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Chlorothalonil-Biotransformation by Glutathione *S*-Transferase of *Escherichia coli*

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It has recently been reported that one of the most important factors of yeast resistance to the fungicide chlorothalonil is the glutathione contents and the catalytic efficiency of glutathione *S*-transferase (GST) (Shin *et al.*, 2003). GST is known to catalyze the conjugation of glutathione to a wide variety of xenobiotics, resulting in detoxification. In an attempt to elucidate the relation between chlorothalonil-detoxification and GST, the GST of *Escherichia coli* was expressed and purified. The drug-hypersensitive *E. coli* KAM3 cells harboring a plasmid for the overexpression of the GST gene can grow in the presence of chlorothalonil. The purified GST showed chlorothalonil-biotransformation activity in the presence of glutathione. Thus, chlorothalonil is detoxified by the mechanism of glutathione conjugation catalyzed by GST.

Key words: glutathione *S*-transferase, chlorothalonil, biotransformation

One of the most important factors of yeast resistance to the fungicide chlorothalonil is the content of endogenous thiol compounds, mainly glutathione, and the catalytic efficiency of glutathione *S*-transferase (GST) (Shin *et al.*, 2003). Glutathione is an especially abundant non-proteinous thiol compound, found in most aerobic organisms, and has many physiological functions in cells (Meister and Anderson, 1983; Meister, 1985). GST catalyzes the conjugation of the sulfur atom of glutathione to a large variety of electrophilic compounds of both endobiotic and xenobiotic origin, resulting in detoxification (Jakobi *et al.*, 1976; Wilce and Parker, 1994). Therefore, it was hypothesized that GST may be involved in the detoxification (or degradation) of the fungicide chlorothalonil.

Chlorothalonil (2, 4, 5, 6-tetrachloroisophthalonitrile) is a broad-spectrum chlorinated fungicide, but is considered a potential pollutant due to its high application rate, persistence, and toxicity to human and other species. Biological treatment of toxic pollutants (bioremediation), using microorganisms or the enzymes produced from microorganisms, is often considered as an environmentally favorable method. To date, however, there have been no unambiguous reports about the bioremediation of soil contaminated by chlorothalonil and its metabolites.

The objective of current study was to evaluate the possibility of biological treatment for the biotransformation of chlorothalonil. In this paper, the relationship between the biotransformation efficiency of chlorothalonil and the activity of bacterial GST are reported.

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Materials and Methods

Materials

Chlorothalonil was purchased from Wako Pure Chemicals (Japan), and prepared by dissolving in dimethyl sulfoxide (DMSO). It was then added to the medium at the concentrations indicated. For the purification of the GST of *Escherichia coli*, GST bind kits were used (Novagen, USA). All other reagents were of reagent grade and purchased from commercial sources.

Construction of expression vector and purification

In order to construct an expression plasmid for the GST of *E. coli*, a PCR was carried out using two synthetic oligonucleotides based on the report of Nishita *et al.* (1994): (sense) 5'-GCGAATTCATGAAATTGTTCTACAAACCG-3' and (antisense) 5'-GCGGATCCTTACTTTAAGCCTTCCGC-3'. The PCR was carried out using intact *E. coli* DH5α cells as a template. The thermal profile used was 25 cycles consisting of denaturation of 1 min at 94°C, annealing of 1 min at 55°C, and extension of 1 min at 72°C. A final extension step consisting of 5 min at 72°C was included. The PCR product was digested by *Eco*RI-*Bam*HI restriction enzymes and then ligated into pTrc99A (Pharmacia Biotech, USA), which had been digested with *Eco*RI-

*Bam*HI. The nucleotide sequence of the inserted DNA was determined by DNA sequencing (Sanger *et al.*, 1977). The nucleotide sequences reported here were registered in the GenBank database, accession number AE000259. The resulting plasmid was designated pTEcGST. *E. coli* JM105 cells (Reha-Krantz, 1985) harboring pTEcGST were grown overnight at 37°C in Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin. After overnight culture, the cells were diluted 50-fold into fresh medium and grown to an A_{600} of 0.6, at which point the GST expression was induced by the addition of 1 mM IPTG and incubated for an additional 6 h. After harvesting, the cells were resuspended in 100 mM potassium phosphate buffer (pH 6.5) and sonicated 3 times for 30 sec at 95 μA with an ultrasonicator (Ultrasonic Ltd., UK). The unbroken cells were removed by centrifugation at 100,000×g for 10 min, and the supernatant was taken for purification. The purification of the GST was carried out using GST bind Kits, according to the recommendations of the manufacturer (Novagen, USA). The crude extracts and each fraction throughout the purification procedures were collected and analyzed by SDS-PAGE. The proteins were stained with Coomassie Brilliant blue (CBB) R-250. The GST activity was measured as described previously (Shin *et al.*, 2003), and the protein content was determined by the Bradford method (1976).

Effect of the GST gene on the cell growth

To investigate the effect of the GST on cells growth, *E. coli* KAM3 cells, which lack major multi-drug efflux pumps (Δ*acrB*) (Morita *et al.*, 1998), harboring pTEcGST were grown on M9 minimal medium (Sambrook *et al.*, 1989) agar plates containing 1% glucose, 0.2 mM IPTG and 0.37 mM chlorothalonil, and then incubated at 37°C for 3 days.

The biotransformation of chlorothalonil by GST

To investigate the biotransformation of the chlorothalonil by the purified GST, 500 μl of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence or absence of the purified GST (1 μg/ml) and 5 mM reduced glutathione. The reaction was performed for 60 min at 25°C and stopped by the addition of 20 μl of 20% trichloroacetic acid. The remaining chlorothalonil in the reaction mixture was extracted with the same volume of hexane. The extract was evaporated and then dissolved in acetonitrile, prior to analysis. To determine the chlorothalonil in the reaction mixture, high performance liquid chromatography (HPLC) analysis was performed, using a μBondapak C_{18} column (3.9×150 mm; Waters, USA) with a eluent of water : acetonitrile (4 : 6) for 10 min at a flow rate of 1 ml/min. The HPLC effluent was detected in series by UV monitoring at 235 nm.

To investigate the effect of the biotransformation of chlorothalonil by thiol compounds, several thiol compounds were added to a reaction buffer containing 0.37 mM chlorothalonil. The reaction and analysis conditions were as described above.

Results and Discussion

Expression and purification of the E. coli GST

The *E. coli* GST is present at very low amounts in the cells, similarly to other bacterial GSTs (Sheehan and Casey, 1993; Nishita *et al.*, 1994), which limits the study of the physical/chemical properties. Therefore, the first step involved the overproduction of the enzyme to enable the study of the bacterial GST properties. For this overproduction, a gene encoding the *E. coli* GST was subcloned into an IPTG inducible expression vector, pTrc99A, as described in Materials and Methods. The overproduced and purified enzymes were monitored by CBB staining of SDS-PAGE gels (Fig. 1). After induction, an increase in the intensity of the band corresponding to about 22.8 kDa was observed, which corresponded to the molecular mass calculated from the deduced amino acid sequences of the gene (Fig. 1, lane 1), but no band was detected in the control cells, *E. coli* JM105/pTrc99A (data not shown). Thus, it was concluded that the 22.8 kDa band represented the overproduced GST. The overproduced GST was purified using the GST bind kits. The purified GST band had an apparent molecular mass near

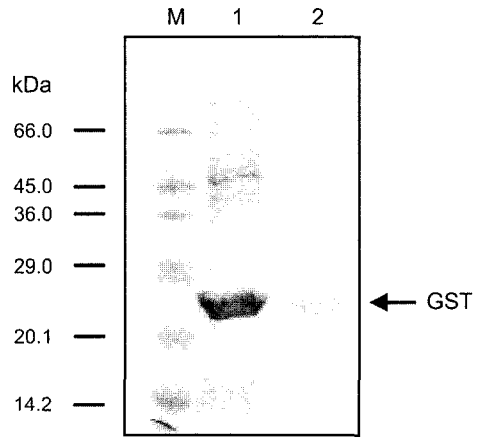

Fig. 1. The expression and purification of GST from *Escherichia coli*. *E. coli* JM105/ pTEcGST cells were induced with 1 mM IPTG for 6 h and harvested. The GST was purified as described in Materials and Methods. M, standard protein marker; lane 1, crude enzymes; lane 2, purified GST.

Table 1. Purification of glutathione *S*-transferase

Fraction	Protein (mg)	Specific activity (μmol/min/mg protein)	Purification rate (fold)
Crude enzyme	0.89	0.31±0.03*	1
Eluate	0.13	2.53±0.31*	8

*The data are the averages of triplicate experiments.

22.8 kDa, similar to the crude enzyme fraction (Fig. 1, lane 2). Table 1 summarizes the GST purification. The specific activity of the purified protein increased 8-fold compared to that of the crude enzyme. 0.15 mg of purified protein was obtained from about 1 mg of crude extract.

Enhancement of cell growth by GST

Although several bacterial GSTs have been cloned and purified (Di Ilio *et al.*, 1988; Di Ilio *et al.*, 1991; Di Ilio *et al.*, 1993; Nishita *et al.*, 1994), little is known about their biological functions, structure, and regulation. It was recently reported that one of the most important factors of yeast resistance to the fungicide chlorothalonil is the content of endogenous thiol compounds, especially glutathione, and the catalytic efficiency of GST (Shin *et al.*, 2003). Therefore, it was presumed that GST may be involved in the detoxification of the fungicide chlorothalonil. To investigate this possibility, *E. coli* KAM3 cells (Morita *et al.*, 1998), which are not able to grow on M9 minimal medium agar plates containing 1% glucose, 0.2 mM IPTG and 0.37 mM chlorothalonil, were transformed with the plasmid, pTEcGST, and incubated for 3 days at 37°C. As shown in Fig. 2, normal growth was observed on the agar plate of the *E. coli* KAM3 cells transformed with pTEcGST, but not of the control cells with the vector plasmid. Thus, the drug-hypersensitive *E. coli* KAM3 cells transformed with the GST gene showed elevated levels of resistance to chlorothalonil, suggesting that GST may be involved in the detoxification of the chlorothalonil.

The biotransformation of chlorothalonil by GST

In plants, the GST activity is an important factor in determining the resistance to various 2-chloroacetanilide herbicides (Scarponi *et al.*, 1991). Conjugation with glutathione, to displace chlorine by the thiol group of glutathione, has been recognized as a major detoxification pathway in plants. Glutathione is an especially abundant non-proteinous thiol compound found in most aerobic organisms, and has many physiological functions in cells (Meister and Anderson, 1983; Meister, 1985). Based on the reports and our results of Fig. 2, it was hypothesized that chlorothalonil is detoxified via a mechanism of glutathione *S*-conjugate formation catalyzed by intracellular GST. In order to test our hypothesis, the biotransformation of chlorothalonil by the purified GST was investigated *in vitro* in the presence of glutathione. As shown in Fig. 3, the biotransformation of chlorothalonil by GST was observed in the presence of glu-

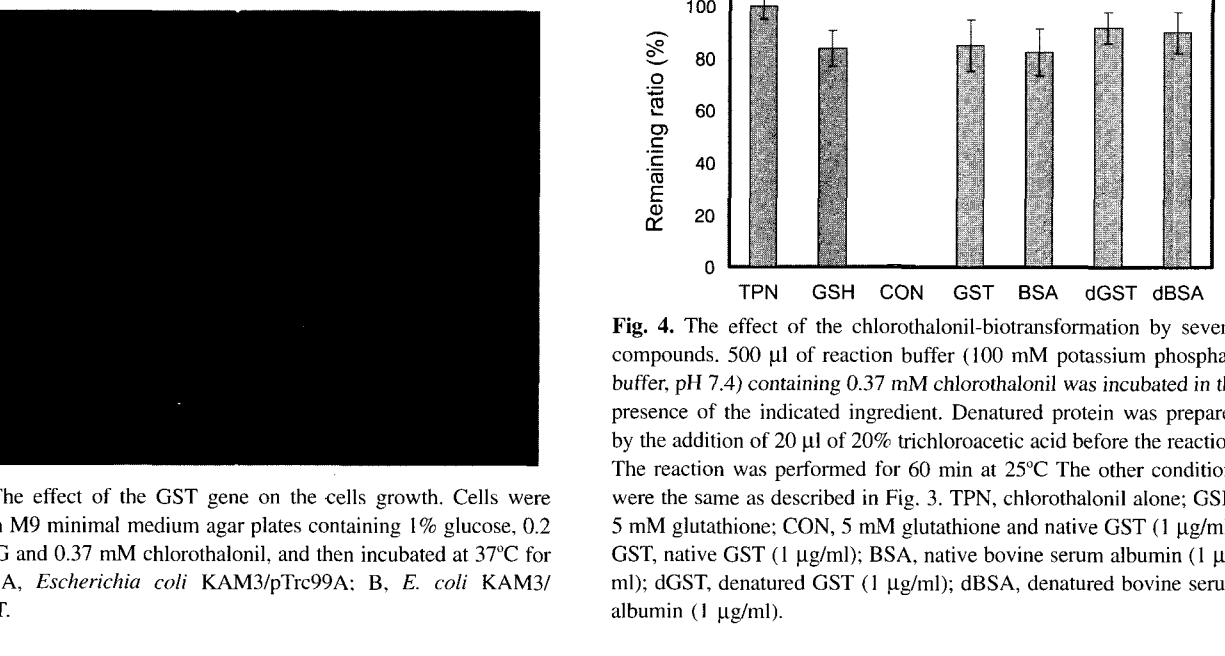

Fig. 2. The effect of the GST gene on the cells growth. Cells were grown on M9 minimal medium agar plates containing 1% glucose, 0.2 mM IPTG and 0.37 mM chlorothalonil, and then incubated at 37°C for 3 days. A, *Escherichia coli* KAM3/pTrc99A; B, *E. coli* KAM3/pTEcGST.

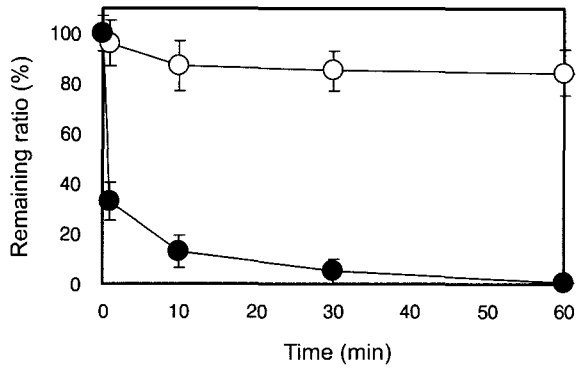

Fig. 3. The biotransformation of chlorothalonil by GST in the presence of glutathione. 500 μl of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of the purified GST (1 μg/ml) and 5 mM glutathione. The reaction was performed at 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 in Materials and Methods. ○-○, chlorothalonil alone; ●-●, in the presence of 5 mM glutathione and the purified GST.

Fig. 4. The effect of the chlorothalonil-biotransformation by several compounds. 500 μl of reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil was incubated in the presence of the indicated ingredient. Denatured protein was prepared by the addition of 20 μl of 20% trichloroacetic acid before the reaction. The reaction was performed for 60 min at 25°C The other conditions were the same as described in Fig. 3. TPN, chlorothalonil alone; GSH, 5 mM glutathione; CON, 5 mM glutathione and native GST (1 μg/ml); GST, native GST (1 μg/ml); BSA, native bovine serum albumin (1 μg/ml); dGST, denatured GST (1 μg/ml); dBSA, denatured bovine serum albumin (1 μg/ml).

tathione. The chlorothalonil content in the reaction mixture rapidly decreased. No chlorothalonil was detected after 60 min of incubation at 25°C (Fig. 3). An additional experiment was performed to determine whether protein or glutathione affected the dissipation of chlorothalonil (Fig. 4). In a reaction mixture containing only glutathione, a degree of chlorothalonil-dissipation was observed, due to the nonenzymatic conjugation of the thiol group of glutathione (Leavitt and Penner, 1979). The effect of the interaction (adsorption) between chlorothalonil and protein was investigated. The proteins tested (native GST, denatured GST, native bovine serum albumin, and denatured bovine serum albumin) did not significantly affect the dissipation of the chlorothalonil, and there were no significant difference among the proteins tested (Fig. 4). Thus, the chlorothalonil was mainly dissipated with the conjugation of the glutathione, which was catalyzed by GST.

The effect of thiol compounds on the chlorothalonil-biotransformation

The growth of *Saccharomyces cerevisiae* was recently reported to be restore by the addition of several thiol compounds when the cells were cultured in the presence of chlorothalonil (Shin *et al.*, 2003). It was presumed that the thiol compounds could be involved in reducing or removing the toxicity caused by the chlorothalonil. In order to test our hypothesis, the biotransformation rate of chlorothalonil, caused by several thiol compounds, was investigated. Several thiol compounds (cysteine, reduced glutathione, and β-mercaptoethanol) were added to a reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil. Fig. 5 showed the positive effects of the thiol compounds on the chlorothalonil-biotransformation in the presence of the purified GST. However, there was no significant difference in the chlorothalonil-biotransformation effect among the thiol compounds tested, which was consistent with our recent

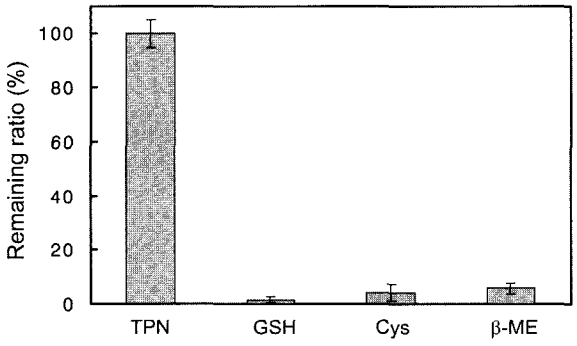

Fig. 5. The effect of thiol compounds on the chlorothalonil-biotransformation. Thiol compounds were added to a reaction buffer (100 mM potassium phosphate buffer, pH 7.4) containing 0.37 mM chlorothalonil, and the reaction then performed in the presence of GST (1 μg/ml) for 60 min at 25°C The other conditions were the same as described in Fig. 3. TPN, chlorothalonil alone; GSH, 5 mM glutathione; Cys, 5 mM L-cysteine; β-ME, 5 mM β-mercaptoethanol.

results obtained in yeasts (Shin *et al.*, 2003). A degree of chlorothalonil-dissipation was also observed in the absence of GST by thiol compounds such as glutathione (data not shown). The above result was supposed to be due to the nonenzymatic conjugation of the thiol group (Leavitt and Penner, 1979).

The results presented in this paper suggest that chlorothalonil was detoxified by conjugation with the cellular free thiol groups, especially glutathione, and catalyzed by bacterial GST. The structure of glutathione *S*-conjugates formed by the bacterial GST has, however, not been elucidated. In order to study these issues in more detail, it will be necessary to confirm the conjugate structures and elucidate the pathway of the chlorothalonil metabolism.

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References

Bradford, M.M. 1976. A rapid and sensitive method for the quantitification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248-254.

Di Ilio, C., A. Aceto, R. Piccolomini, N. Allocati, A. Faraone, L. Cellini, G. Ravagnan, and G. Federici. 1988. Purification and characterization of three forms of glutathione transferase from *Proteus mirabilis*. *Biochem. J.* 255, 971-975.

Di Ilio, C., A. Aceto, R. Piccolomini, N. Allocati, A. Faraone, T. Bucciarelli, D. Barra, and G. Federici. 1991. Purification and characterization of a novel glutathione transferase from *Serratia marcescens*. *Biochim. Biophys. Acta* 1077, 141-146.

Di Ilio, C., A. Aceto, N. Allocati, R. Piccolomini, T. Bucciarelli, B. Dragani, A. Faraone, P. Sacchetta, R. Petruzzelli, and G. Federici. 1993. Characterization of glutathione transferase from *Xanthomonas campestris*. *Arch. Biochem. Biophys.* 305, 110-114.

Jakobi, W.B., W.H. Habig, J.H. Keen, J.N. Ketley, and M.J. Pabst. 1976. Glutathione *S*-transferases: Catalytic aspects. In: Glutathione: Metabolism and Function. pp. 189-211. Raven Press, New York.

Leavitt, J.R.C. and D. Penner. 1979. 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.* 27, 533-536.

Meister, A. 1985. Methods for the selective modification of glutathione metabolism and study of glutathione transport. *Methods Enzymol.* 113, 571-583.

Meister, A. and M. E. Anderson. 1983. Glutathione. *Annu. Rev. Biochem.* 52, 711-760.

Morita, Y., K. Kodama, S. Shiota, T. Mine, A. Kataoka, T. Mizushima, and T. Tsuchiya. 1998. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. *Antimicrob. Agents Chemother.* 42,1778-1782.

Nishida, M., K.H. Kong, H. Inoue, and K. Takahashi. 1994. Molecular cloning and site-directed mutagenesis of glutathione *S*-

transferase from *Escherichia coli*. The conserved tyrosyl residue near the N terminus is not essential for catalysis. *J. Biol. Chem.* 269, 32536-32541.

Reha-Krantz L.J. 1985. The *Escherichia coli* strain JM105 contains partial *supE* activity. *Gene* 38, 275-276.

Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.

Scarponi, L., P. Perucci, and L. Martinetti. 1991. Conjugation of 2-chloroacetanilide herbicides with glutathione: Role of molecular structures and of glutathione *S*-transferase enzymes. *J. Agric. Food. Chem.* 39, 2010-2013.

Sheehan, D. and J.P. Casey. 1993. Microbial glutathione *S*-transferases. *Comp. Biochem. Physiol. B.* 104, 1-6.

Shin, J.H, Y.M. Kim, J.W. Park, J.E. Kim, and I.K. Rhee. 2003. Resistance of *Saccharomyces cerevisiae* to fungicide chlorothalonil. *J. Micobiol.* 41, 219-223.

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