Glutathione Transferase from *Mucor javanicus*Kazunori ANDO, Mamoru HONMA, Seiya CHIBA,
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Glutathione transferase was purified from *Mucor javanicus* and characterized as a dimer of identical subunits (M_r ; 22,000). Kinetics and K_m values of *M. javanicus* glutathione transferase, 0.48 mM and 1.0 mM (at pH 6.5) for glutathione and 1-chloro-2,4-dinitrobenzene, are close to those of rat liver enzyme, but occurrence of isoenzymes was not recognized in *M. javanicus*. The best substrate of *M. javanicus* glutathione transferase was 1-chloro-2,4-dinitrobenzene. On the other hand, 2,3-, 2,4-, 2,5-, and 3,4-dichloronitrobenzenes were converted to the related conjugates by 0.17 to 0.35% of the velocity toward the best substrate. This is correlated to the previous result that 2,3- and 2,4-dichloronitrobenzenes were metabolized to 2-methylthio derivatives by the incubation with *M. javanicus*.

Previous papers^{1,2)} based on interest in the degradation of pesticides in soil showed that fungi including *Mucor javanicus* metabolized several dichloronitrobenzenes to form dichlorobenzamines and chloro-methylthio-nitrobenzenes, and discussed possible mechanisms for the methylthio compound formation. Recent evidence^{3,4)} showed the possibility that the methylthio compounds were formed through glutathione conjugates as products of glutathione transferase (EC 2.5.1.18). Glutathione transferase is regarded as an important enzyme in xenobiotic metabolism and its existence was shown in rat,⁵⁾ mouse,⁶⁾ bovine,⁷⁾ monkey,⁸⁾ and human⁹⁾ liver, house fly,¹⁰⁾ a bacterium (*Escherichia coli*),¹¹⁾ and fungi (*Cunninghamella elegans* and *Fusarium*).^{12,13)} Especially, the rat liver enzymes were characterized as a dimer of 3 kinds of subunits (M_r : 22,000, 23,000, and 25,000).¹⁴⁾ Purification and properties^{5,15~19)} of these different forms of the enzyme and the amino acid sequence^{20,21)} of one subunit were reported. Knowledge about the enzymes of microorganisms is less than about the animal enzymes.

This paper describes the purification of the glutathione transferase from a fungus, *Mucor*

javanicus, and its reaction toward several substrates. *M. javanicus* glutathione transferase catalyzed the formation of glutathione conjugates of several dichloronitrobenzenes including ones metabolized to chloromethylthionitrobenzenes by *M. javanicus*.

METHODS

Materials. The fungus, *Mucor javanicus* AHU 6010, was cultured in liquid medium (5% glucose, 1% peptone, and 0.1% dried yeast extract) as described in a previous paper¹⁾ and stored at -20°C .

Substrates tested for the enzyme were purchased from Tokyo Kasei Kogyo Co. and Wako Pure Chemical Ind. except for 2,4-dinitrophenyl benzenesulfonate, which was prepared by base-catalyzed coupling²²⁾ of 2,4-dinitrophenol and benzenesulfonyl chloride from Tokyo Kasei Kogyo Co.

Measurement of enzyme activity and protein. The activity of glutathione transferase was measured with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione by a method based on the description of Habig *et al.*⁵⁾ The enzyme and both substrates were incubated in 1 ml of 0.1 M potassium phosphate, pH 6.5, containing 2.5% ethyl alcohol to dissolve 1-chloro-2,4-dinitrobenzene at 30°C . After an addition of 3 ml of 0.33 N HCl to stop the reaction, absorbance at 340 nm was measured ($E=9,600$).

Protein was measured by the method of Lowry *et al.*²³⁾

and by absorbance at 280 nm with bovine serum albumin as the standard protein.

Enzymatic activity toward 3,4-dichloronitrobenzene, 4-nitropyridine-*N*-oxide, *p*-nitrophenethylbromide, *p*-nitrobenzylchloride, and 4-phenyl-3-buten-2-one were measured spectrophotometrically. The concentration of substrates, pH of 0.1 M potassium phosphate buffer, and wavelength used to follow the reaction were described by Habig *et al.*⁵⁾ The activity on 2,4-dinitrophenyl benzene-sulfonate was assayed by the increase of absorbance at 340 nm at pH 5.5 because it formed the same product as 1-chloro-2,4-dinitrobenzene¹¹⁾ and it made a larger spectral change nonenzymatically than the enzymatic change at higher pH.

The activity on some chloronitrobenzenes was estimated by measurement of chloride ion²⁴⁾ released from the substrate. After incubation of the enzyme with 1 mM glutathione and 1 mM chloronitrobenzene, the reaction was stopped by the addition of 1 ml of 2 mM *N*-ethylmaleimide to 1 ml of the reaction mixture. The resulting solution was mixed with 0.3 ml of 0.5% Hg (SCN)₂ in methyl alcohol and 0.4 ml of 34.61% Fe (ClO₄)₃·6H₂O in 26.4% HClO₄. After the mixture was left for 10 min, the absorbance at 460 nm was measured.

All enzymatic reactions were done at 30°C.

Glutathione conjugates. Products of glutathione transferase reaction were identified by thin layer chromatography on Kieselgel 60 F₂₅₄ (Merck) with chloroform-methanol-acetic acid-water (10:7:1:2). Products were detected by the appearance at 254 nm UV and with ninhydrin. Authentic conjugates were prepared by reaction of each chloronitrobenzene (1.24 g) and glutathione (1.0 g) in 100 ml of water-methanol-acetone (43:40:17) containing K₂CO₃ (2.25 g).³⁾

Electrophoresis. Homogeneity of the enzyme preparation was tested by 7.5% polyacrylamide gel electrophoresis (pH 8.0) by the method of Davis.²⁵⁾ Molecular weight of the enzyme was estimated from the mobility in different concentrations of gel²⁶⁾ using myoglobin (*M_r*: 18,000), ovalbumin (*M_r*: 45,000, 90,000), and bovine serum albumin (*M_r*: 67,000, 134,000) as standard proteins. Sodium dodecyl sulfate gel electrophoresis of the enzyme was done by the method of Weber *et al.*²⁷⁾ with standard proteins of cytochrome *c* (*M_r*: 12,500), myoglobin (*M_r*: 18,000), ovalbumin (*M_r*: 45,000), bovine serum albumin (*M_r*: 67,000), and γ -globulin (*M_r*: 25,000, 55,000).

Purification of *Mucor javanicus* glutathione transferase. Mycelia were suspended in 5 volumes of 0.1 M potassium phosphate buffer, pH 6.5, with a Polytron (Kinematica) and homogenized with a French press (Ohtake Works). The homogenate was centrifuged at 12,000 $\times g$ for 20 min. The precipitation was resuspended, homogenized, and centrifuged in the same way.

Supernatants (300 ml) were collected and mixed with 3

volumes of 1.33% Triton X-100 and divided into 6 samples of 200 ml. Five of them were put on 5 different columns (1.5 $\times$ 2.5 cm) of DEAE-cellulose equilibrated with 0.02 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM mercaptoethanol (1), 10% sucrose (2), 10% ethanol (3), 10% acetone (4) or nothing (5). The last aliquot was put on a column (1.5 $\times$ 2.5 cm) of DEAE-Toyopearl (Toyosoda Co.) equilibrated with 0.02 M potassium phosphate buffer, pH 6.5. Each column was washed with 150 ml of each buffer and eluted with a linear gradient (100 ml) of 0 to 0.3 M potassium chloride. The enzyme activity was eluted as one peak from every column with yields of 42 to 59%. The active fractions were collected and rechromatographed on a DEAE-cellulose column (1.5 $\times$ 5.5 cm) by the same method except for use of the buffer containing 1 mM EDTA, 1 mM mercaptoethanol, and 10% ethanol.

The eluted enzyme fraction was dialyzed against 0.02 M potassium phosphate buffer, pH 6.5, and put on an aminohexyl-Sepharose column (1.5 $\times$ 13.0 cm) equilibrated with the same buffer and eluted by a linear gradient of 0 to 0.5 M potassium chloride (400 ml). The active fractions were collected and concentrated with polyethyleneglycol 20000.

The concentrated enzyme solution was passed through a Bio-Gel P-150 column (1.5 $\times$ 60 cm) with 0.02 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA, 1 mM mercaptoethanol, and 10% ethanol. Fractions with enzyme activity were collected and dialyzed against 0.005 M phosphate buffer, pH 6.5, and put on a hydroxylapatite column (1.0 $\times$ 5.0 cm) equilibrated with the same buffer. Protein absorbed on the column was eluted with a linear gradient of 0.005 to 0.1 M phosphate buffer (100 ml).

RESULTS

Purification of glutathione transferase

The results of purification of glutathione transferase from *M. javanicus* are summarized in Table I. The enzyme from this microorganism was absorbed by DEAE-cellulose and eluted as one peak unlike the liver enzyme.^{5,8,9)} Elution profiles of the enzyme from DEAE-cellulose were not changed by the additions of 1 mM EDTA and 1 mM mercaptoethanol, 10% sucrose, 10% ethanol, or 10% acetone, but recovery of the enzyme activity increased with EDTA and mercaptoethanol, or ethanol from 42% to 58% or 54%. An enzyme preparation from the hydroxylapatite column showed a single band by polyacrylamide disc gel electrophoresis. A molecular weight of 45,000 was estimated for *M. javanicus* glutathione trans-

TABLE I. PURIFICATION OF GLUTATHIONE TRANSFERASE

Step	Protein (mg)	Activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Yield (%)
Crude extract	1040	219.5	0.211	100
DEAE-cellulose	52.1	42.2	0.81	19.2
Aminoethyl-Sepharose	12.5	35.2	2.82	16.0
Bio-Gel P-150	3.6	9.3	2.58	4.2
Hydroxylapatite	0.71	9.2	13.0	4.2

FIG. 1. Effect of Glutathione (GSH) Concentration on Glutathione Transferase.

Different fixed concentrations of 1-chloro-2,4-dinitrobenzene are 0.2, 0.22, 0.25, 0.27, 0.33, 0.4, 0.5, and 0.67 mM from top to bottom.

ferase by electrophoresis²⁶⁾ with different concentrations of gel. Sodium dodecyl sulfate gel electrophoresis showed that the enzyme was a dimer of identical subunits (M_r : 22,000).

Reaction of glutathione transferase to 1-chloro-2,4-dinitrobenzene

Among the tested compounds, 1-chloro-2,4-dinitrobenzene was converted to a glutathione conjugate with the highest rate, as described in a following section. Kinetics of the enzymatic reaction (pH 6.5) between glutathione and 1-chloro-2,4-dinitrobenzene is shown in Fig. 1 with various concentrations of glutathione under several fixed concentrations of another substrate, and in Fig. 2 with various concentrations of 1-chloro-2,4-nitrobenzene under several fixed concentrations of glutathione. Both figures have crossing points near the abscissa

FIG. 2. Effects of 1-Chloro-2,4-dinitrobenzene (CDNB) Concentration on the Glutathione Transferase Reaction.

Different fixed concentrations of glutathione are 0.4, 0.5, 0.67, 1.0, and 2.0 mM from top to bottom.

and give K_m values of 0.48 mM for glutathione and 1.0 mM for 1-chloro-2,4-dinitrobenzene. Similar analysis at pH 7.0 gave figures with similar patterns and K_m values of 0.33 mM for glutathione and 0.71 mM for 1-chloro-2,4-dinitrobenzene. The maximum rates of catalysis were $34 \mu\text{mol}/\text{min}/\text{mg}$ at pH 6.5 and $39 \mu\text{mol}/\text{min}/\text{mg}$ at pH 7.0.

Reaction toward the other substrate

Several compounds were tested by the method of Habig *et al.* (Table II). The best substrate for *M. javanicus* glutathione transferase was 1-chloro-2,4-dinitrobenzene, and 2,4-dinitrophenyl benzenesulfonate was also a better substrate than the other compounds. Formation of a conjugate from 3,4-dichloronitrobenzene was detectable by relative rate of about 0.2%, but preliminary tests showed that the conjugate formation of the other dichloronitrobenzenes made little spectral change.

TABLE II. ACTIVITY OF GLUTATHIONE TRANSFERASE TOWARD SOME SUBSTRATES

Substrate (mM)	Relative activity
1-Chloro-2,4-dinitrobenzene (1.0)	100
3,4-Dichloronitrobenzene (1.0)	0.17
4-Nitropyridine- <i>N</i> -Oxide (0.2)	0.00
<i>p</i> -Nitrophenethyl bromide (0.1)	0.00
<i>p</i> -Nitrobenzylchloride (1.0)	0.00
4-Phenyl-3-buten-2-one (0.05)	0.05
2,4-Dinitrophenyl benzenesulfonate (1.0)	9.5

FIG. 3. Comparison of Amount of Chloride Ion Released from 1-Chloro-2,4-dinitrobenzene (●) with Conjugate Formation (○).

Enzyme was incubated with 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in 1 ml of 0.1 M potassium phosphate, pH 6.5. Chloride ion and conjugate formation were measured as described in METHODS.

For the test of the enzyme reactivity toward the other dichloronitrobenzenes, measurement of chloride ion liberated from the substrate was compared with the method of Habig *et al.* (Fig. 3). Formation of chloride ion was lower than the amount of the conjugate calculated with the spectrophotometric assay, but measurement of chloride ion is useful for the detection of the reaction and for comparison of relative rates among several substrates. Table III shows that 2,3-, 2,4-, and 2,5-dichloronitrobenzenes and 2,4,5-trichloronitrobenzene were active. Glutathione conjugates of these compounds were detected by thin layer chromatography. From 2,6- and 3,5-dichlo-

TABLE III. ACTIVITY OF GLUTATHIONE TRANSFERASE TOWARD THE OTHER DICHLORONITROBENZENES

Glutathione transferase, 1 mM substrate, and 1 mM glutathione were incubated in 0.1 M potassium phosphate, pH 7.5, except for 1-chloro-2,4-dinitrobenzene which was done at pH 6.5. After incubation, chloride ion in medium was measured.

Substrate	Relative activity
1-Chloro-2,4-dinitrobenzene	100
2,3-Dichloronitrobenzene	0.22
2,4-Dichloronitrobenzene	0.35
2,5-Dichloronitrobenzene	0.19
2,4,5-Trichloronitrobenzene	0.45

ronitrobenzenes, 2,4,6-trichloronitrobenzene, 2,3,5,6-tetrachloronitrobenzene, and 2,3,4,5,6-pentachloronitrobenzene, the formation of conjugate did not show on the thin layer chromatogram.

DISCUSSION

Our previous papers^{1,2)} presented evidence of 3- and 4-chloro-2-methylthio-1-nitrobenzene formation from 2,3- and 2,4-dichloronitrobenzenes by *Mucor javanicus*. In view of the possibility that the replacement of chlorine by a methylthio group is due to a mechanism including a glutathione conjugate, glutathione transferase was purified from *M. javanicus*. The purified enzyme catalyzed the formation of glutathione conjugates from 2,3- and 2,4-dichloronitrobenzenes, showing the possibility of a pathway including a glutathione conjugate. The enzymatic formation of the glutathione conjugate was also observed with 2,5- and 3,4-dichloronitrobenzenes which did not show the formation of their methylthio derivatives²⁾ in the incubation with *M. javanicus*. This indicates a possibility that these two dichloronitrobenzenes are metabolized through a glutathione conjugate by *M. javanicus*. Two isomers, 2,6- and 3,5-dichloronitrobenzenes, the methylthio derivatives of which were not detected,²⁾ were also enzymatically inert.

Gel electrophoresis showed that glutathione transferase from *M. javanicus* was a dimer of identical subunits (M_r : 22,000). Kinetics of glutathione transferase reaction toward different fixed concentrations of 1-chloro-2,4-dinitrobenzene and various concentrations of glutathione gave straight lines crossed near the abscissa of the double reciprocal plot and K_m values of 0.48 mM for glutathione and 1.0 mM for 1-chloro-2,4-dinitrobenzene. These results indicate that *M. javanicus* glutathione transferase has similar properties to the rat liver enzyme.¹⁶⁾

Liver glutathione transferases were absorbed to CM-cellulose at neutral pH and separated into several isozymes,^{5,6,8,9)} but *M. javanicus* glutathione transferase was absorbed by DEAE-cellulose and eluted as one peak. Most of the liver isozymes have been reported to show the highest activity toward 1-chloro-2,4-dinitrobenzene among the tested compounds,^{5~9)} but some isozymes have higher activity to 3,4-dichloronitrobenzene than the other isozymes. *M. javanicus* glutathione transferase is similar to the isozymes^{5,6)} with the lower activity to 3,4-dichloronitrobenzene.

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