds (bioremediation), using microorganisms or enzymes produced from the microorganisms or plants, is often considered as an environmentally favorable method (*4*−*9*). To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by chlorothalonil.

Ochrobactrum anthropi SH35B, capable of efficiently biotransforming the fungicide chlorothalonil, was isolated from soil. A gene responsible for the chlorothalonil biotransformation was cloned from the strain (*10*). The gene was determined to be an open reading frame (ORF) for the glutathione *S*-transferase (GST) by the nucleotide sequence. It has been known that the GST catalyzes the conjugation of the glutathione sulfur atom to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin, resulting in detoxification (*11*, *12*). Therefore, the GST might be involved in the detoxification of the fungicide chlorothalonil.

The experiment reported here was performed to investigate the mechanism of the biotransformation of chlorothalonil by *O. anthropi* SH35B. In this study, we report the glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST, which was expressed in *Escherichia coli* and purified by affinity chromatography.

MATERIALS AND METHODS

Chemicals. Chlorothalonil was purchased from Wako Pure Chemicals (Osaka, Japan) and was prepared by dissolving it in dimethyl sulfoxide (DMSO). All other reagents were of reagent grade and purchased from commercial sources.

Expression and Purification of *O. anthropi* SH35B GST. To construct an expression plasmid for the GST of *O. anthropi* SH35B, a Polymerase Chain Reaction (PCR) was carried out using two synthetic oligonucleotides based on the ORF (GenBank accession no. AY378173) to generate a unique *Eco*RI and *Bam*HI restriction enzyme site, respectively: (forward) 5′-CGGAATTCATCGAAACTGATGGGAG-3′, and (reverse) 5′-GCGGATCCTTAGTTCAGGCCTTC-3′. The PCR product was digested by *Eco*RI−*Bam*HI restriction enzyme and then ligated into pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which had been digested with *Eco*RI−*Bam*HI. The resulting plasmid was designated pTOaGST. *E. coli* JM105 cells, transformed with pTOaGST, were grown overnight at 37 °C in Luria−Bertani medium (LB) containing 100 μg/mL ampicillin. After overnight culture, the cells were diluted 50-fold into a fresh medium and grown to an A_{600} of 0.6, at

* Corresponding author (telephone 82-53-950-5718; fax 82-53-953-7233; e-mail ikrhee@knu.ac.kr).
† Institute of Agricultural Science and Technology, Kyungpook National University.
§ Department of Agricultural Chemistry, Kyungpook National University.
# National Agricultural Products Quality Management Service.

10.1021/jf040047u CCC: $27.50
Published on Web 05/27/2004

which point the GST expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional 6 h. After harvesting, the cells were resuspended in 100 mM potassium phosphate buffer (pH 6.5) and disrupted by sonication. The unbroken cells were removed by centrifugation at 100000*g* for 10 min, and the supernatant was taken for purification. Purification of the GST was carried out using GST Bind Kits according to the recommendations of the manufacturer (Novagen, Madison, WI). Protein purity was tested by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining. GST activity was measured as described previously (*13*), and the protein content was determined according to the Bradford method (*14*).

Biotransformation of Chlorothalonil by the GST. To investigate the biotransformation of chlorothalonil by the purified GST, 0.5 mL of a buffer solution (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated at 25 °C in the presence and absence of the purified GST (1 μg/mL) and 5 mM reduced glutathione. At a specific time, the enzymatic reaction was stopped by the addition of 60 μL of 20% trichloroacetic acid. The mixture was extracted with the same volume of hexane for the detection of the remaining chlorothalonil and with the same volume of methanol for the metabolites that resulted from the enzymatic reaction. The extract was filtered through a 0.45 μm membrane filter and analyzed by high-performance liquid chromatography (HPLC).

Analytical Methods. The content of the remaining chlorothalonil was determined using a Younglin-M930 HPLC (Younglin Co., Seoul, South Korea) equipped with a Waters μBondapak C_{18} column (3.9 × 150 mm; Waters Co., Milford, MA). An elution of water/acetonitrile (2:3) was used at a flow rate of 1 mL/min for 10 min. The eluate was monitored at 254 nm using a M720 UV−visible absorbance detector (Younglin Co.).

To isolate and identify the metabolites that formed from the biotransformation of chlorothalonil, liquid chromatography−mass spectral (LC-MS) analyses were performed using an Agilent 1100 LC/MSD mass spectrometer equipped with an Agilent 1100 HPLC system and a diode array detector (LC-DAD; Agilent Technologies, Palo Alto, CA). The mass spectrometer was operated in the positive atmosphere pressure ionization electrospray (API-ES) mode with the capillary exit voltage at 100 V, the high-energy dynode at 10 kV, and the multiplier at 2.1 kV. Full scans were acquired from *m*/*z* 50 to 1200 at 1.67 scans/s. Agilent chemstation software was used to collect concurrent LC-DAD and positive API-ES data. Chlorothalonil metabolites were purified using a Zorbax C_{18} column (4.6 × 150 mm; i.d., 3.5 μm; Agilent Technologies). For the isolation of chlorothalonil, metabolites **A** and **B**, a linear gradient elution of 60% water with 1% acetic acid/40% methanol (v/v) to 100% methanol was used at a flow rate of 0.7 mL/min for 45 min. For the isolation of metabolite **C**, the mobile phase was changed to a flow rate of 0.4 mL/min.

RESULTS AND DISCUSSION

Purification and Characterization of *O. anthropi* SH35B GST. Bacterial GSTs are present in very low amounts (*15*), resulting in limited study of their physical/chemical properties. Therefore, the first step is overproduction of the enzyme to study the properties of the *O. anthropi* SH35B GST. The GST was overexpressed and purified as described under Materials and Methods. An increase in the intensity of the band corresponding to ~22 kDa, which corresponds to the molecular mass calculated from its gene, was observed (**Figure 1**, lane 1). The GST purified by the GST Bind Kits also appeared at the same molecular mass (**Figure 1**, lane 2). The specific activity of the purified protein increased 16-fold compared with that of the crude enzyme. A total of 0.12 mg of purified protein from ~1.2 mg of crude extract was obtained.

Biotransformation of Chlorothalonil by the GST. In plants, the GST activity is an important indicator for determining the resistance to various 2-chloroacetanilide herbicides (*16*). The conjugation with glutathione, to displace chlorine by the thiol group of glutathione, has been recognized as a major detoxification pathway in plants. Recently, it was also reported that the drug-hypersensitive *E. coli* KAM3 cells (*17*), into which the GST gene was transferred, showed elevated levels of resistance to chlorothalonil, suggesting that the GST may be involved in the detoxification of the fungicide chlorothalonil (*10*). On the basis of the above results, it was hypothesized that chlorothalonil will be detoxified via the mechanism of the conjugation with glutathione catalyzed by the intracellular GST in *O. anthropi* in vivo. To verify our hypothesis, the biotransformation of chlorothalonil by the GST was investigated in the presence of glutathione in vitro. The chlorothalonil content in the reaction mixture rapidly decreased, and a negligible amount of chlorothalonil was observed at 30 min after the reaction (**Figure 2**). A control experiment was also performed to determine whether GST or glutathione affected the dissipation of chlorothalonil. To some extent, chlorothalonil dissipation (10−15%) was observed due to the nonenzymatic conjugation with the thiol group (*18*) and the interaction (adsorption) between the chlorothalonil and GST (active GST and denatured GST by boiling) in the absence of glutathione. However, there was no significant difference in the chlorothalonil biotransformation effect among thiol compounds tested (cysteine, reduced glutathione, and β-mercaptoethanol; data not shown). Thus, the

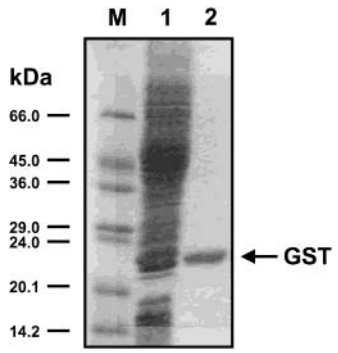

Figure 1. Expression and purification of glutathione *S*-transferase (GST) from *O. anthropi* SH35B. *E. coli* JM105/pTOaGST cells were induced with 1 mM IPTG for 6 h and harvested. Crude enzymes were prepared from the cells. GST was purified as described under Materials and Methods. M, standard protein marker; lane 1, crude enzymes; lane 2, purified GST.

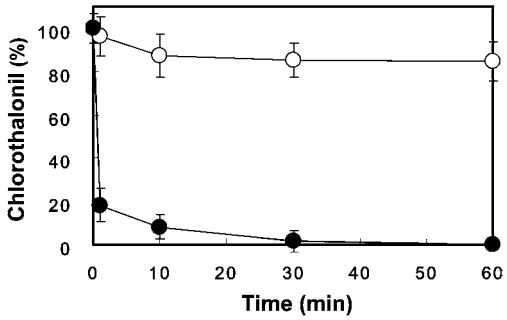

Figure 2. Biotransformation of chlorothalonil by glutathione *S*-transferase (GST) in the presence of glutathione: (○) chlorothalonil alone; (●) chlorothalonil in the presence of 5 mM glutathione and purified GST. Half a milliliter of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of purified GST (1 μg/mL) and 5 mM glutathione. The reaction was performed at 25 °C and stopped by the addition of 20 μL of 20% trichloroacetic acid at the indicated times. The amount of chlorothalonil was analyzed by HPLC as described under Materials and Methods.

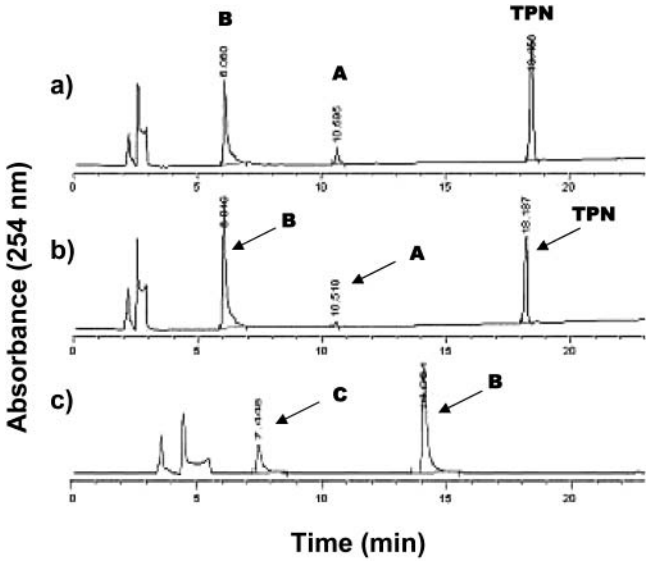

Figure 3. HPLC elution profile of chlorothalonil (TPN) and metabolites **A**–**C** formed from the enzymatic reaction of glutathione *S*-transferase. Metabolites **A**–**C** were isolated as described under Materials and Methods. The enzymatic reaction was performed for 30 s (**a**), for 5 min (**b**), and for 30 min (**c**).

chlorothalonil was mainly dissipated by a mechanism of the conjugation with glutathione catalyzed by the GST.

Identification of Chlorothalonil Metabolites. HPLC analysis showed that chlorothalonil was metabolized to three metabolites (**A**−**C**) by the GST in the presence of glutathione (**Figure 3**). The formation of the metabolites was dependent upon the presence of glutathione, incubation time, and the GST concentration (data not shown). After short incubation periods (<30 s), metabolites **A** and **B** were observed as major metabolites (**Figure 3a**). After 5 min of the reaction, a negligible content of metabolite **A** was detected and mainly metabolite **B** was formed (**Figure 3b**), suggesting that metabolite **A** is an intermediate in the biotransformation of chlorothalonil. After 30 min of reaction, only metabolites **B** and **C** were detected as major products in the enzymatic reaction (**Figure 3c**). The content of metabolite **C** was apt to enhance with an increase of the reaction time; however, metabolite **B** did not completely convert to metabolite **C** under the current conditions, even though the reaction time was increased to 24 h (data not shown). The retention time of metabolite **B** was at ∼6 min in **Figure 3a**,**b** and at ∼14 min in **Figure 3c** due to the use of different HPLC condition as described under Materials and Methods. No other HPLC peak was detected.

It was proposed that metabolites **A**−**C** are glutathione conjugates because they originated from the reaction catalyzed by the purified GST in the presence of glutathione. Considering the different elution times of the metabolites (**Figure 3**), metabolites may differ in the number of glutathione conjugations, because hydrophathy changes with increasing conjugation. A compound with more conjugations generally elutes more quickly than a less conjugated compound due to increased hydrophilicity under the HPLC separation conditions.

To clarify this hypothesis, a LC-MS analysis was performed as described under Materials and Methods. The metabolites were identified by comparing the electron impact mass fragmentation patterns with those of the expected reaction molecules. The mass spectrum of chlorothalonil showed sodiated molecules at *m*/*z* 289 $[M + Na]^+$ (**Figure 4a**). The mass spectrum of metabolite **A** consisted of molecules at *m*/*z* 536 [M − GS] and protonated

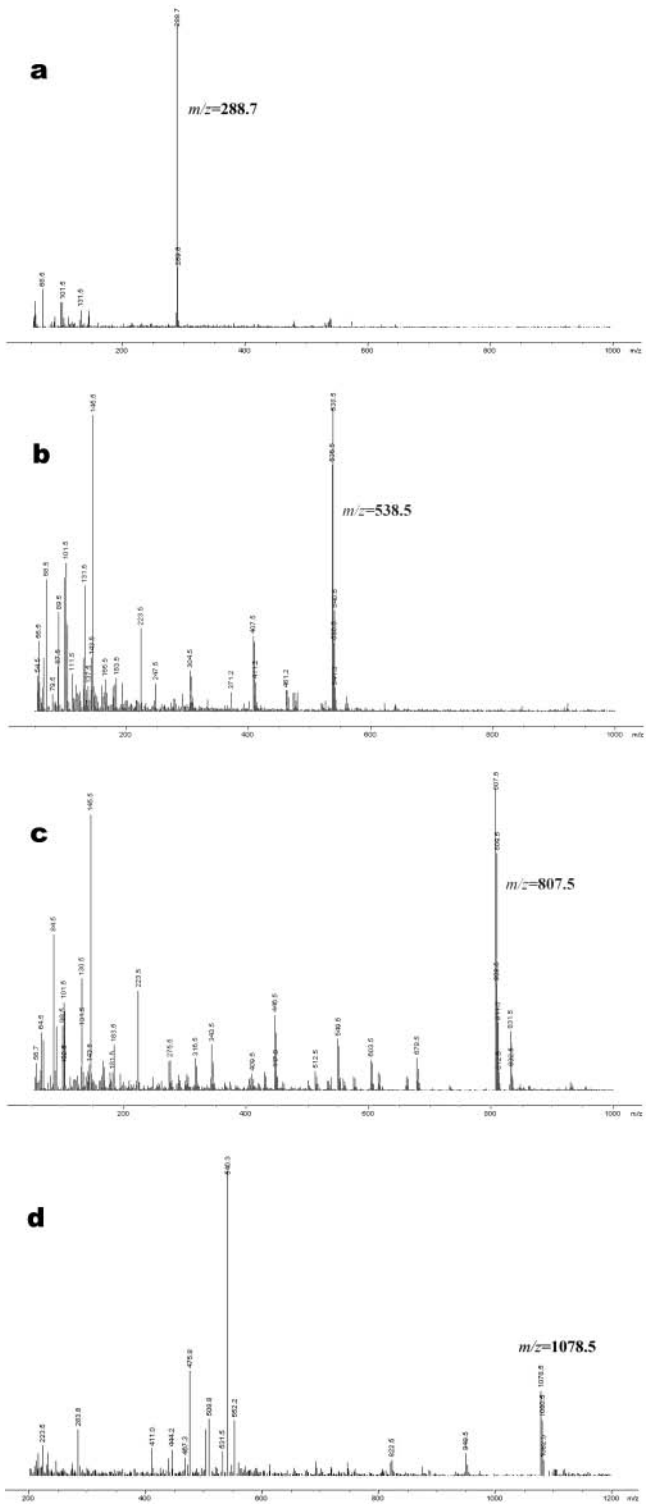

Figure 4. Mass spectra of chlorothalonil (**a**) and its metabolites formed from the enzymatic reaction of glutathione *S*-transferase: (**b**) metabolite **A**; (**c**) metabolite **B**; (**d**) metabolite **C**.

molecules at *m*/*z* 538 $[M - GS + 2H]^+$ (**Figure 4b**), which were consistent with the structure of the one-glutathione conjugated to chlorothalonil after the loss of chlorine. The mass spectrum of metabolite **B** consisted of molecules at *m*/*z* 807 [M − 2GS] and protonated molecules at *m*/*z* 809 $[M - 2GS + 2H]^+$ (**Figure 4c**), suggesting that metabolite **B** is the structure of the two-glutathione conjugated to chlorothalonil after the loss of two chlorines. The ion detected at *m*/*z* 831 was consistent with the sodiated molecules at *m*/*z* 807 (**Figure 4c**). The mass

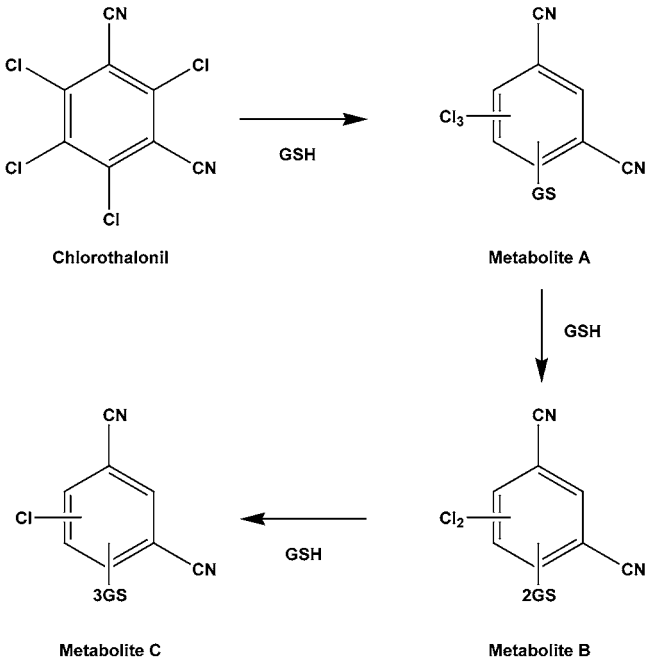

Figure 5. Proposed pathway of glutathione-dependent biotransformation of chlorothalonil catalyzed by glutathione *S*-transferase. GSH indicates glutathione.

spectrum of metabolite **C** showed a peak at *m*/*z* 1078 corresponding to the structure of the triglutathione conjugate [M − 3GS] and a peak at *m*/*z* 1080 corresponding to the protonated molecular ion $[M - 3GS + 2H]^+$ (**Figure 4d**). However, the major peak was detected at *m*/*z* 540 (**Figure 4d**), not at *m*/*z* 1078 or 1080 in the mass spectrum of metabolite **C**. We attributed this to cleavage of the protonated molecule ion at *m*/*z* 1080. Considering the results from the HPLC elution and the mass spectrum profiles, metabolite **C** was proposed to be the structure of the three-glutathione conjugated to chlorothalonil. No other conceivable metabolites of chlorothalonil were detected.

Although identification of the reaction products in this study was tentative, it appeared to be reasonable. On the basis of the results obtained in this study, a reaction pathway for the glutathione-dependent biotransformation of chlorothalonil catalyzed by the GST is proposed (**Figure 5**). In the enzymatic reaction, the one-glutathione conjugate was formed initially, but it seems to be a substrate for the GST, resulting in the substitution of a second chlorine atom to give the two-glutathione conjugate. The two-glutathione conjugate also seems to be a substrate for the enzymatic reaction to produce the three-glutathione conjugate, which appears to be the final metabolite in the glutathione-dependent biotransformation of chlorothalonil.

A similar mechanism has been discussed for the biotransformation of chlorothalonil in the rat liver cytosol, and two metabolites, 4-(glutathion-*S*-yl)-2,5,6-trichloroisophthalonitrile and 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophthalonitrile, were reported (*19*). Metabolite **B** reported here showed the same UV spectrum profiles, absorption maxima at 264, 309, and 340 nm, as 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophthalonitrile. Indeed, 4-(glutathion-*S*-yl)-2,5,6-trichloroisophthalonitrile was also reported as an intermediate (*19*) such as metabolite **A** reported in this study. Thus, the structures of metabolites **A** and **B** correspond to 4-(glutathion-*S*-yl)-2,5,6-trichloroisophthalonitrile and 4,6-bis(glutathion-*S*-yl)-2,5-dichloroisophthalonitrile, respectively. To our knowledge, the triglutathione conjugate (metabolite **C**) has not been previously reported.

In general, the formation of glutathione conjugates is associated with the detoxification of xenobiotics (*11*, *12*). The glutathione conjugates represent the biotransformation pathway for chlorothalonil in *O. anthropi*, resulting in the detoxification of chlorothalonil. Thus, we believe the results reported in this study are the first evidence on the glutathione-dependent biotransformation of chlorothalonil catalyzed by the bacterial GST. Several questions, however, are still unanswered: Which compound is the final metabolite in the biotransformation of chlorothalonil, and how is the final glutathione conjugate excreted from the cells? It has been reported that glutathione conjugates formed in the liver are efficiently excreted in the bile due to a high-affinity transport for glutathione conjugates in the canalicular membrane (*20*). The glutathione conjugate reported in this study is also likely excreted by a specific transporter after several enzymatic reactions such as γ-glutamyl transpeptidase in vivo (*21*). To address these issues, it will be necessary to confirm a structure that results from the conjugation with glutathione in the bacterial medium.

ACKNOWLEDGMENT

We thank Dr. T. Tsuchiya of the Faculty of Pharmaceutical Science at Okayama University, Japan, for the gift of *E. coli* KAM3.

LITERATURE CITED

(1) Spencer, J. R.; Bissell, S. R.; Sanborn, J. R.; Schneider, F. A.; Margetich, S. S.; Kriger, R. I. Chlorothalonil exposure of workers on mechanical tomato harvesters. *Toxicol. Lett.* **1991**, *55*, 99−107.

(2) Davies, P. E.; White, R. W. G. The toxicology and metabolism of chlorothalonil in fish, I. Lethal levels for *Salmo gairdueri*, *Gclaxias maculatus*, *G. truttaceous* and *G. auratus* and fate of ^{14}C-TCIN in *S. gairdneri*. *Aquat. Toxicol.* **1985**, *7*, 93−105.

(3) Cox, C. Chlorothalonil. *J. Pestic. Reform.* **1997**, *17*, 14−20.

(4) Kim, J. E.; Fernandes, E.; Bollag, J. M. Enzymatic coupling of the herbicide bentazon with humus monomer and characterization of reaction products. *Environ. Sci. Technol.* **1997**, *31*, 2392−2398.

(5) Lee, J. E.; Park, J. W.; Kim, J. E. Use of plant materials for decontamination of wastewater polluted with 2,4-dichlorophenol. *Korean J. Environ. Agric.* **1999**, *18*, 292−297.

(6) Lee, J. E.; Park, J. W.; Kim, J. E. Decontamination of wastewater polluted with phenolic and anilinic compounds using plant materials. *Korean J. Environ. Agric.* **2000**, *19*, 228−233.

(7) Behki, R.; Topp, E.; Dick, W.; Germon, P. Metabolism of the herbicide atrazine by *Rhodococcus* strains. *Appl. Environ. Microbiol.* **1993**, *59*, 1955−1959.

(8) Kilbane, J. J.; Chatterjee, D. K.; Karns, J. S.; Kellogg, S. T.; Chakrabarty, A. M. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia. Appl. Environ. Microbiol.* **1982**, *44*, 72−78.

(9) Levanon, D. Roles of fungi and bacteria in the mineralization of the pesticides atrazine, alachlor, malathion and carbofuran in soil. *Soil Biol. Biochem.* **1993**, *25*, 1097−1105.

(10) Kim, Y. M.; Park, K.; Choi, J. H.; Kim, J. E.; Rhee, I. K. Biotransformation of the fungicide chlorothalonil by bacterial glutathione *S*-transferase. *J. Microbiol. Biotechnol.* **2004**, in press.

(11) Jakobi, W. B.; Ziegler, D. M. The enzymes of detoxification. *J. Biol. Chem.* **1990**, *265*, 20715−20719.

(12) Wilce, M. C. J.; Parker, M. W. Structure and function of glutathione *S*-transferase. *Biochim. Biophys. Acta* **1994**, *1205*, 1−18.

(13) Shin, J. H.; Kim, Y. M.; Park, J. W.; Kim, J. E.; Rhee, I. K. Resistance of *Saccharomyces cerevisiae* to fungicide chlorothalonil. *J. Microbiol.* **2003**, *41*, 219−223.

(14) Bradford, M. M. A rapid and sensitive method for the quantitification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(15) Sheehan, D.; Casey, J. P. Microbial glutathione *S*-transferases. *Comp. Biochem. Physiol. B* **1993**, *104*, 1−6.
(16) Scarponi, L.; Perucci, P.; Martinetti, L. Conjugation of 2-chloroacetanilide herbicides with glutathione: Role of molecular structures and of glutathione *S*-transferase enzymes. *J. Agric. Food Chem.* **1991**, *39*, 2010−2013.
(17) Morita, Y.; Kodama, K.; Shiota, S.; Mine, T.; Kataoka, A.; Mizushima, T.; Tsuchiya, T. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. *Antimicrob. Agents Chemother.* **1998**, *42*, 1778−1782.
(18) Leavitt, J. R. C.; Penner, D. In vitro conjugation of glutathione and other thiols with acetanilide herbicides and EPTC sulfoxide and the action of the herbicide antidote R-25788. *J. Agric. Food Chem.* **1979**, *27*, 533−536.
(19) Rosner, E.; Klos, C.; Dekant, W. Biotransformation of the fungicide chlorothalonil by glutathione conjucation. *Fundam. Appl. Toxicol.* **1996**, *33*, 229−234.
(20) Vore, M. Canalicular transporter: Discovery of ATP-dependent mechanisms. *Appl. Pharmacol.* **1993**, *118*, 2−7.
(21) Lau, S. S.; Jone, T. W.; Sioco, R,; Hill, B. A.; Pinon, R. K.; Monk, T. J. Species differences in renal γ-glutamyl transpeptidase activity do not correlate with susceptibility to 2-bromo-(diglutathion-*S*-yl)-hydroquinone nephrotoxicity. *Toxicology* **1990**, *64*, 291−311.

Received for review February 2, 2004. Revised manuscript received April 12, 2004. Accepted April 13, 2004. This work was supported by a grant from the Korea Research Foundation (KRF2000-005-G00001).

JF040047U