Studies on Yeast Glutathione S-Transferase

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Contents

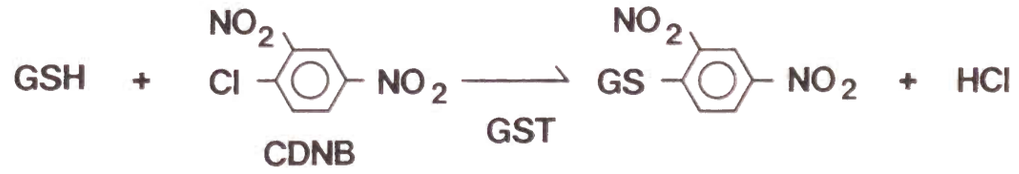
Abbreviations

aa.	amino acid
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CM	carboxymethyl
dCTP	deoxy cytidine triphosphate
DEAE	diethylaminoethyl
DNB	o-dinitrobenzene
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
dT	deoxythymidine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GSH	glutathione(reduced form)
GST	glutathione S-transferase
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
IgG	immunoglobulin G
Km	michaelis constant
mRNA	messenger ribonucleic acid
OD	optical density
PAGE	polyacrylamidegel electrophoresis
PMR	proton magnetic resonance
poly $(A)^+$RNA	poly adenylated ribonucleic acid

PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
SIMS	secondary ion mass spectrum
TBS	tris buffered saline
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
U	unit

Introduction

Glutathione (γ-glutamylcysteinylglycine) is the most prevalent low molecular weight thiol compound widely present in all living organisms (13) and plays many important physiological roles. It provides reducing capacity for cells and protects against many toxic substances including heavy metals, various electrophiles and free radicals (11). In addition, glutathione acts as a coenzyme for a variety of enzymes (11). Detoxification is one of the most important roles of glutathione. Electrophilic xenobiotics react with nucleophilic groups of proteins and nucleic acids in the cells and then chemically modify them causing loss of cell function, tissue necrosis, mutation and carcinogenesis. Glutathione, which is a strong nucleophile, reacts with these electrophiles and detoxifies them.



Glutathione S-transferase (GST)(EC 2.5.1.18) catalyzes the conjugation of glutathione to a large variety of electrophilic

substrates. Glutathione S-transferase is widely distributed from mammals to bacteria (6) and many isoenzymes have been isolated and characterized in mammals (9). Glutathione S-transferase has also been studied in higher plants (4,12) and insects (2) in relation to resistance to herbicides and insecticides, respectively.

Recently, glutathione S-transferase has been studied by genetic engineering. Many cDNA clones for glutathione S-transferase have been isolated and the deduced amino acid sequences have been reported in mammals and plants (10). Although so many progress has been made with regard to glutathione S-transferases in mammals and plants, there have been few investigations in microorganisms.

Recently glutathione S-transferase has been purified from Mucor javanicus (1) and Escherichia coli (5). However in yeast, the existence of glutathione S-transferase activity has been reported in only some strains (7,8). Although glutathione was first isolated from yeast cells (3) in abundance, the physiological role of glutathione and glutathione S-transferase in yeast cells remains largely unknown. The author studied glutathione S-transferase in the yeast Issatchenkia orientalis which had the highest glutathione S-transferase activity in all yeast strains investigated, in order to elucidate the properties and physiological roles of the enzyme and the glutathione related detoxification system.

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Chapter I. Distribution, Formation and Stabilization of Yeast Glutathione S-Transferase.

Glutathione S-transferase (GST) catalyzes the conjugation of various electrophiles with glutathione (GSH). These electrophiles include xenobiotics as well as endogenous compounds in the organism. In general, the GSH conjugates produced are metabolized *via* the mercapturic acid biosynthesis pathway in the mammalian liver (4). GST has been found in a variety of higher organisms (9), and a number of species-specific and tissue-specific GST isoenzymes were detected and studied in detail (21). There have been few reports on GST in microorganisms, although glutathione was reported to be relatively abundant in bacteria and yeasts (28). In this study, the author investigated the distribution of GST in various yeasts, the culture conditions for the optimum production of GST by *I. orientalis* and the stabilization of the solubilized enzyme.

Materials and Methods

Reagents.

Glutathione was a generous gift from Kirin Brewery Co. 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Wako Pure Chemical Co. *o*-Dinitrobenzene (DNB) was from Nakarai Tesque Co.

Other chemicals were analytical grade reagents from commercial sources.

Yeast strain.

Issatchenkia orientalis and other yeast strains used in this study were from the culture collection of the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto. I. orientalis IFO 1279 was selected to establish the optimum culture conditions.

Cultivation for screening.

Each yeast strain was grown on a basal medium consisting of 2 % glucose, 0.5 % peptone, 0.2 % yeast extract, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4 and 0.02 % $MgSO_4 7H_2O$, pH 7.0. Precultures were carried out at 28°C for 24 hr under continuous shaking in test tubes (1.65 x 16.5 cm) containing 5 ml of medium. The precultures were then transferred to 500-ml Sakaguchi flasks containing 100 ml of the same medium, followed by culturing at 28°C for 1-2 days with reciprocal shaking (114 rpm).

Culture conditions.

Cells were grown on a basal medium in which one or more of the components were replaced or their concentrations were changed.

Preparation of cell-free extracts.

The cultured broths were centrifuged at 6,000 xg for 30 min. The harvested cells were washed with 0.05 M potassium phosphate buffer, pH 7.0, and then suspended in the same buffer. The suspended cells were disrupted with a Kaijo Denki ultrasonic

oscillator (20 kHz) for 10 min and then the supernatant was obtained by centrifugation at 7,000 xg for 20 min.

Enzyme assays for GST activity.

GST activity was assayed by the following two methods:

(1) DNB method. The enzyme activity was determined by measuring the nitrite released enzymatically from the substrate, DNB, using the diazo-coupling method of Asaoka and Takahashi (3) with a slight modification. The reaction mixture contained 50μmol of sodium phosphate buffer, pH 7.0, 1μmol of DNB dissolved in ethanol, 1μmol of GSH and enzyme solution in a total volume of 0.5 ml. The reaction was started by the addition of GSH and after incubation at 30°C for 15 min, the reaction was terminated by the addition of 0.05 ml of acetic anhydride. The released nitrite was determined as the absorbance of diazo-coupling products with N-(1-naphthyl) ethylenediamine dihydrochloride and sulfanilamide at 540 nm. Blank reactions in which the enzyme solution or GSH solution was replaced by distilled water were carried out and the absorbance values were subtracted from the corresponding assay values. This method was mainly used for the screening of yeasts showing high GST activity and the formation of yeast GST. One enzyme unit was defined as the amount of enzyme which produced 1μmol of nitrite per min.

(2) CDNB method. The enzyme activity was also assayed spectrophotometrically with CDNB as the substrate according to Habig et al. (12). The reaction mixture contained 100μmol of potassium phosphate buffer, pH 6.5, 1μmol of CDNB, 1μmol GSH and

enzyme solution in a total volume of 1 ml. Enzyme activity was determined by monitoring the change in absorbance at 340 nm, at 25°C, with a double beam spectrophotometer, Hitachi Model 100-50. The reaction mixture without any enzyme was used as a control. One unit was defined as the amount of enzyme which produced 1μmol S-2,4-dinitrophenyl glutathione per min.

Determination of protein.

Protein concentrations were determined by the method of Lowry et al. (20) with ovalbumin as a standard.

Results

Distribution of GST activity in yeasts.

GST activity was measured in crude extracts of 168 yeast strains with DNB as a substrate. The activity was widely distributed in various yeasts belonging to the genera Saccharomyces, Shizosaccharomyces, Pichia, Issatchenkia, Hansenula, Candida and Trichosporon, as shown in Table I. I. orientalis showed the highest GST activity and so was chosen as the origin of the enzyme for further investigation.

Inductive formation of GST by I. orientalis.

The effect of the addition of DNB to the medium on the induction of GST activity was examined in I. orientalis. The yeast

TABLE I. DISTRIBUTION OF GST IN YEASTS

Strain	Specific activity (mU/mg)
Candida krusei	1.25
Candida vini	1.46
Hanseniaspora valbyensis	0.23
Hansenula anomala	0.16
Hansenula bimundalis	0.29
Hansenula jadinii	0.26
Hansenula silvicola	0.13
Hansenula wingei	0.20
Issatchenkia orientalis	1.58
Pichia aganobii	0.32
Pichia farinosa	0.14
Pichia pseudopolymorpha	0.13
Rhodotorula minuta	0.14
Rhodotorula rubra	0.13
Saccharomyces bisporus	0.17
Saccharomyces chevalieri	0.15
Saccharomyces uvarum	0.13
Saccharomycodes ludwigii	0.22
Saccharomycopsis lipolytica	0.15
Schizosaccharomyces pombe	0.51
Trichosporon cutaneum	0.31

Cultivation of yeasts and preparation of cell-free extracts of yeasts were carried out as described under MATERIALS AND METHODS. Enzyme activity was determined in cell-free extracts by the DNB method, as described under MATERIALS AND METHODS.

cells were cultured in the presence of various concentrations of DNB and the absorbance of the cultures was monitored at 610 nm. Prolongation of the lag phase was observed in proportion to the concentration of added DNB (data not shown). Then, the cells were cultured in the presence and absence of 200μM DNB, and cell growth and GST activity were followed (Fig. 1). Cell growth was suppressed for two days by the addition of 200μM DNB, followed by rapid increases in cell growth and enzyme activity, and at the end of the exponential growth-phase, the enzyme specific activity was about 4-fold in comparison with that in the case of cells cultured without the inducer.

Effects of the glucose, yeast extract and peptone concentrations on GST production.

The effects of the concentrations of glucose, yeast extract and peptone on GST production were investigated in the presence of 200μM DNB. As shown in Table II, 50 g/l of glucose gave the best results in this experiment, but 20 g/l of glucose was selected for further investigation because it was economical for mass cultivation. In the presence of 20 g/l glucose, the concentration of yeast extract did not affect the enzyme production substantially and so a yeast extract concentration of 0.5 g/l was selected. As shown in Table II, five grams per liter of peptone was most effective in increasing the total activity and 2.5 g/l most effective in increasing the specific activity in the presence of 20

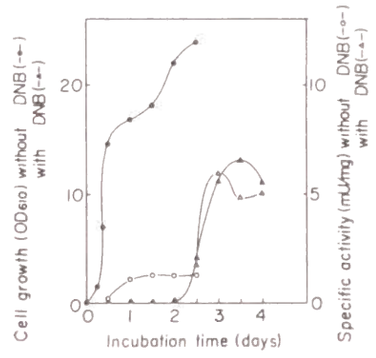

FIG. 1. Cell Growth and Glutathione *S*-Transferase Activity in the Presence and Absence of DNB.

A preculture of *Issatchenkia orientalis* was inoculated into 100 ml of the basal medium, with or without DNB, in a 500-ml Sakaguchi flask, followed by incubation at 28°C with reciprocal shaking. Cell growth in the presence (▲) and absence (●) of 200 μM DNB was followed. Glutathione *S*-transferase activity was assayed with DNB as the substrate and expressed as m units per mg protein in the presence (△) and absence (○) of 200 μM DNB.

TABLE II. EFFECTS OF THE GLUCOSE AND PEPTONE CONCENTRATIONS ON GST ACTIVITY

		Specific activity (mU/mg)	Total activity (mU)
Glucose	5 (g/l)	1.9	180
	10	3.6	260
	20	4.4	350
	35	4.4	430
	50	5.2	460
Peptone	1.25	2.6	110
	2.5	6.1	510
	5	5.2	630
	10	3.2	530

Issatchenkia orientalis was grown in 100 ml of each medium containing 200 μM DNB. The concentration of the yeast extract was 0.5 g/l when the effect of the peptone concentration was examined. Other conditions were the same as under MATERIALS AND METHODS. GST activity was assayed with DNB as the substrate.

g/l of glucose and 0.5 g/l of yeast extract.

Effects of glutathione constituents.

In the presence of 200μM DNB, the effects of glutathione-constituting amino acids were investigated. The concentration of yeast extract in the basal medium was changed to 0.5 g/l, and yeast cells were grown in 100 ml of the modified basal medium containing 200μM DNB and various glutathione-constituting amino acids (single or in combination) at 28°C to the late exponential phase. GST activities were assayed with DNB as the substrate. The addition of L-cysteine + glycine and L-glutamate + L-cystine increased the enzyme production significantly. But the addition of L-cysteine, L-cystine or L-glutamate + L-cysteine + glycine did not affect the enzyme production. The other glutathione constituents did not affect the enzyme production so much.

GST production.

In the presence of 200μM DNB, I. orientalis was cultured at 28°C in the medium containing L-cysteine + glycine or L-glutamate + L-cystine, and the cell-growth and GST activity during cultivation were followed. As shown in Fig. 2, the enzyme activity increased with cell-growth, the highest activity being seen at the end of the exponential phase, and then decreased gradually. The addition of L-cysteine + glycine increased the specific activity about 2-fold in comparison with the addition of L-glutamate + L-cystine. In addition, the cells grew 24 hr-faster in the medium containing L-cysteine + glycine in comparison within the medium containing L-glutamate + L-cystine.

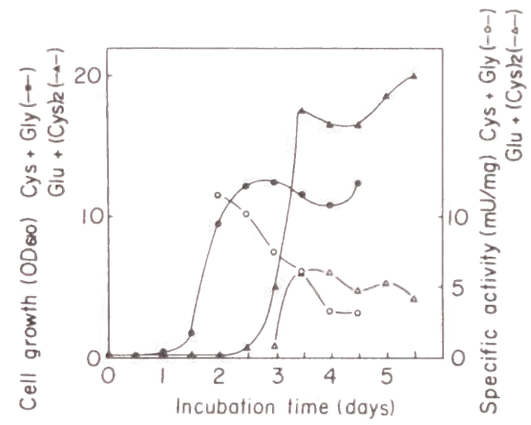

FIG. 2. Time Course of Glutathione *S*-Transferase Formation.

A preculture of *Issatchenkia orientalis* was inoculated into 100 ml of medium, in a 500-ml Sakaguchi flask, containing 2% glucose, 0.5% peptone, 0.05% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 200 μM DNB, followed by incubation at 28°C with reciprocal shaking. Cell growth was followed in medium containing 0.1% L-cysteine+glycine (●) or 0.1% L-glutamate+L-cystine (▲). GST activity was assayed with DNB as the substrate and expressed as m units per mg protein in medium containing 0.1% L-cysteine+glycine (○) and 0.1% L-glutamate+L-cystine (△).

Stabilization of GST.

(1) Effects of chemicals.

The GST of I. orientalis was very unstable and in a 0.05 M potassium phosphate buffer (pH 7.0) at 4°C for 1 week, the enzyme lost more than 50 % of its activity. Stabilization of the enzyme

activity was then examined, by means of the addition of various chemicals to the solution. As the addition of each chemical scarcely affected the stability, the combined addition of two or three chemicals was examined (data not shown). The combined addition of 20 % glycerol, 1mM EDTA and 2mM DTT stabilized the enzyme for at least 1 week, but 3 weeks later, the enzyme activity had decreased to below 25 % of that at the start.

(2) Effect of N_2 substitution.

Because the addition of DTT affected the enzyme stability to a great extent in the presence of glycerol and EDTA, the effect of N_2 substitution was examined. The air above the enzyme solution in a test tube was substituted with N_2 gas and then the sample was preserved at 4°C in 0.05 M potassium phosphate buffer, pH 7.0, with or without the stabilizing agents mentioned above, and the activity was followed (Fig. 3.). With N_2, the crude enzyme solution was stable for 1 month in 0.05 M potassium phosphate buffer, pH 7.0, containing 20 % glycerol, 1 mM EDTA and 2 mM DTT.

(3) Effects of antioxidative additives on GST stability.

N_2 substitution was effective in keeping the GST active but it was rather difficult to apply it at each purification step for the enzyme. The effects of the addition of some other antioxidants were examined and the results are shown in Table III. The addition of ethylene glycol, sodium sulfite or sodium hydrosulfite stabilized the enzyme as much as N_2 substitution and CO_2 substitution did.

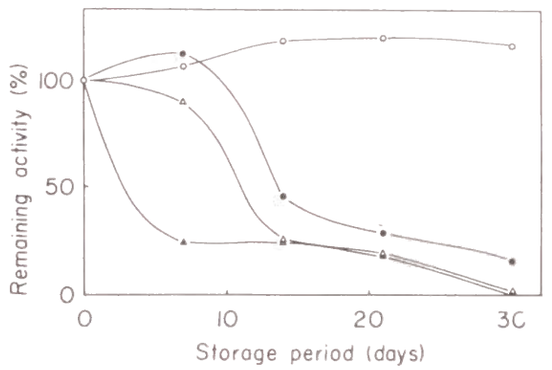

FIG. 3. Effect of N_2 Substitution on Glutathione *S*-Transferase Stability.

Various chemicals were added to cell-free extracts of *Issatchenkia orientalis* and then the air above the cell-free extracts in the test tubes was substituted with N_2 gas. GST activity was assayed with CDNB as the substrate. ▲, none; △, N_2 substituted; ●, addition of 20% glycerol, 1 mM EDTA and 2 mM DTT; ○, addition of 20% glycerol, 1 mM EDTA and 2 mM DTT, and N_2 substituted.

TABLE III. EFFECTS OF ANTIOXIDATIVE ADDITIVES ON GST STABILITY

Stabilizer added	Remaining activity (%)
10 mM Ascorbic acid	24
10% Ethylene glycol	102
10 mM Sodium sulfite	106
10 mM Sodium hydrosulfite	87
N_2 Substitution	104
CO_2 Substitution	101
None	24

Various antioxidative reagents were added to cell-free extracts of *Issatchenkia orientalis* or the air above the cell-free extracts in test tubes was substituted with N_2 gas or CO_2 gas, followed by storing at 4°C for 3 weeks. Each cell-free extract contained 20% glycerol, 1 mM EDTA, 2 mM dithiothreitol and 50 mM potassium phosphate (pH 7). GST activity was assayed with CDNB as the substrate.

Discussion

Glutathione S-transferase activity has been found in mammals such as rat (27), mouse (19), monkey (2), cow (1), rabbit (8) and man (29). GST has been studied in detail in the rat and tissue-specific GST isoenzymes were found in the liver (13,17), kidney (10), brain (26), testis (11) and heart (14). Many mammalian GST isoenzymes have been purified and characterized, and recently, species-independent classification has been attempted on the basis of structural and catalytic properties (22). GST activity was also found in other animals (15), plants such as corn (6) and peas (7), and insects such as flies (5). GST activity in plants and insects is related to resistance to herbicides and insecticides, respectively.

There have been many reports about GST in higher organisms, however, there have been few about GST in microorganisms. Meijer et al. (23) reported that Salmonella typhimurium can enzymatically conjugate GSH with 1-chloro-2,4-dinitrobenzene. Lau et al. (18) reported that several microorganisms, including a bacterium such as Escherichia coli and a yeast such as Candida lypolytica, exhibit GST activity, although they did not detect GST activity in Saccharomyces cerevisiae. However, Jaspers and Penninckx reported the occurrence of GST in Saccharomyces cerevisiae (16). In our experiment, GST activity could not be detected in Saccharomyces cerevisiae with DNB as an electrophilic

substrate.

In Issatchenkia orientalis, GST activity was increased by DNB, a GST substrate, and other substrates such as CDNB and 1,2-dichloro-4-nitrobenzene could also induce GST activity (data not shown) but not as much as DNB did. Also, it was reported that GST was induced by phenobarbital or 3-methylcholanthrene in rat liver (24,25), but yeast GST was not induced by phenobarbital (data not shown). During cultivation in the presence of DNB, CDNB or 1,2-dichloro-4-nitrobenzene, a prolonged lag period was observed, depending on the concentrations of these chemicals. On the other hand, in the presence of phenobarbital, a lag period was not observed at all. These results may indicate that the nitrobenzene analogues act as GST inducers in this strain and that GST is not induced in the presence of phenobarbital because of its low toxicity for the cells.

The GST activity and the concentration of intracellular glutathione in this strain in the inducing medium (containing DNB, cysteine and glycine) were higher than those in the basal medium at the exponential phase and the stationary phase, respectively (data not shown). So the addition of L-cysteine + glycine to the medium triggers the biosynthesis of glutathione and the succeeding conjugation reaction in this strain, and therefore the lag period of this culture may be shortened, as shown in Fig. 2.

Yeast GST was very unstable but was stabilized by N_2 substitution of the air above the enzyme solution containing 20%

glycerol, 1 mM EDTA and 2 mM DTT. The addition of an antioxidant, such as sodium sulfite, sodium hydrosulfite or ethylene glycol, also had an effect on the enzyme stability like N_2 substitution. These results will facilitate further investigation of the enzyme after purification and suggest that the GST has an active site that is sensitive to oxidation.

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Chapter II. Purification and Properties of Glutathione S-Transferase from Issatchenkia orientalis.

Glutathione S-transferase (GST) catalyzes the conjugation of various electrophilic substrates including xenobiotics such as carcinogens and mutagens with glutathione (GSH). In the mammalian liver, these GSH conjugates are metabolized further by γ-glutamyl-transpeptidase, cysteinylglycinase and N-acetyl transferase through the mercapturic acid biosynthesis pathway (7). The distribution of GST has been reported from bacteria to mammals (14) and many GST isoenzymes have been isolated and characterized in various mammals (2, 3, 9, 16, 21). Some GSTs have been reported to have alternative functions other than detoxification, such as the reduction of organic hydroperoxides by GSH peroxidase activity (20) and the hypothesis of leukotriene C formation from leukotriene A (5).

GSH was first isolated from yeasts and is abundant in yeast cells (27), but there are a few reports of yeast GST (15, 19) and GST has not been purified from yeast cells to a homogeneous state. Recently the author reported the distribution of GST in yeasts, the formation and the stabilization of GST of Issatchenkia orientalis that showed the highest GST activity of all yeast strains investigated (17).

In this study, GST was purified from Iss. orientalis and characterized to determine the physiological role of GSH and GST in

yeast cells.

Materials and Methods

Reagents.

Bromosulfophthalein, 1,2-epoxy-3-(p-nitrophenoxy)propane, ethacrynic acid, and hematin were purchased from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene (CDNB), o-dinitrobenzene (DNB), p-nitrobenzyl chloride, p-nitrophenethyl bromide, and DEAE-cellulose were from Wako Pure Chemical Co. 4-Nitropylidine-N-oxide was from Nakarai Chemical Co. 1,2-Dichloro-4-nitrobenzene, tributyltin acetate, and triphenyltin chloride were from Tokyo Kasei Co. trans-4-phenyl-3-buten-2-one was from Aldrich Chemical Co., and Cibacron Blue 3G-A was from Fluka AG. Sephadex G-100, Phenyl-Sepharose CL-4B, CM-Sepharose CL-4B, PBE 94, and Polybuffer 96 were from Pharmacia. Reduced glutathione was a generous gift from Kirin Brewery Co. Other chemicals were purchased from commercial sources.

Yeast strain and culture.

Issatchenkia orientalis was obtained from stock cultures of Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan. Cells were picked up from a basal medium slant culture containing 2% glucose, 0.5% peptone, 0.05% yeast extract, 0.05%

KH_2PO_4, 0.05% K_2HPO_4, 0.02% $MgSO_4$, inoculated into a test tube containing 5 ml of the basal medium, and grown at 28°C overnight with reciprocal shaking. Each test tube culture was transferred to a 2-liter Sakaguchi flask containing 500 ml of basal medium and then grown at 28°C overnight with reciprocal shaking. Two such subcultures were inoculated into a 30-liter jar fermentor (type MSJ-U 301, Marubishi Co.) containing 25 liters of inducing medium consisting 200μM DNB, 0.1% glycine and 0.1% L-cysteine besides the basal medium as described before (17). Cultivation was carried out at 30°C with aeration (1 liter per liter of medium per min) and agitation (200 rpm). The grown cells were harvested at the late exponential phase with a refrigerated continuous-flow centrifuge (type GLE; Carl Padberg Gmbh).

Enzyme assay for GST activity.

During the purification, GST activity was assayed spectrophotometrically with a substrate CDNB as described previously (17). For the substrate specificities, the enzyme activity was determined according to Habig et al.(13). GST activity was also determined by measuring nitrite released enzymatically from DNB, using a diazo-coupling method according to Asaoka and Takahashi (4) with a slight modification (17).

Protein determination.

Protein concentrations were determined by the method of Lowry et al. (22), with ovalbumin as a standard, and by the method of Lowry modified by Bensadoun and Weinstein (6) when the enzyme

solution contained glycerol.

Electrophoresis.

Disc-polyacrylamide gel electrophoresis (disc-PAGE) was performed by the method of Tamura et al. (26). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (18). Proteins of native gel were stained with amide black 10B and those of Laemmli gel were stained with coomassie blue R-250.

Molecular weight estimation.

Molecular weights were determined by gel filtration on HPLC equipped with a TSK-GEL 2,000 SW column (7.5 by 30 mm) equilibrated with 0.05 M potassium phosphate (pH 7.0). Elution was performed with the same buffer and the absorbance at 280 nm was measured. The subunit molecular weights were estimated on an SDS-PAGE, using an LMW molecular weight calibration kit purchased from Pharmacia.

Chromatofocusing.

The enzyme solution was applied on a PBE 94 column (0.8 by 20 cm) equilibrated with 0.25 M ethanolamine-HCl (pH 9.4) containing 20 % glycerol, 1 mM EDTA and 10 mM sodium sulfite. The enzyme was eluted in 2 ml fractions with a descending linear gradient of Polybuffer 96-HCl (eleven fold dilution, pH 7.0), from pH 9 to 7.

Purification of GST from I. orientalis.

All operations were carried out at 0 to 4°C but Phenyl-Sepharose CL-4B column chromatography was carried out at room temperature (15-20°C). In all column chromatographies,

equilibration and elution was carried out with a buffer solution containing 20% glycerol, 1 mM EDTA and 10 mM sodium sulfite. GST solution was concentrated by Ultrafiltration, if necessary, with a Labo cassette using the UF membrane NMWL 1,000 (Millipore Co.)

Purification step.

(i) Preparation of cell extracts.

Cells harvested from 50 liters of culture (wet weight, 600g) were suspended in 2 liters of 0.05 M of potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 mM sodium sulfite. The cells were disrupted with a Dyno-Mill (agitation rate, 3,000 rpm with glass beads of 0.25 to 0.50 mm in diameter) (Willy A. Bachofen, Maschinenfabrik, Basel, Switzerland), and the supernatant solution was obtained by centrifugation.

(ii) Protamine sulfate treatment.

A 2 % protamine sulfate solution was added to cell extracts up to 10 % of its total protein, and the precipitate formed was removed by centrifugation. The supernatant solution was dialyzed for 48 hr against three changes of 20 liters of 0.05 M of potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and sodium sulfite.

(iii) DEAE-cellulose column chromatography.

Glycerol was added to the dialyzed solution to make a 20% concentration. Then this enzyme solution was applied to a DEAE-cellulose column (10 by 50 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. The enzyme was eluted with the same buffer. Active fractions were combined and concentrated by

Ultrafiltration.

(iv) Sephadex G-100 gel filtration.

The enzyme solution was divided into three portions and each portion was applied to a Sephadex G-100 column (1.8 by 125 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, and eluted with the same buffer. The active fractions were combined and concentrated by Ultrafiltration. The enzyme solution was dialyzed against the 0.05 M potassium phosphate buffer, pH 7.0, containing 0.25 M NaCl.

(v) First Phenyl-Sepharose CL-4B column chromatography.

The enzyme solution was divided into two portions and each portion was applied to a Phenyl-Sepharose CL-4B column (2 by 25 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.25 M NaCl. After the column was washed with the same buffer, the elution was carried out with 0.05 M potassium phosphate buffer containing 30% ethylene glycol. Two peaks of activity were eluted and the fractions containing the activity were combined separately for each peak. The second eluted peak was used for the study of enzymatic properties without further purification (GST Y-2).

(vi) CM-Sepharose CL-4B column chromatography.

The first eluted active peak of Phenyl-Sepharose CL-4B column chromatography was applied to a CM-Sepharose column (0.9 by 12 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. The enzyme activity was eluted with the same buffer. The active

fractions were combined and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0, containing 0.25 M NaCl.

(vii) Second Phenyl-Sepharose column chromatography.

The enzyme solution was applied to a Phenyl-Sepharose column (1.4 by 43 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.25 M NaCl. After the column was washed with the same buffer, the enzyme was eluted with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.25 M NaCl, 25 % ethylene glycol. The active fractions were combined and used for the study of enzymatic properties (GST Y-1).

Preparation of antisera.

Antiserum was raised against GST Y-2 in white rabbits. GST Y-2 obtained by first Phenyl-Sepharose CL-4B column chromatography was applied to SDS-PAGE to have further purified preparation. After staining, the band of GST Y-2 was cut out and eluted electrophoretically. About 1 mg of eluted protein was dialysed thoroughly against distilled water and freeze dried. This preparation gave a single band on SDS-PAGE. Prepared GST Y-2 antigen was dissolved in phosphate-buffered saline (12.5mM Na-phosphate, pH 7.4, 150mM NaCl) and emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly into male rabbits on 1st. and 22nd. days. The rabbits were bled from ear vein 7days after the second injection. The serum was stored at 4 C containing 0.1% NaN_3. Stained SDS-polyacrylamidegel contained no protein was also eluted and injected into rabbit in the same

way, and obtained serum did not formed precipitin line against GST Y-1, Y-2 and cell-free extract as well as control sera which were obtained from rabbits before immunization.

Immunodiffusion.

The Ouchterlony immunoprecipitin tests (25) were performed in 1% purified agar gel (DIFCO) containing 0.1% NaN and 0.05M tris-HCl,pH 8.0.

Results

Enzyme purification.

The purification of GST was carried out from the cell-free extract of Issatchenkia orientalis by protamine treatment and five column chromatographic fractionations (Table 1). At the 1st Phenyl-Sepharose column chromatography, two peaks having GST activity were eluted (Fig. 1) and were named fraction A and fraction B. Fraction B gave a single band on disc-PAGE (Fig. 2A-b) but fraction A gave several bands including the same band as fraction B. Fraction B was designated as GST Y-2 and characterized, and fraction A was further purified. Disc-PAGE of fraction A after CM-Sepharose CL-4B column chromatography gave two protein bands (Fig. 2A-a) and each band extracted from the gel before staining showed GST activity. The heavy band showed the same migration with GST Y-2 on SDS-PAGE. At the final step, 2nd

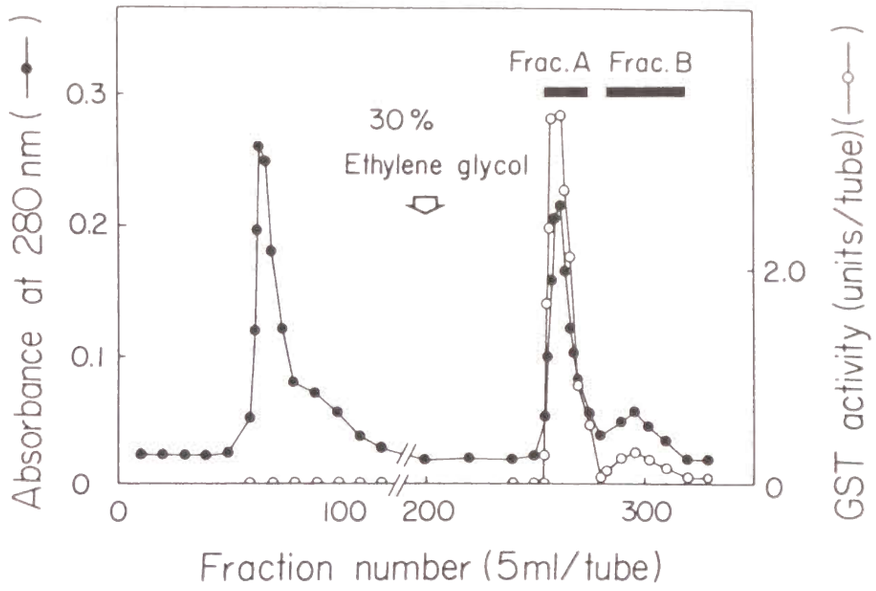
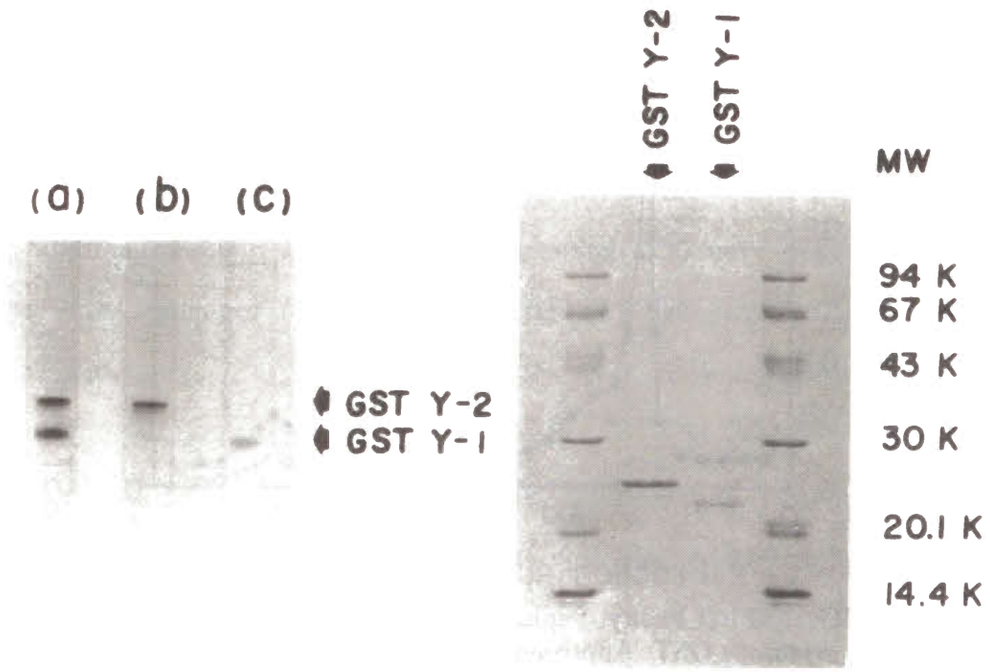


FIG. 1. First Phenyl-Sepharose CL-4B column chromatography of *I. orientalis* GST. Approximately 30 U of enzyme was applied on a Phenyl-Sepharose column, and chromatography was performed as described in Materials and Methods. Symbols: ○, GST activity; ●, A_{280}.

Table 1.
Purification step

Purification procedure	Total protein (mg)	Total activity (mU)	Sp activity (mU/mg)	Yield (%)
1. Cell extract	38,400	239,000	6.2	100
2. Protamine	16,400	361,000	22.0	151
3. DEAE-cellulose	3,950	130,000	32.9	54.4
4. Sephadex G-100	130	79,200	609	33.1
5. First Phenyl-Sepharose CL-4B				
Fraction A*	22.8	42,000	1,840	17.6
Fraction B (GST Y-2)	2.3	1,400	609	0.59
6. CM-Sepharose CL-4B	14.2	36,300	2,560	15.2
7. Second Phenyl-Sepharose CL-4B (GST Y-1)	0.09	570	6,330	0.24

*Only fraction A was submitted to steps 6 & 7.



(A) DISC-PAGE (B) SDS-PAGE

FIG. 2. PAGE of *I. orientalis* GST Y-1 and GST Y-2. (A) Disc PAGE. Electrophoresis and staining were carried out as described in Materials and Methods. Lanes: a, 40 μg of active fraction from CM-Sepharose; b, 20 μg of fraction B (GST Y-2); c, 10 μg of active fraction eluted from the second Phenyl-Sepharose column (GST Y-1). (B) SDS-PAGE. Approximately 15 μg of GST Y-1 and GST Y-2 were applied.

Phenyl-Sepharose column chromatography, the active fraction was eluted by 0.05 M potassium phosphate buffer, pH 7.0, containing 25% ethylene glycol and 0.25 M NaCl. It gave only the light protein band on disc-PAGE (Fig. 2A-c) and was named GST Y-1.

Molecular properties of GST Y-1 and GST Y-2.

The molecular weights of GST Y-1 and GST Y-2 were determined to be ca. 37,500 and 40,000 by gel filtration with HPLC. SDS-PAGE of GST Y-1 and GST Y-2 gave a single band of 22,000 and 23,500, respectively (Fig. 2B), indicating that each isoenzyme is a homodimer. Isoelectric points of GST Y-1 and GST Y-2 were determined to be pH 8.40 and 8.55, respectively, from the results of Chromatofocusing.

Immunological properties.

To demonstrate the immunological characters of GST Y-1 and Y-2, immunodiffusion experiments were performed. The antiserum to GST Y-2 reacted with two kinds of cell free extract of I. orientalis cultured with or without DNB, and both formed a precipitin line that showed identity with that of purified GST Y-2 (Fig. 3). On the other hand, the antiserum to GST Y-2 did not react with purified GST Y-1 (Fig. 3). These results suggest that GST Y-1 is immunologically distinguishable from GST Y-2.

Catalytic properties.

The properties of the purified isoenzymes were investigated with CDNB as an electrophilic substrate. The optimum pH for the conjugation of GSH with CDNB by GST Y-1 and GST Y-2 was 7.0 and 7.0-7.5, respectively (Fig. 4). The optimum temperature for the conjugation of GSH with CDNB by GST Y-1 and GST Y-2 was 45°C and 35°C, respectively (Fig. 5). The pH stability of GST Y-1 and GST

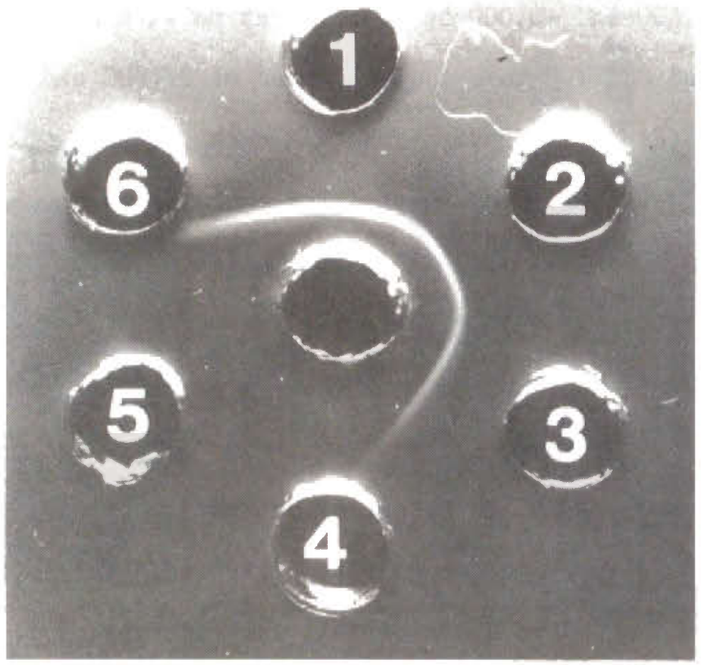


FIG. 3. Immunodiffusion of GST Y-1 and GST Y-2 with antiserum to GST Y-2. Wells: 1, homogeneous GST Y-2 (0.13 mg of protein per ml); 2, cell extract of *I. orientalis* cultured with *o*-DNB; 3, cell extract of *I. orientalis* cultured without *o*-DNB; 4 through 6, homogeneous GST Y-1 (wells 4, 5, and 6 contained 0.29, 0.09, and 0.02 mg of protein per ml, respectively); center well, GST Y-2 antiserum.

Y-2 were investigated by preservation of these isoenzymes in various buffers containing stabilizing agents at 4℃ for 1 week. GST Y-1 retained more than 90% activity in the pH range of 6.5 to 7.5, but lost ca. 40% of its activity below pH 5.0 and above 9.0. GST Y-2 retained more than 85% activity in the pH range of 6.5 to 11.7, but lost 60% of its activity at pH 3.8 (Fig. 6). The temperature stability of these isoenzymes were investigated by

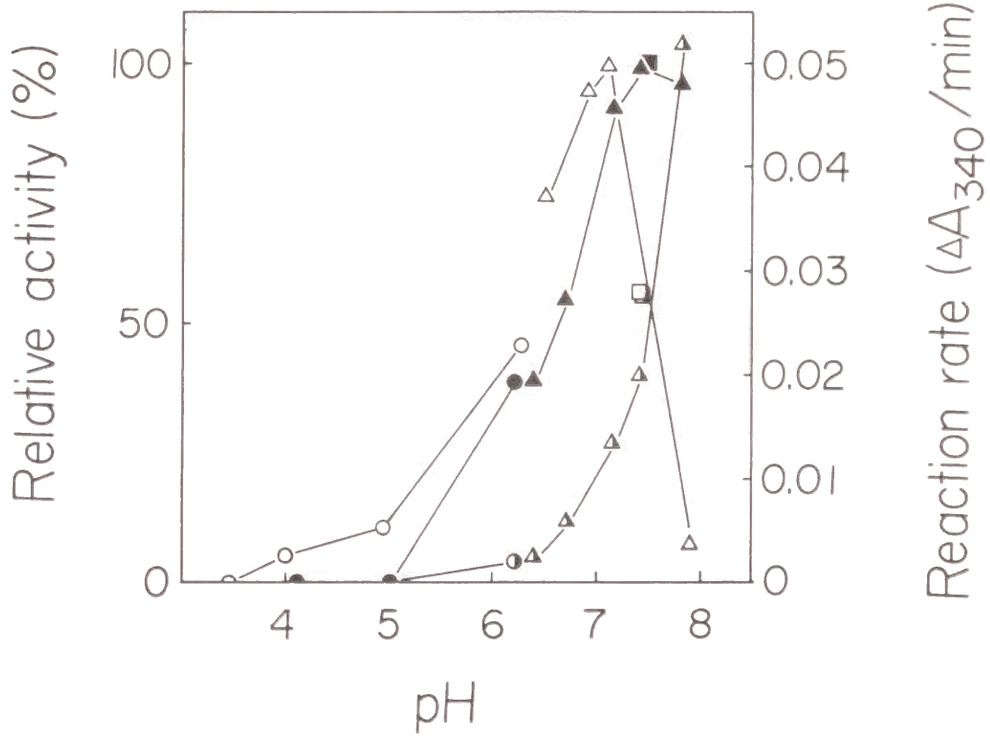


Fig. 4 Effect of pH on GST Y-1 and GST Y-2.
Enzyme activities were measured by the CDNB method as described under Materials and Methods at the indicated pH. GST Y-1 is shown by open symbols and GST Y-2 by closed symbols. The nonenzymatic reaction (half closed symbols) was also measured and subtracted from the corresponding values. The pH of the reaction mixture was measured with a pH meter and plotted. Sodium acetate buffer (○), potassium phosphate buffer (△), Tris-HCl buffer (□).

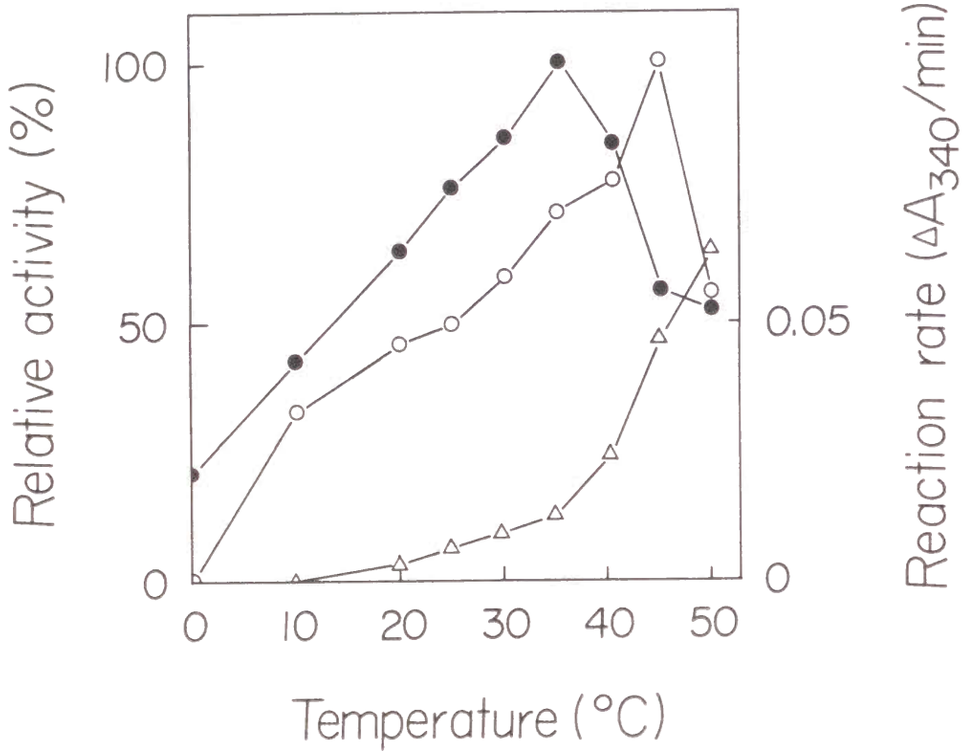


Fig. 5 Effect of temperature on GST Y-1 and GST Y-2.
Enzyme activities were measured at the indicated temperature by the CDNB method as described under Materials and Methods. The nonenzymatic reaction was subtracted from the corresponding value. GST Y-1 (○), GST Y-2 (●), nonenzymatic reaction (△).

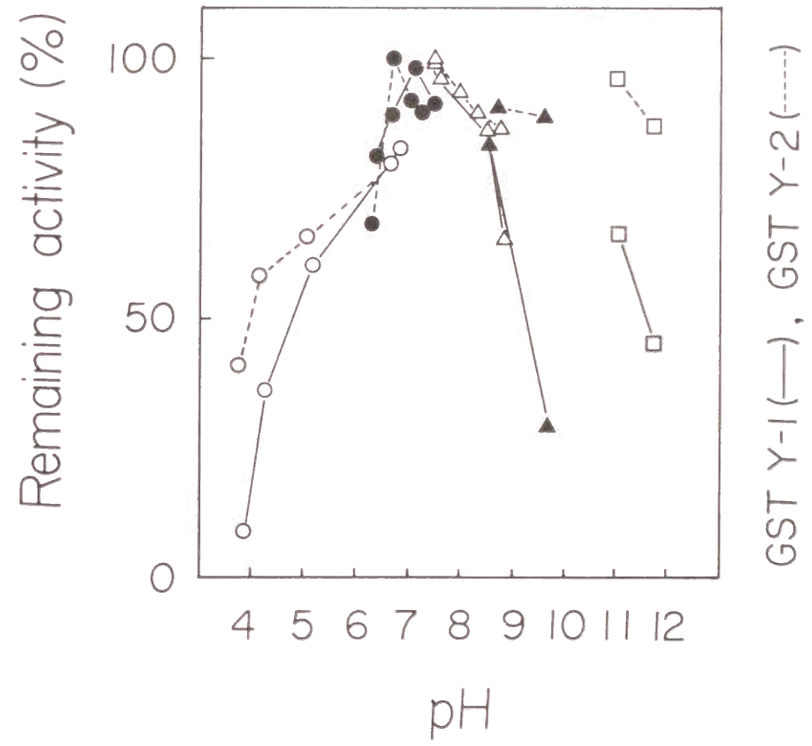


Fig. 6 pH stability of GST Y-1 and GST Y-2 .
Each isoenzyme was stored at 4°C for 1 week in the buffers with different pH containing 20 % glycerol, 2mM dithiothreitol, 1mM EDTA, and the air above the enzyme solution in the test tube was substituted with N_2 gas. The pH of the mixture was measured with a pH meter and plotted. Enzyme activities were measured by the CDNB method as described under Materials and Methods. GST Y-1 is shown by the solid line and GST Y-2 by the broken line. Buffers used were sodium acetate (○), glycine-HCl (▲), potassium phosphate (●), sodium phosphate (□), Tris-HCl (△).

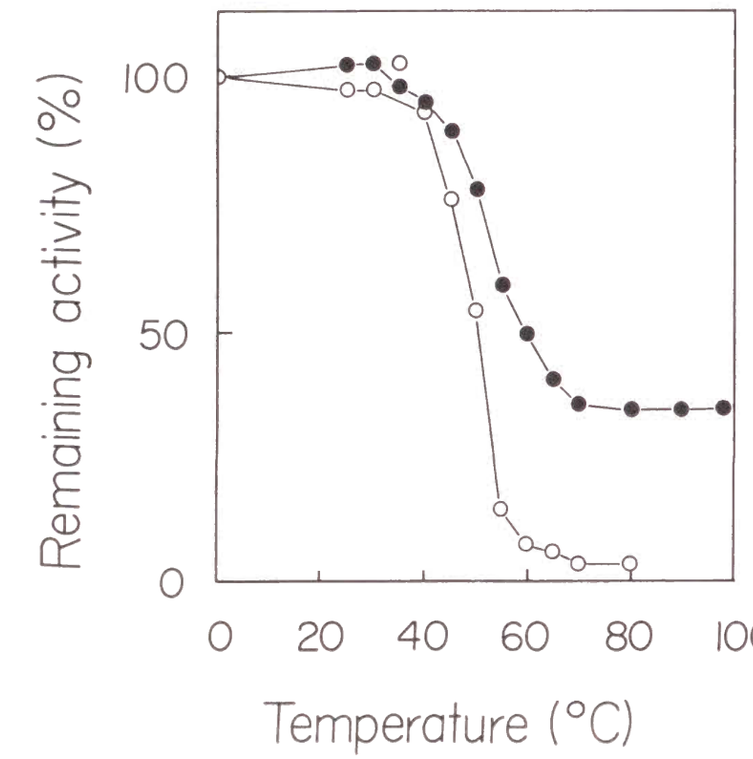


Fig. 7 Temperature stability of GST Y-1 and GST Y-2.
Each isoenzyme was incubated at various temperature for 10 min in 0.1 M potassium phosphate buffer, pH 7.0, containing 20 % glycerol, 1 mM EDTA, 10 mM sodium sulfite. Then enzyme activity was measured by the CDNB method as described under Materials and Methods. GST Y-1 (○), GST Y-2 (●).

incubation of these isoenzymes at various temperatures. GST Y-1 retained 92% of the initial activity on incubation at 40°C for 10 min and lost 96% of the activity on incubation at 70°C. GST Y-2 retained 95% of the initial activity on incubation at 40°C for 10 min and retained 34% of the activity after boiling for 10 min (Fig. 7).

Substrate specificities.

The conjugation of GSH with various electrophilic substrates was measured spectrophotometrically (Table 2). GST Y-1 showed higher specific activity (6.33 unit/mg of protein) than GST Y-2 (0.61 unit/mg of protein) when CDNB was used as an electrophilic substrate. When DNB was used as the substrate, GST Y-1 also showed

TABLE 2. Substrate specificities of GST Y-1 and GST Y-2

Substrate	Sp act in mU/mg[a]	
	GST Y-1	GST Y-2
1-Chloro-2,4-dinitrobenzene	6,300 (100)	609 (100)
o-Dinitrobenzene	490 (7.7)	81 (13.3)
1,2-Dichloro-4-nitrobenzene	ND	ND
Bromosulfophthalein	ND	ND
p-Nitrobenzoyl chloride	ND	ND
1,2-Epoxy-3-(*p*-nitrophenoxy)-propane	ND	ND
Ethacrynic acid	ND	ND
p-Nitrophenethyl bromide	ND	ND
trans-4-Phenyl-3-buten-2-one	ND	ND
4-Nitropyridine-*N*-oxide	ND	ND

[a] GST activity was measured by a spectrophotometric method as described in Materials and Methods. Relative rates are shown in parentheses. ND, Not detectable under assay conditions used.

higher specific activity (0.49 unit/mg of protein) than GST Y-2 (0.08 unit/mg of protein), but when other substrate such as bromosulfophthalein and trans-4-phenyl-3-buten-2-one were used, GST activity was not detected for either isoenzyme. Km values of GST Y-1 for GSH and CDNB were 0.51 mM and 0.16 mM and those of GST Y-2, 0.75 mM and 4.01 mM, respectively.

Effect of inhibitors.

GST activities were measured in the presence of various GST inhibitors using CDNB as an electrophilic substrate (Table 3). Both GST Y-1 and Y-2 were inhibited by hematin rather than tributyltin acetate and triphenyltin chloride. Cibacron blue 3G-A inhibited GST Y-1 significantly, whereas GST Y-2 was strongly inhibited by bromosulfophthalein.

TABLE 3. Inhibition of GST Y-1 and GST Y-2

Inhibitor	I_{50} value (μM)[a]	
	GST Y-1	GST Y-2
Cibacron blue 3G-A	0.9	>40
Tributyltin acetate	>50	40
Triphenyltin chloride	17	37
Bromosulfophthalein	27	3.2
Hematin	3.6	5

[a] I_{50} value is the concentration of inhibitor giving 50% inhibition of GST activity. CDNB was used as a substrate.

Discussion

Glutathione S-transferase activity is widely distributed from mammals to microorganisms (14). Especially in mammals, many tissue-specific (11, 12, 16) and species-specific (2, 3, 9, 21) GST isoenzymes have been purified and their properties and functions in cells have been studied extensively. GST activities have also been found in plants (10) and insects (8) and they are related to resistance to herbicides and insecticides. For microorganisms, there are only a few reports about GST activity (15, 19, 24), and GST was purified and characterized only from Mucor javanicus (1) but it has not been purified from bacteria or yeasts. Recently, the author reported that GST activities were widely distributed in yeast and was induced by DNB, one of the GST's substrates in Issatchenkia orientalis (17). Thus, the author used a culture medium containing 200μM DNB for purification of GST in this study.

GST of Issatchenkia orientalis is very unstable and it loses almost all of its activity by ammonium sulfate precipitation or by standing at 4℃ for one week without stabilizing agents (17). During purification, the author could not use ammonium sulfate fractionation and all operations had to be carried out in the presence of stabilizing agents in the pH range of 6.5 to 7.5, and the author also could not use affinity gel such as S-hexylglutathione-agarose because both isoenzymes did not adsorbed to the affinity column. Under these very restricted condition, two

GST isoenzymes were isolated to homogenity for the first time from yeast. The yield of GST Y-1 was low because it was isolated by a slight difference of hydrophobicity between GST Y-1 and Y-2. And when gradient was used in the Phenyl-Sepharose column chromatography, GST activity was eluted gradually and no separation of GST Y-1 was observed.

Multiple forms of GST in an organism is a prominent feature in the occurrence of the enzyme (23) and the author have obtained two isoenzymes GST Y-1 and Y-2 from the yeast. Both GST isoenzymes are homodimers containing the subunits with a molecular weight of 22,000 (Y-1) and 23,500 (Y-2), respectively.

Antiserum to GST Y-2 related with two kinds of cell-free extracts of I. orientalis and each formed single line of precipitation and these lines showed identity with homogeneous GST Y-2. On the contrary, antiserum to GST Y-2 did not react with homogeneous GST Y-1. From these results, two kinds of GST were distinguishable from each other. And it is very interesting to study the role and relation of two isoenzymes in the cell.

Molecular properties of GST Y-1 and Y-2 are similar to GSTs from mammals (23), but hybridization of subunits, which underlies the occurrence of multiple isoenzymes in a mammal, was not observed in our isoenzymes. Isoelectric points of the isoenzymes, pH 8.40 for Y-1 and pH 8.55 for Y-2, correspond to those of basic GSTs of mammals. Both isoenzymes showed high substrate specificity toward CDNB as is reported for mammalian and Mucor (1) GSTs. GST Y-1

showed higher specific activities than GST Y-2 when DNB or CDNB was used as a substrate. Although DNB was used as an inducer of GST, the relative rates of GST reaction toward DNB were low as compared with CDNB. Relation of the sort of inducer and the molecular species of GST synthesized in the yeast cells is interesting and is a problem to be elucidated. GSH peroxidase activity, which has been reported to coexist in some mammalian GST (20), was not detected in our isoenzymes.

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Chapter III. Detoxification Metabolism of o-Dinitrobenzene by Yeast Issatchenkia orientalis.

Glutathione(GSH) is widely distributed in organisms and plays many physiological roles in living cells. Detoxification is one of the most important roles of GSH and has been studied extensively in relation to glutathione S-transferase(GST) (7). In mammals, it has been reported that xenobiotics were excreted as mercapturic acid and it has been suggested that the initial conjugation reaction was catalyzed by GST (3). It has also been reported that there are GST activities in plants (4,8) and insects (9) and they are related to the detoxification of herbicides and insecticides, respectively. In microorganisms, metabolism of 2,4-dichloro-1-nitrobenzene by Mucor javanicus was examined and reported to be also related to GSH conjugation (1,10). However, there are few reports about a GSH-related detoxification system in yeast, in which GSH is abundant.

Recently, the author reported the distribution of GST in yeasts (6) and the purification and properties of two kinds of GST isoenzymes from I. orientalis (11). In this study the author report the detoxification of o-dinitrobenzene(DNB), a GST substrate (2), by GSH conjugation and the metabolism of GSH conjugate in yeast I. orientalis.

Materials and Methods

Reagents.

Glutathione was a generous gift from Kirin Brewery Co. Carboxypeptidase Y was from Takara Shuzo Co. o-Dinitrobenzene (DNB) was from Nacalai Tesque Chemical Co. Other reagents were of analytical grade from commercial sources.

Yeast strain and culture.

Issatchenkia orientalis was obtained from the culture collection of the laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan. In a preculture, I. orientalis was grown on 5 ml basal medium in a test tube(1.65x16.5 cm) consisting of 2 % glucose, 0.5 % peptone 0.2 % yeast extract, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4 and 0.02 % $MgSO_47H_2O$, pH 7.0, at 28°C for 24 hr with continuous shaking. The preculture was then transfered to a 500 ml Sakaguchi flask containing 100 ml basal medium described above supplemented with 200μM DNB (solubilized in ethanol), 0.1 % glycine and 0.1 % L-cysteine (6). Cultivation was carried out at 28°C with reciprocal shaking.

Preparation of cell free extract.

The cultured broth was centrifuged at 6,000 xg for 30 min. The harvested cells were washed with 0.05 M potassium phosphate buffer, pH 6.5, and then suspended in the same buffer. The suspended cells were disrupted using a Kaijo Denki ultrasonic

oscillator(20 KHz) for 10 min and a supernatant was obtained after centrifugation at 7,000 xg for 20 min.

GSH assay.

Cell culture (3ml) was centrifuged and cells were washed with water. After washing, cells were resuspended in the same volume of water and intracellular GSH was extracted by incubation at 70°C for 3 min. Extracted GSH was assayed with glutathione reductase as described by Fahey et al. (5) and expressed as the concentration (μM) per optical density of cell cultures at 610 nm.

Chemical synthesis and isolation of S-(2-nitrophenyl)glutathione and S-(2-nitrophenyl)cysteine.

S-(2-Nitrophenyl)glutathione, GSH conjugate, was chemically synthesized from DNB and GSH. The reaction mixture containing 5 mM GSH, 5 mM DNB (solubilized in ethanol) and 0.03 % ammonia was incubated at 50°C for 12 hr. After incubation, the reaction mixture was concentrated by evaporation and subjected to paper chromatography with a solvent of 1-butanol, acetic acid and water(4:1:1). After development, a yellow spot which absorbed at 254 nm was cut off and extracted with water. After concentration by evaporation, this substance was further purified using Cosmosil C18-OPN column (4x45 cm) chromatography with a mobil phase of methanol and water(1:8) at a flow rate of 1 ml/min or using HPLC as described below. S-(2-Nitrophenyl)cysteine, Cys conjugate, was also synthesized in the same way using L-cysteine in place of GSH.

Enzymatic degradation of GSH conjugate.

The reaction mixture, containing 5 mM GSH conjugate and 100 mM potassium phosphate buffer(pH 6.5) and enzymes, 10 units carboxypeptidase Y or an appropriate amount of cell-free extract, was incubated at 37°C for 12 hr. After incubation, the reaction mixture was analyzed by HPLC and TLC.

HPLC.

High performance liquid chromatography(HPLC) was performed with an M6000A pump, a U6K injector and an M440 UV detector(fixed wave length at 254 nm)Waters Assoc. (Milford, MA, USA) or a multichannel, photodiode-array UV-VIS detector(SPD-M6A) Shimadzu Co. equipped with a TSK-gel ODS-80 TMCTR column(4.6x100 mm)(Tosoh Co.) and with mobile phase of methanol, water, acetic acid (80:30:0.14) at a flow rate of 1ml per min.

In order to isolate the GSH conjugate and Cys conjugate, HPLC was performed using the same conditions as described above using a μBondapac C18 semipreparative column.

TLC.

Thin-layer chromatography(TLC) was performed using a 20x20cm TLC aluminum sheet with a 0.2 mm layer of silica gel 60F254(Art. 5735) Merck Co.. It was developed with a solvent of 1-butanol, acetic acid and water(4:1:1). The sheet was exposed to ultraviolet light(254 nm) after chromatography and GSH conjugate and its metabolites were detected.

Instruments.

A secondary ion mass spectrum (SIMS) was recorded using a

Hitachi M-80 mass spectrometer at 8 kV (primary beam gas, xenon; matrix, glycerol; silver sample stage). Proton magnetic resonance (PMR) spectra were measured using a JEOL JNM FX-90Q(90MHz) spectrometer with DSS as the internal standard. The letters s, d, t and m in the spectral data represent singlet, doublet, triplet and multiplet, respectively.

Results

Identification of chemically synthesized GSH conjugate and Cys conjugate.

The molecular weights of GSH conjugate and Cys conjugate were determined to be 428 and 242, respectively, since a protonated molecular ion was observed at m/z 429 and m/z 243 on each secondary ion mass spectrum (Fig. 1 a,b). Proton magnetic resonance (PMR) spectrum of GSH, GSH conjugate, Cys, and Cys conjugate were shown in Fig. 2-1 and 2-2, and PMR assignments of GSH conjugate and Cys conjugate were made in comparison with those of GSH and cysteine (Table I). The signals generated by the 2-nitrophenyl moiety were observed in both GSH conjugate and Cys conjugate(δ 8.2-7.4). For GSH conjugate, many signals found in the GSH moiety gave values consistent with the corresponding signals for GSH. An extreme difference was observed for the b(b1,b2)-proton signals between GSH (b:δ 2.94) and GSH conjugate(b1:δ 3.37, b2:δ 3.7), which clearly

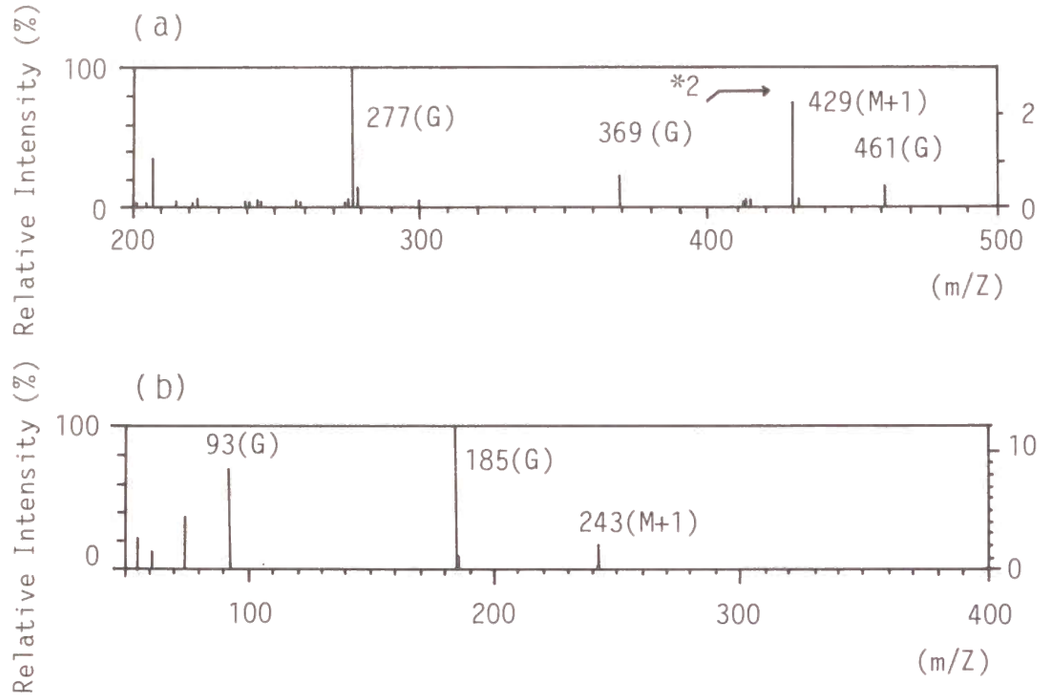


Fig. 1. Mass spectrum of GSH conjugate (a) and Cys conjugate (b).

verified that the 2-nitrophenyl moiety was attached to the sulfur atom in GSH. The a-proton of GSH conjugate showed an upfield shift ($\Delta\delta$=0.18ppm) which also suggested the attachment of the 2-nitrophenyl moiety to the sulfur atom in GSH. For Cys conjugate, a downfield shift(b1:$\Delta\delta$= 0.32ppm, b2:$\Delta\delta$=0.71ppm)and an upfield shift ($\Delta\delta$=0.32ppm) were also observed with the b1,b2-proton and the 1-proton, respectively. From these results, chemically synthesized GSH conjugate and Cys conjugate were identified as S-(2-nitrophenyl)glutathione and S-(2-nitrophenyl) cysteine, respectively. The molar absorption coefficients of S-(2-nitrophenyl)glutathione

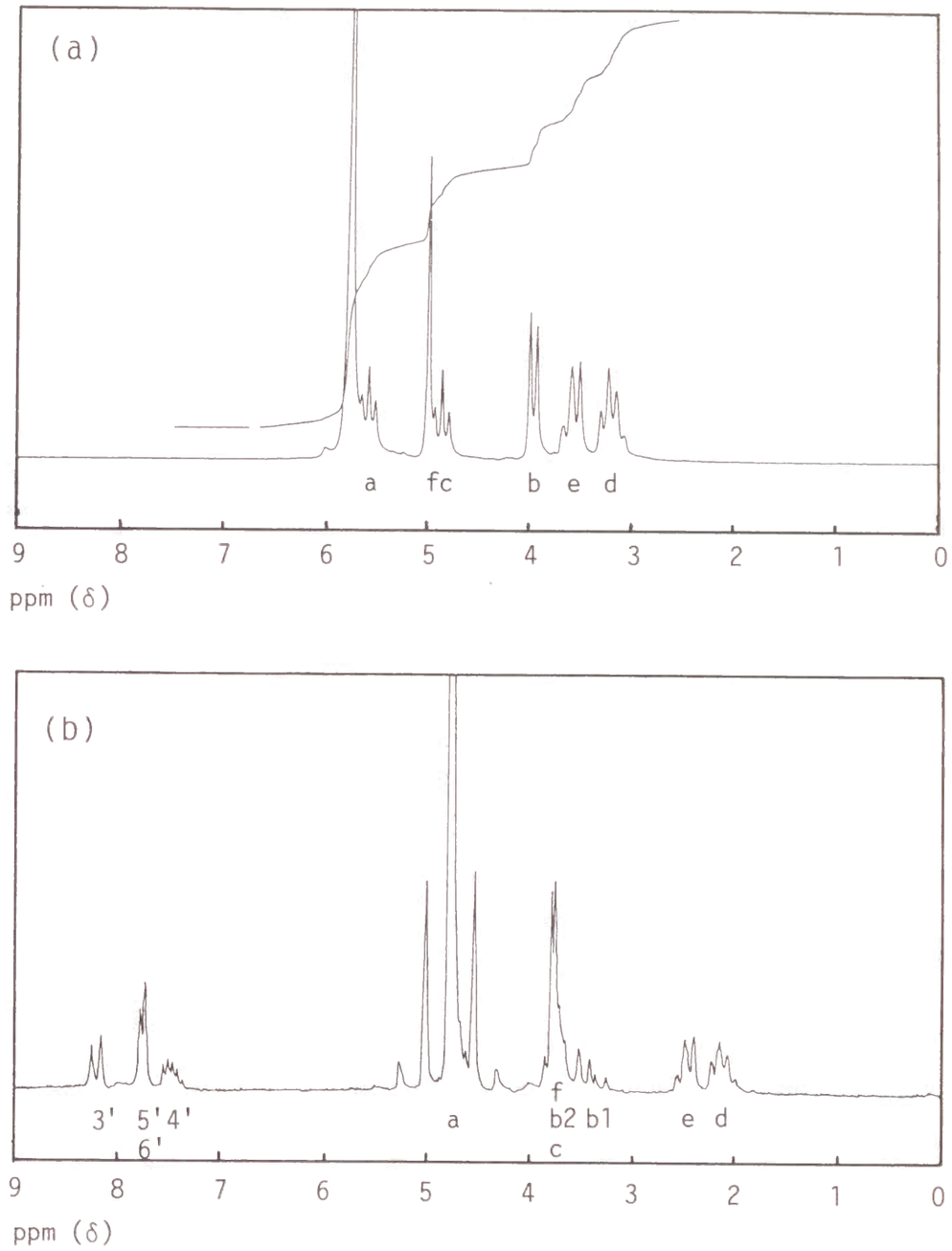
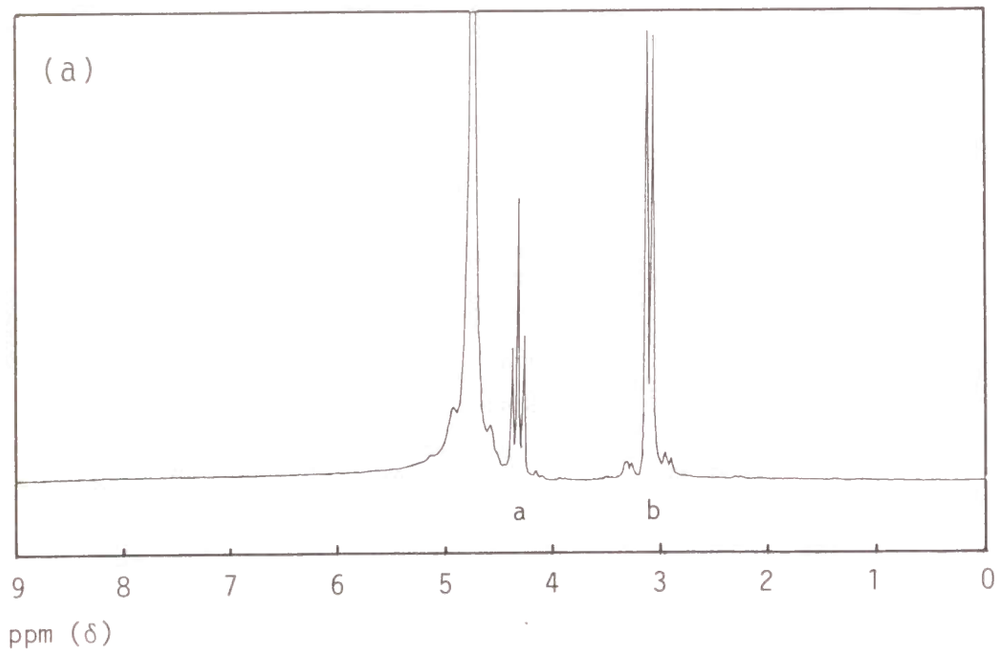


Fig. 2-1. PMR spectrum of GSH (a) and GSH conjugate (b).



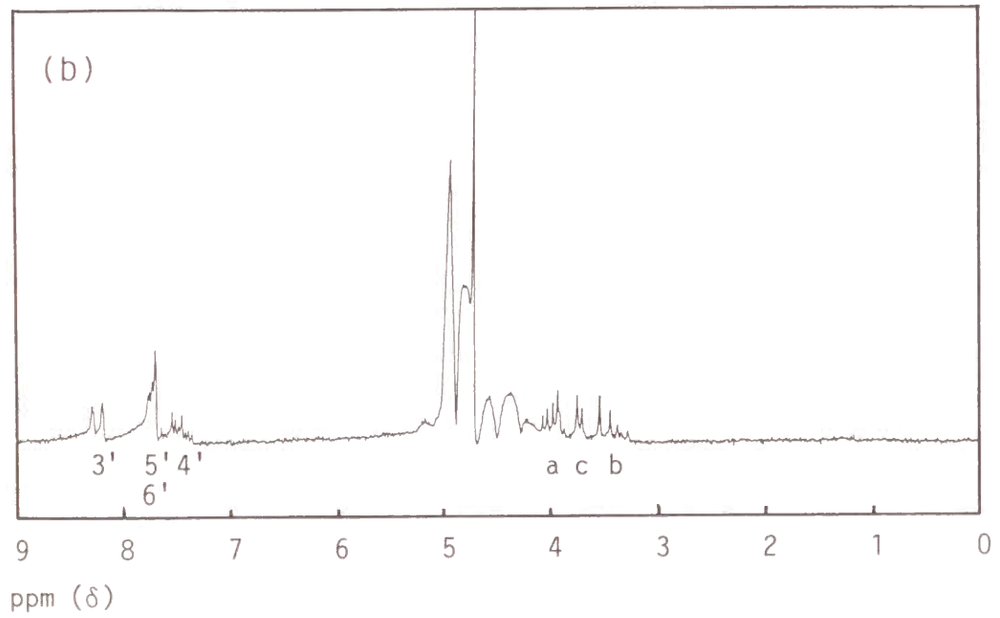
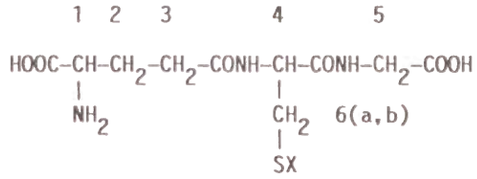
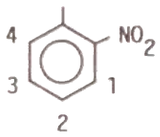


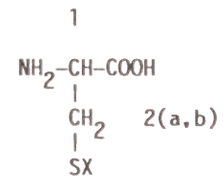
Fig. 2-2. PMR spectrum of Cys (a) and Cys conjugate (b).



GSH ; X=H
(Glutathione)

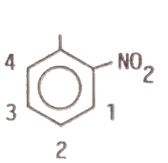
GSH conjugate ; X=
(S-(2-Nitrophenyl)glutathione)





Cys ; X=H
(L-Cysteine)

Cys conjugate ; X=
(S-(2-Nitrophenyl)cysteine)



PMR Spectral data of GSH, GSH conjugate, Cys, Cys conjugate
(chemical shifts, multiplicities and J-values in Hz)

	GSH		GSH conjugate		Cys		Cys conjugate	
	δ	Coupling(J)	δ	Coupling(J)	δ	Coupling(J)	δ	Coupling(J)
a	4.57	t(6.2)	4.75	dd(5.7, 9.3)*	4.30	t(5.0)	3.98	dd(4.0, 8.6)
b (b1)	2.94	d(5.8)	3.37	dd(9.3, 14.1)	3.08	d(5.0)	3.40	dd(8.6, 14.7)
(b2)			~3.7	dd(5.7, 14.1)*			3.79	dd(4.0, 14.7)
c	3.84	t(6.7)	~3.7	m				
d	2.17	dt(6.6, 7.1)	2.09	m				
e	2.57	t(7.1)	2.47	m				
f	3.97	s	~3.7	m				
3'	-	-	8.20	d(7.9)	-	-	8.23	dd(1.1, 7.9)
5',6'	-	-	7.76	d(4.0)	-	-	7.72	m
4'	-	-	7.46	m	-	-	7.43	m

* Coupling patterns could not be observed because of an overlap with other signals.

($\Delta\varepsilon$=2.3 $mM^{-1}cm^{-1}$) and S-(2-nitrophenyl)cysteine ($\Delta\varepsilon$=2.7 $mM^{-1}cm^{-1}$) were obtained at 373 nm and 368 nm, respectively.

GSH conjugation of DNB by yeast glutathione S-transferase.

GSH conjugate was produced by GST from yeast I. orientalis. Purified GST(100 munits) of I. orientalis was added to the reaction mixture containing 1 mM GSH, 1 mM DNB and 50 mM potassium phosphate(pH 7), and incubated for 10 min at 30°C. After incubation, the reaction mixture was analyzed by HPLC and TLC in comparison with the authentic GSH conjugate. By HPLC analysis, the enzymatically produced GSH conjugate was detected at the same elution time as the authentic substance, i.e. 8 min.

Metabolism of DNB in I. orientalis cells.

Issatchenkia orientalis was cultured in the presence of 200μM DNB and culture broth was sampled every 6 or 12 hr followed by HPLC analysis(Fig.3). As reported before, DNB suppresses the cell growth of I. orientalis for about 24 hr and after a lag-phase cells increased exponentially with induced GST activity (6). In the early log phase, DNB in the culture broth began to decrease in contrast to cell growth and finally disappeared(Fig.3). At the same time a peak having the same retention time as Cys conjugate emerged(Fig.3) but the peak of GSH conjugate was barely detected. The peak showed the same elution time (4.5 min) as authentic Cys conjugate on HPLC and was analyzed in detail by using multiple-

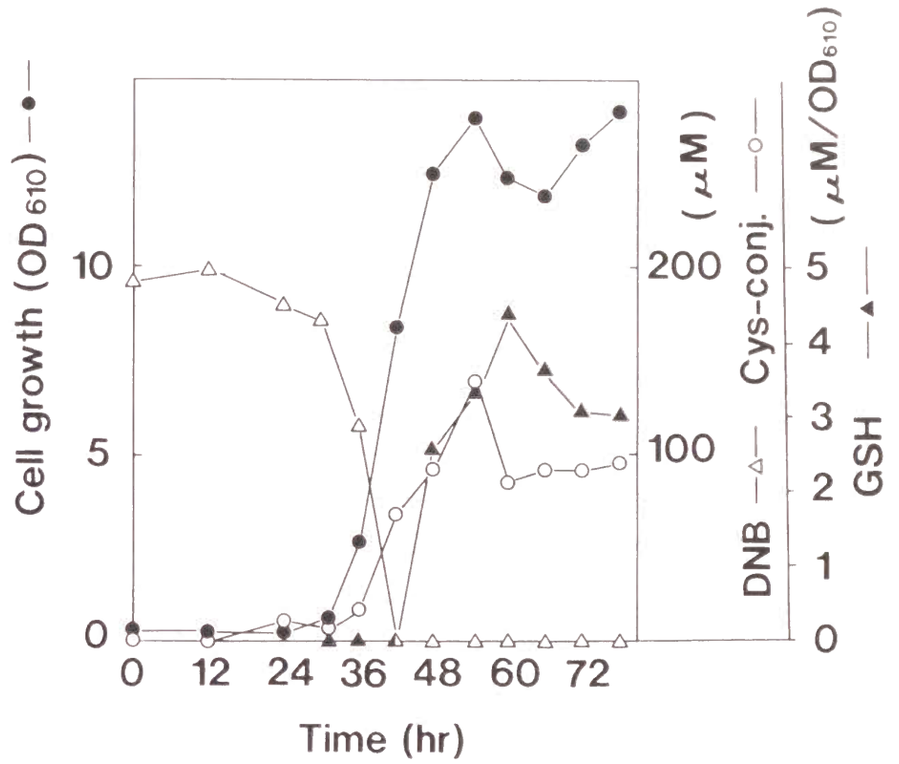


Fig. 3 Metabolism of DNB during cultivation. *Issatchenkia orientalis* was cultured in the presence of 200μM DNB at 28°C with reciprocal shaking. Cell growth at OD_{610}(●) and intracellular GSH concentration(▲ ;μM/OD_{610}) were examined. The concentration of DNB(△ ;μM) and Cys conjugate (○ ;μM) in the culture broth were measured by HPLC.

wavelength data obtained from a photodiode array detector. The multiple-wavelength data of the new substance was compared with that of Cys conjugate, and the new substance showed a 99.5 % similarity with S-(2-nitrophenyl)cysteine. When culture medium containing DNB was incubated without inoculation, the peak for the Cys conjugate was not observed. These result suggest that DNB was

at first GSH conjugated by GST and then the produced GSH conjugate was immediately metabolized and finally released into the cultured broth as a Cys conjugate.

Effect of DNB to the intracellular GSH.

Intracellular GSH was extracted from cultured cells and quantified by the glutathione reductase method as described in MATERIALS AND METHODS. Issatchenkia orientalis was cultured in the presence of 200μM DNB and after lag phase cells were collected every 6 hr and the intracellular GSH concentration was determined (Fig.3). In the early log phase, intracellular GSH was not detected in cultured cells, but it began to accumulate just after DNB in the cultured broth had completely disappeared. After that, intracellular GSH increased until cells reached a stationary phase and then slightly decreased and settled at the concentration of 3μM/OD610(Fig.3).

Effect of DNB, GSH conjugate, Cys conjugate to the cell-growth of I. orientalis.

I. orientalis was cultured in the presence of various concentrations(0-1 mM)of DNB, GSH conjugate or Cys conjugate at 37℃ for 24 hr and cell growth was examined by measuring optical density at 610 nm(Fig.4). DNB, 100μM, suppressed cell growth to 1/6 of the control and 200μM-1 mM DNB suppressed cell growth completely for 24 hr. On the other hand, GSH conjugate of DNB and

its second metabolite, Cys conjugate, did not suppress cell growth at the concentration of 1mM, and there was no difference between these culture and the control(no addition) culture(Fig.4). GST activity was measured in the cell-free extract of I. orientalis cultured in the presence of DNB, GSH conjugate or Cys conjugate. Though cell-free extract from the cells cultivated with DNB showed

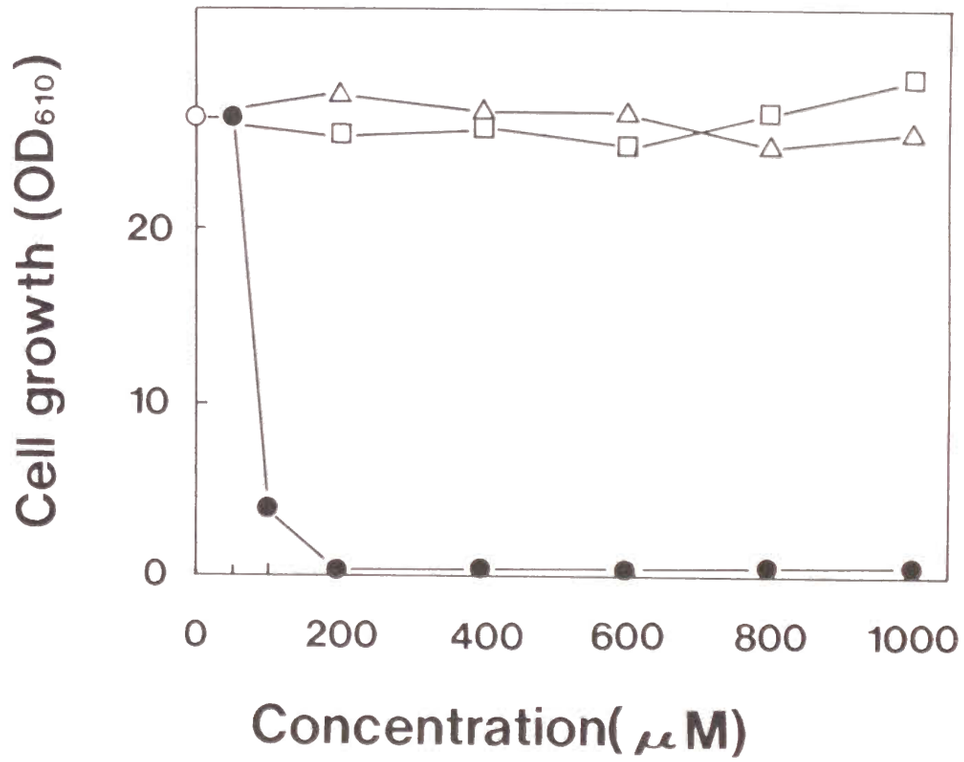


Fig. 4 Effect of DNB, GSH conjugate and Cys conjugate on the cell growth of *Issatchenkia orientalis*. *I. orientalis* was cultured in the presence of various concentrations(0-1mM) of DNB (●), S-(2-nitrophenyl)glutathione (△) and S-(2-nitrophenyl) cysteine(□) for 24hr at 28℃ with reciprocal shaking, and cell growth was monitored.

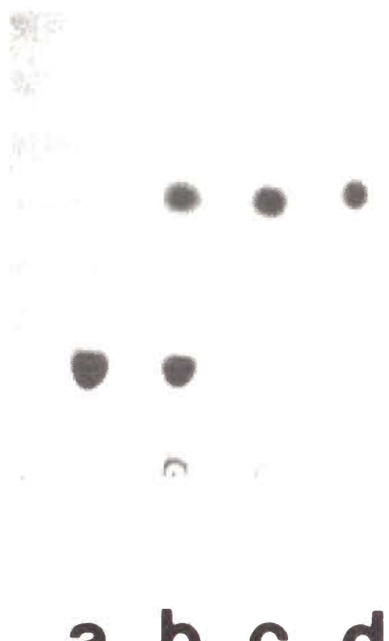


Fig. 5 Thin-layer chromatography of GSH conjugate and its metabolites. Enzymatic degradation of GSH conjugate and thin-layer chromatography were carried out as described in MATERIALS AND METHODS. Lane a: GSH conjugate, lane b: GSH conjugate degradated by cell free extract of *I. orientalis* at pH 6.5, lane c: GSH conjugate degraded by carboxypeptidase Y, lane d: Cys conjugate.

higher GST activity than control(no addition), both GSH conjugate and Cys conjugate did not show inducible effects on GST activity (data not shown).

Enzymatic degradation of GSH conjugate.

In order to identify the metabolic pathway of GSH conjugate, it was treated with cell-free extract of I. orientalis and

carboxypeptidase Y and metabolites were compared by HPLC and TLC analysis. When GSH conjugate was incubated with cell-free extract of I. orientalis at pH 6.5, a new spot besides GSH conjugate was detected at a position above the GSH conjugate. And the same result was obtained when GSH conjugate was incubated with carboxypeptidase Y. Each new spot coincided with the spot for the authentic Cys conjugate(Fig.5). And the same result was observed by HPLC analysis(data not shown).

Discussion

In the early log phase, intracellular GSH was not detected in the cells cultured with DNB and just after DNB was metabolized, it began to accumulate. Recently, the author reported that GST activity was induced by DNB and the highest activity was observed in the log phase (6). From these result, it was strongly suggested that in I. orientalis cells cultured with DNB, intracellular GSH was mainly used for conjugation of DNB by induced GST in a process of detoxification, so that intracellular GSH was not detected in the cells in the early log phase. Just after the conjugation reaction was completed, GSH began to accumulate in the cells. In the early log phase GSH synthesis may also be induced and overproduced GSH accumulated at 60 hr. Following this, because of end product feedback inhibition, over produced GSH might be

metabolized and settled at a concentration of 3μM/OD610.

Electrophiles such as DNB react with nucleophilic groups in proteins and nucleic acids of organisms and this reaction results in chemical modification of such nucleophilic groups. Such chemical modification of proteins and nucleic acids cause loss of cell function, tissue necrosis, mutations and carcinogenesis. GSH reacts with these electrophiles by the catalysis of GST and detoxifies them. The cytotoxicity of DNB completely disappeared after GSH conjugation at the concentration of 1 mM and, in addition, the toxicity of S-(2-nitrophenyl)cysteine, the second metabolite of GSH conjugate, was not observed. From these results, there appears to be a GSH-related detoxification system in yeast I. orientalis.

In the rat liver, it has been reported that xenobiotics were conjugated with GSH by GST and produced GSH conjugates were further metabolized by γ-glutamyltranspeptidase, cysteinylglycinase, N-acetyl transferase through the mercapturic acid biosynthetic pathway (3). In I. orientalis, peptidase activity were detected in the cell-free extract. From enzymatic degradation experiments, it can be suggested that an enzyme such as a carboxypeptidase-like enzyme are associated with the metabolism of generated GSH conjugate. In contrast to mammals, S-(2-nitrophenyl)cysteine was released into the cultured broth without N-acetylation.

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Chapter IV. Glutathione S-Transferase in Yeast: Induction of mRNA, cDNA Cloning and Expression in Escherichia coli.

Glutathione S-transferase(EC 2.5.1.18) catalyzes the conjugation of glutathione to a large variety of electrophilic substrates. Electrophilic xenobiotics which are mutagens and carcinogens are detoxified by glutathione conjugation. Glutathione S-transferase is widely distributed from bacteria to mammals and many isoenzymes have been isolated and characterized in detail (8,9). Transcriptional induction of glutathione S-transferase by drugs and herbicides were also reported in mammals and plants (13,19). The induction of glutathione S-transferase by toxic xenobiotics indicates the existence of a glutathione related detoxification system. Therefore it is very important to study the mechanism of glutathione S-transferase regulation. Recently the author reported the glutathione related detoxification and metabolism of o-dinitrobenzene(DNB) in yeast Issatchenkia orientalis (17). DNB, which suppressed cell growth of I. orientalis for about 24 h, was detoxified by glutathione conjugation and further metabolized.

Recently, genetic engineering approaches to glutathione S-transferase have resulted in many cDNA clones coding for glutathione S-transferases being isolated and the deduced amino acid sequences for these subunits have been reported (9).

Expression of glutathione S-transferase cDNA in E. coli has also been reported in mammals and plants (1,12,18).

Although much progress has been made in studies of glutathione S-transferase in mammals and plants, there has been little information concerning glutathione S-transferase in microorganisms. Especially, glutathione is abundant and is manufactured mainly from yeast, but information about the physiological role of glutathione and glutathione S-transferase in yeast cells is also lacking.

The author have previously reported the distribution of glutathione S-transferase in yeasts (6) and isolated and characterized two glutathione S-transferase isoenzymes from I. orientalis (16). For further investigations such as enzyme structural and functional studies, a large quantity of enzyme is necessary. One way to achieve this is to isolate a cDNA encoding the glutathione S-transferase subunit which expresses large quantities of enzyme. Here, the author report the induction of glutathione S-transferase Y-2 mRNA by DNB in yeast I. orientalis, isolation of cDNA clones coding for glutathione S-transferase Y-2 and its expression in E. coli.

Materials and Methods

Materials.

Restriction enzymes, other DNA modifying enzymes and plasmid pUC118 were purchased from TAKARA Shuzo(Kyoto, Japan). Oligo (dT)-cellulose was from Collaborative Res.(USA). Wheat germ extract, cDNA synthesis kit, blotting detection kit, (α-^{32}P)dCTP, (γ-^{32}P) ATP and (^{35}S)methionine were purchased from Amersham(UK). Eco RI /Not I adaptor was from Pharmacia (Sweden). Lysylendopeptidase was from Wako pure chemicals(Japan).

Yeast and Bacterial culture.

The cultivation condition of I. orientalis was described before(16). Transformants harboring plasmid pUC118 derivatives were cultured in LB broth with 50μg/ml ampicillin.

Protein sequence analysis.

Purified glutathione S-transferase Y-2(1mg) was reacted with 3.4 μg of lysyl endopeptidase in 4 M urea, 0.01 M tris-HCl(pH 9) at 30 °C for 2 h. The reaction mixture was subsequently eluted by HPLC equipped with a Cosmosil 5C18-p column in a 0-60% CH_3CN gradient containing 0.1% trifluoroacetic acid and each peptide fragment was collected. The amino acid sequence was determined in 100μg samples by a gas phase protein sequencer, Model 477A (Applied Biosystems, USA).

Isolation of poly (A)$^+$RNA.

Poly (A)$^+$RNA was isolated from cells at early log phase by

phenol extraction and LiCl precipitation after Zymolyase treatment (15) and oligo dT-cellulose chromatography.

Translation of mRNA in a cell-free system.

Poly $(A)^+$RNA obtained from I. orientalis was translated in a wheat germ extract system for 1 h at 25℃ (10). The reaction mixture (145 µl) contained 75 µl wheat germ extract, 20 µl 1 M potassium acetate, 10µl 1mM amino acids (without methionine), 13.9 MBq (^{35}S)methionine (1000mCi/mmol) and 5µg of poly $(A)^+$RNA. Incorporation of (^{35}S)methionine was determined by spotting 3µl reaction mixture on Whatman 3MM filter papers, which were subsequently soaked in 10 % trichloroacetic acid for 10 min and boiled in 5 % trichloroacetic acid for 3 min. After rinsing twice in ethanol, the paper were dried and the radio-activity was counted in a liquid scintillation counter. For analysis of total translated protein, 20 µl 1:10 diluted reaction mixtures were subjected directly to SDS-poly acrylamide gel electrophoresis (SDS-PAGE) followed by fluorography of dried gels to visualize the total protein.

Immunoprecipitation.

Glutathione S-transferase Y-2 was recovered from the translation mixtures by immunoprecipitation with anti-glutathione S-transferase Y-2 immunoglobulin(Ig) G and Pansorbin cells, Calbiochem(USA). Translation mixtures were diluted 1:10 with immuno buffer containing 50 mM tris-HCl(pH 7.5), 0.15M NaCl, 0.1 % Triton X-100 and 5 mM EDTA, and centrifuged at 10,000 xg for 5 min. Pre-

immune rabbit IgG(5µl) was added to the supernatant and incubated for 1 h at room temperature followed by the addition of 130µl Pansorbin cells and another 1 h incubation. After centrifugation, 5µl of rabbit anti-glutathione S-transferase Y-2 IgG was added to the supernatant followed by addition of 130µl Pansorbin cells and incubated as described above. The immuno-complex was pelleted by centrifugation and washed five times with immuno buffer. Antigen was eluted from immunocomplexes by boiling in SDS-PAGE sample buffer. After centrifugation, supernatants containing antigen were subjected to SDS-PAGE (7). Radiolabeled glutathione S-transferase Y-2 was identified by fluorography (2) using Amplify(Amersham) and quantified by densitometric analysis of the fluorogram.

Construction of a cDNA library.

Poly $(A)^+$RNA isolated from I. orientalis cells cultured in the presence of DNB was fractionated on a 5-20 % sucrose gradient (Fig.3). Each fraction was used for in vitro translation of wheat germ extract followed by immunoprecipitation, and after SDS-PAGE enriched glutathione S-transferase Y-2 mRNA fractions were identified by fluorography (Fig.4). Complementary DNA (cDNA) to I. orientalis mRNA was synthesized by AMV reverse transcriptase and the second strand DNA was synthesized by DNA polymerase I(3). Both 3'- and 5'- ends of cDNA were blunted by T4-DNA polymerase and Eco RI/Not I Adaptors (Pharmacia) were ligated to both sites of the double-stranded cDNA. After ligation, double-stranded cDNAs were ligated to the Eco RI site of pUC118 and transformed in E. coli

DH5α(5).

Enzyme assay for glutathione S-transferase.

Glutathione S-transferase activity was assayed spectrophotometrically with 1-chloro-2,4-dinitrobenzene as the substrate according to Habig et al. (4). One enzyme unit was defined as the amount of enzyme which produced 1μmole of S-(2,4-dinitrophenyl) glutathione per min.

Immunoblot analysis.

E. coli lysate or purified yeast glutathione S-transferase Y-2 were applied to SDS-PAGE then electroblotted onto polyvinylidene difluoride(PVDF) membranes (11). The PVDF membranes were blocked with TBS-T(tris-buffered saline, pH 7.6, and 0.1 % Tween 20) containing 5 % dried milk, for 1 h at room temperature then incubated with a 1:400 dilution of rabbit antiserum to glutathione S-transferase Y-2 (16) for 1 h at room temperature. After washing membranes once more with TBS-T, they were incubated with a 1:500 dilution of anti-rabbit immunoglobulin biotinylated species-specific whole antibody (from donkey) for 20 min at room temperature, washed and incubated with a 1:3000 dilution of streptavidin-alkaline phosphatase conjugate and washed again. Glutathione S-transferase Y-2 was detected by adding nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in diethanolamine-HCl buffer(pH9.5), after which, the membranes were washed with water and air dried.

In vitro translation and quantitation of glutathione S-transferase Y-2 mRNA.

The author reported previously that when Issatchenkia orientalis was cultured in the presence of 200μM DNB, cell-growth was suppressed for about 24 h and after this lag phase, cells began to increase exponentially with a 4-fold higher glutathione S-transferase activity (6). In order to investigate the induction mechanism, poly $(A)^+$RNA was isolated from I. orientalis cells cultured in the presence or absence of DNB and each poly $(A)^+$RNA was translated in the wheat germ cell-free protein synthesizing system using (^{35}S)methionine as the labeling amino acid. Each translation mixture was subjected to immunoprecipitation utilizing anti-glutathione S-transferase Y-2 IgG. The immunoprecipitable products and total translated products were subjected to SDS-PAGE followed by fluorography. The total translation products from poly $(A)^+$RNA from cells cultured with DNB(Fig.1, lane B) were less than 1/3 of those from normally cultured cells as determined by (^{35}S)methionine incorporation. In spite of this translational difference, the immunoprecipitated translation products from poly $(A)^+$RNA of cells cultured with DNB were detected as one polypeptide band with a molecular weight identical to the purified glutathione S-transferase Y-2 subunit(Fig.1,lane B-2). This band incorporated approximately 37 times more (^{35}S)methionine than that from normally

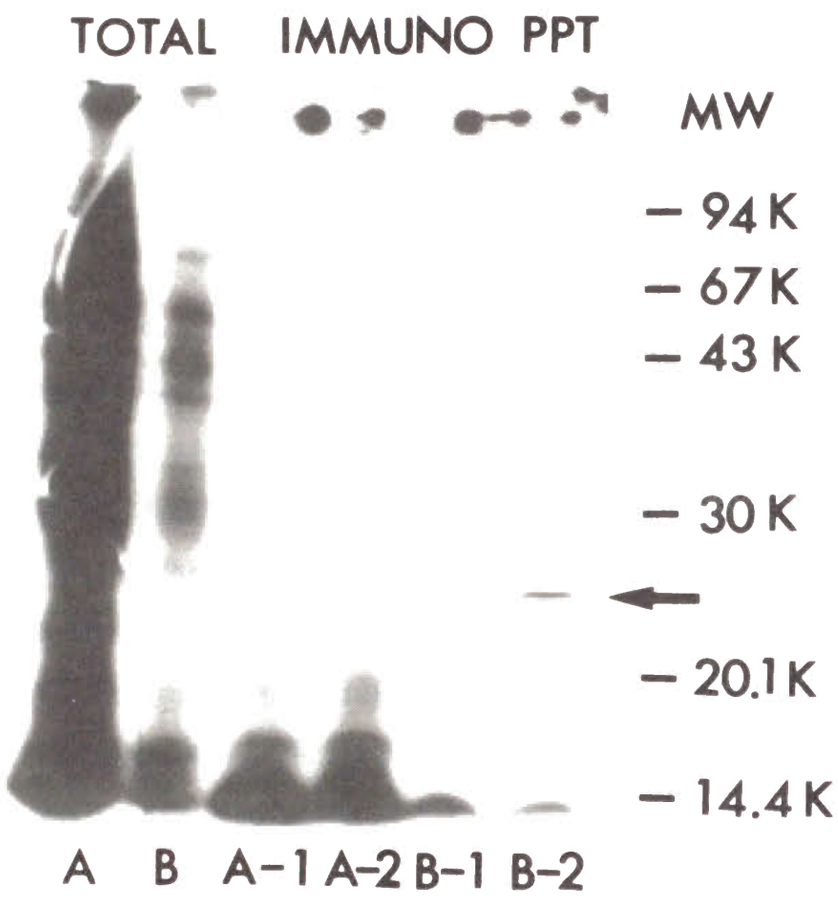

Fig. 1. Fluorogram of SDS-poly acrylamide gel of total and immunoprecipitated translation products using poly (A)⁺RNA from *I. orientalis*. Translation was carried out with equal amount of poly(A)⁺RNA and equal volume of total translation products or immuno precipitated products were layered on the 12.5 % SDS-poly acrylamide gel. Total translation products(20 μl of 1:10 dilution) of poly (A)⁺RNA from *I. orientalis* grown without and with DNB were shown in lane A and B. Lane A-1 and A-2 represent the immunoprecipitated translation products of poly (A)⁺RNA from *I. orientalis* grown without DNB using rabbit pre-immun (lane A-1) and anti-glutathione S-transferase Y-2 (lane A-2) Ig G and Lane B-1 and B-2 represent that with DNB using pre-immun (lane B-1) and anti-glutathione S-transferase Y-2 (lane B-2) Ig G.

cultured cells(Fig.1, lane A-2,). When pre-immune IgG was used in place of glutathione S-transferase Y-2 IgG, no immuno-precipitable products were observed(Fig.1, lane A-1,B-1). These results suggest that glutathione S-transferase Y-2 mRNA in I. orientalis was strongly induced by DNB at the level of transcription. Electrophiles such as DNB are thought to chemically modify the nucleophilic groups of proteins and nucleic acids of organisms. Because of chemical modification of nucleophilic groups in mRNA by DNB, translation might decrease to less than 1/3 that of normal cells which may cause cell suppression in I. orientalis. The author have no information as to whether glutathione S-transferase Y-2 mRNA is resistant to chemical modification by DNB or whether the induction level of glutathione S-transferase Y-2 mRNA is superior to the level of chemical modification of mRNA.

Partial amino acid sequence of glutathione S-transferase Y-2 and synthesized oligonucleotide probes.

Purified glutathione S-transferase Y-2 and a lysyl endopeptidation glutathione S-transferase Y-2 fragment were sequenced and 15 and 26 amino acids from each N-terminus were determined, respectively(Fig. 2). Two kinds of oligonucleotide probes(probe 1, probe 2) were synthesized complementary to the sequences corresponding to amino acids 6-10 in glutathione S-transferase Y-2 <1> and amino acids 5-10 in the lysylendo-peptidation glutathione S-transferase Y-2 fragment <2>,

respectively(Fig. 2), using DNA synthesizer Model 381A(Applied Biosystems). Probe 1 (14 mer) and 2 (17 mer) were mixtures of 48 and 64 different oligonucleotides, respectively.

```
                    5              10              15
(1)  N-Thr-Phe-Ala-Thr-Val-Tyr-Ile-Lys-Pro-His-Thr-Pro-Arg-Gly-Asp

                  5'-UAU-AUU-AAA-CCU-CA-3'
                       C   C   G   C
                           A       A
                                   G

Probe 1           3'-ATA-TAA-TTT-GGA-GT-5'
                       G   G   C   G
                           T       T
                                   C

                    5              10              15              20              25
(2)  N-Gln-Phe-Gly-Val-Asp-Phe-Thr-His-Tyr-Pro-Asn-Val-Glu-Arg-Phe-Thr-Gly-Glu-Val-Ser-Gln-His-Pro-Ile-Ile-Lys

                5'-GAU-UUU-ACU-CAU-UAU-CC-3'
                   C   C   C   C   C
                           A
                           G

Probe 2         3'-CTA-AAA-TGA-GTA-ATA-GG-5'
                   G   G   G   G   G
                           T
                           C
```

Fig. 2. N-Terminal amino acid sequence of glutathione S-transferase Y-2 and lysyl endopeptidation fragment of glutathione S-transferase Y-2, and synthetic oligonucleotides used as the probes. N-Terminal amino acid sequences were determined as described in MATERIALS AND METHODS. Oligonucleotide probes were synthesized complementary to the all possible DNA sequences corresponding to amino acids 6-10 in glutathione S-transferase Y-2 (probe 1) and 5-10 in lysyl endopeptidation fragment of glutathione S-transferase Y-2 (probe 2).

CDNA cloning and expression in E. coli.

A cDNA library was constructed as described in Materials and methods. Poly $(A)^+$RNA isolated from I. orientalis cells cultured in the presence of DNB was fractionated on a 5-20 % sucrose gradient (Fig.3). Each fraction was used for in vitro translation of wheat germ extract followed by immunoprecipitation, and after SDS-PAGE enriched glutathione S-transferase Y-2 mRNA fractions were identified by fluorography (Fig.4). Complementary DNA (cDNA) to I. orientalis mRNA was synthesized by AMV reverse transcriptase and ligated to the Eco RI site of pUC118 and transformed in E. coli

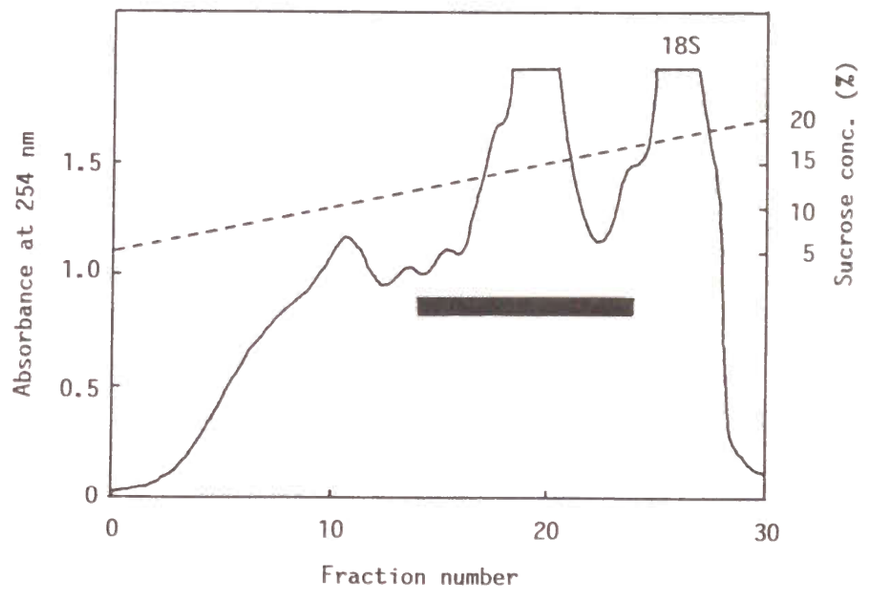

Fig. 3. Sucrose gradient fractionation of poly $(A)^+$RNA. 500μg poly $(A)^+$RNA isolated from *I. orientalis* was fractionated on 5-20% sucrose gradient. Ultracentrifugation was performed at 40,000 rpm for 15 h and then it was fractionated 400μl each.

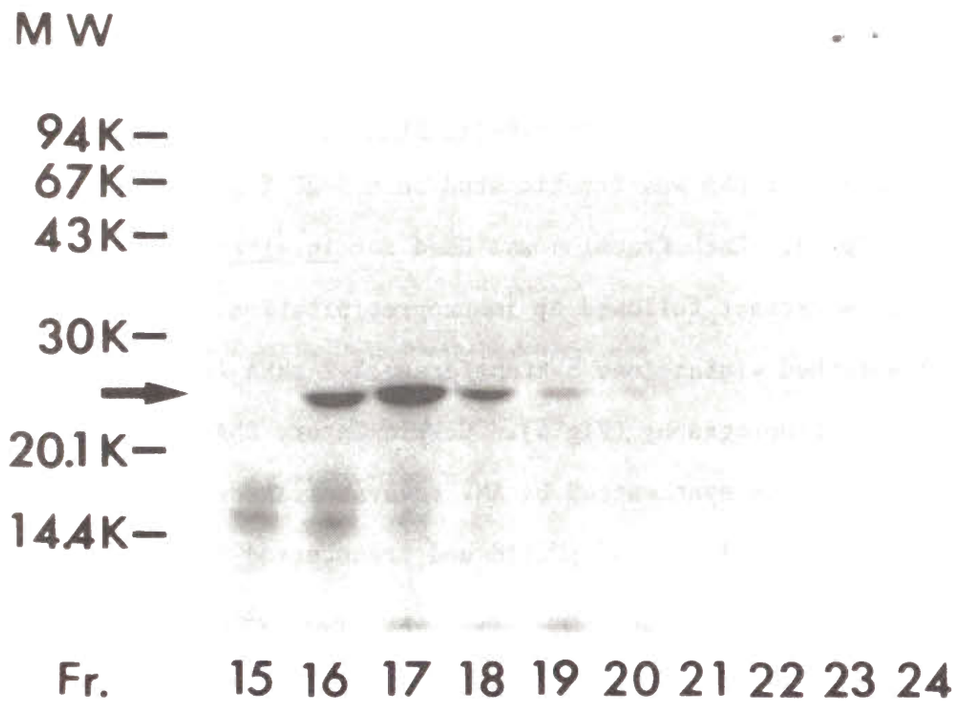

Fig. 4. Fluorogram of SDS-PAGE of immunoprecipitated translation products using poly (A)⁺RNA of each fraction. Translation was carried out with poly (A)⁺RNA of each fractions and immuno-precipitated products were layered on to 12.5% SDS-poly acrylamide gel. The synthesized GST Y-2 protein was seen in Fr. 16-18.

DH5α(5). The library was screened by colony hybridization (14) using two kinds of (^{32}P)labeled synthesized oligo-nucleotide probes. Twenty positive clones were obtained that hybridized with both probes from about 6,000 clones screened (Fig. 5). Cell-free extracts from seven of 20 positive clones expressed glutathione S-transferase activity. Antiserum to glutathione S-transferase Y-2

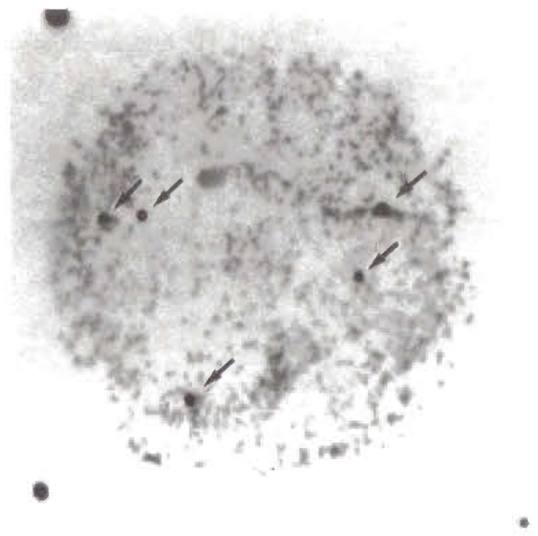

Fig. 5. Colony hybridization. Arrows indicate the positive clones.

reacted with these and formed a precipitin line that was identical to that of purified glutathione S-transferase Y-2 in the immuno-diffusion test (Fig.6). One of the positive clones, E. coli strain DH5α containing plasmid pHT108 (DH5α/pHT108) expressed approximately 28 times more glutathione S-transferase activity than DH5α containing plasmid pUC118 (DH5α/ pUC118) after IPTG induction(Table I). Cell lysates of DH5α/ pHT108, DH5α/pUC118 and purified glutathione S-transferase Y-2 were run on 12.5 % SDS-PAGE followed by western blotting, and total protein was detected by Coomassie blue staining(Fig. 7-B). DH5α/ pHT108 lysates had a new

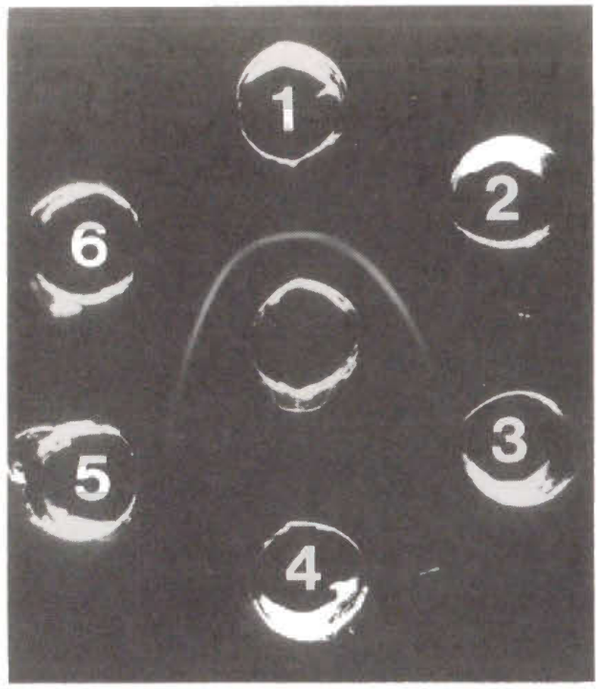

Fig. 6. Immunodiffusion of GST Y-2 and cell lysate of *E. coli* with antiserum to GST Y-2. Wells: 1. GST Y-2, Cell lysate of *E. coli* harboring plasmide pHT108: 2, pUC118: 3, pHT110: 5, pHT120: 6, and E. coli DH5α : 4.

Table I. Enzyme activity of cloned glutathione S-transferase.

	Specific activity (mU/mg)	
	DH5α/pHT108	DH5α/pUC118
$IPTG^+$	78.7	2.8
$IPTG^-$	20.1	2.9

E. coli strain DH5α harboring plasmid pHT108 (DH5α/pHT108) and plasmid pUC118 (DH5α/pUC118) were grown in the presence ($IPTG^+$) and absence ($IPTG^-$) of 1mM IPTG for 12 h at 37°C. Glutathione S-transferase activity was assayed with 1-chloro-2,4-dinitrobenzene as the substrate.

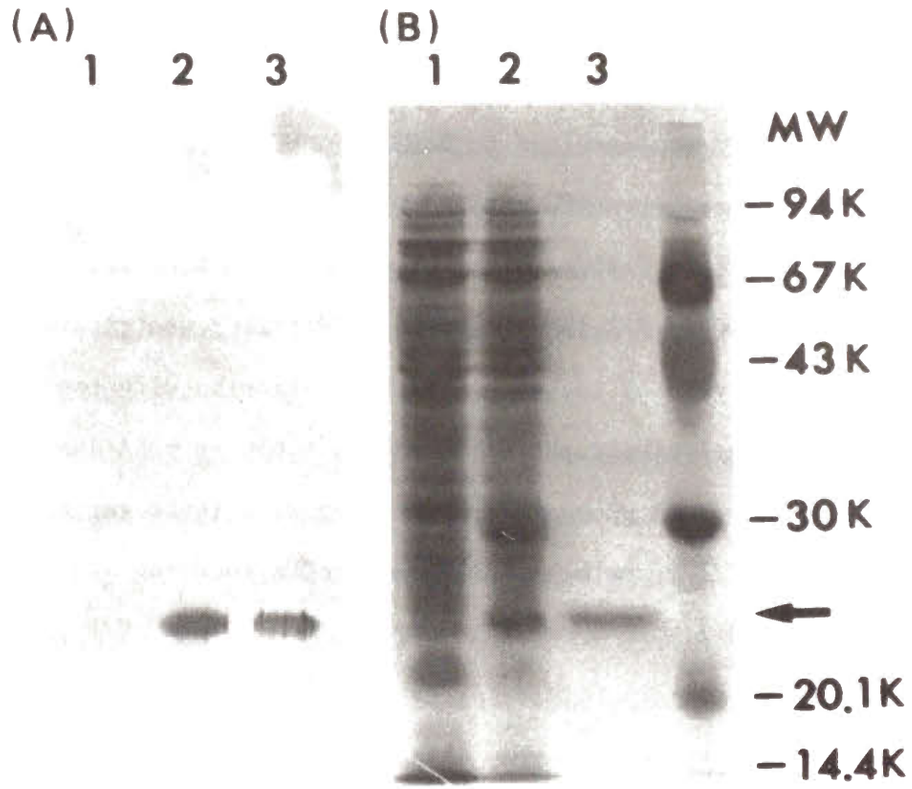

Fig. 7. Western blot analysis of cell lysate of *E. coli* strain DH5α and purified yeast glutathione S-transferase Y-2. (A) SDS-polyacrylamide gel was transferred to PVDF membrane and detected by immuno blot analysis with rabbit antiserum to glutathione S-transferase Y-2 . Lane 1.; Cell lysate of *E. coli* DH5α harboring plasmid pUC 118. Lane 2.; Cell lysate of *E. coli* DH5α harboring plasmid pHT 108. Lane 3.; Purified yeast glutathione S-transferase Y-2. (B) SDS-polyacrylamide gel was transferred to PVDF membrane and detected by coomasie brilliant blue staining. For immunoblotting 1/40 diluted samples of coomasie blue staining were used.

band which comigrated with the purified yeast glutathione S-transferase Y-2 subunit(Fig. 7-B,lane 2). The same diluted samples were also examined by immunoblotting using antiserum to glutathione S-transferase Y-2(Fig. 7A). A single band of immuno-reactive protein was detected in both DH5α/pHT108 and purified yeast glutathione S-transferase Y-2 with a molecular weight of 23,500(Fig.7-A,lane 2,3). On the other hand, no band was detected in DH5α/pUC118 (Fig.7-A,lane 1). Plasmid pHT108 was cleaved by Eco RI and analyzed by southern blot hybridization with two probes after agarose gel electrophoresis and about 650 bp cDNA insert hybridized with both probes. From this result, it is suggested that plasmid pHT108 contains full length cDNA encoding glutathione S-transferase Y-2.

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Chapter V. Nucleotide Sequence of the Yeast Glutathione S-Transferase cDNA.

Glutathione S-transferase (GST)(EC 2.5.1.18) catalyzes the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups in xenobiotics. Because of this nucleophilic attack, electrophilic xenobiotics including mutagens and carcinogens are conjugated with glutathione and detoxified. GST is widely distributed from bacteria to mammals and many isoenzymes have been isolated and characterized in detail (11,16). Some types of GSTs have been studied in relation to carcinogenesis in mammals (23,26) and resistance to herbicides and insecticides in plants (9,21) and insects (5), respectively. Recently, many cDNAs of GST have been cloned and nucleotide sequences and deduced amino acid sequences have been determined in rat, mouse, human, maize and Schistosoma japonicum (18). Expression of GST cDNA in Escherichia coli has also been reported in mammals (2,15,31) and plants (19). Although much progress has been made in research of GST in mammals and plants, there has been little information about GST in micro-organisms. Glutathione was first found in abundance in yeast cells, but there have also been few reports about the physiological role of glutathione and GST in yeast.

The author previously reported the distribution of GST in yeasts (12) and purified and characterized two GST isoenzymes (GST

Y-1 and Y-2) from Issatchenkia orientalis (27). The author also investigated the GST related detoxification and metabolism of o-dinitrobenzene in I. orientalis (29). Recently, the author reported the induction of GST Y-2 mRNA by o-dinitrobenzene in I. orientalis, isolation of cDNA clones coding for GST Y-2 and its expression in E. coli (28). One of the positive clones in colony hybridization harboring plasmid pHT108 showed 28-times more GST activity than the control when induced by isopropyl-β-D-thio-galactopyranoside. And a single band comigrating with the yeast GST Y-2 subunit was detected when the cell lysate of the positive clones harboring pHT108 was applied to immunoblot analysis using antibody to GST Y-2.

To obtain information concerning the structure and function of GST Y-2 in yeast I. orientalis, the author sequenced the 650 bp cDNA insert of plasmid pHT108, which is considered to harbor a nearly full length GST Y-2 cDNA.

Materials and Methods

Nucleotide sequence determination.

cDNA fragment carring GST Y-2 cDNA was determined from the plasmid pHT108. To obtain the nucleotide sequence of GST Y-2 cDNA, this region was digested with various restriction enzymes and subcloned into pUC118 vectors. The single stranded phage DNA

prepared from the resulting clones by M13KO7 was subjected to the chain termination tequnique of DNA sequencing (22, 30, 32). The sequencing strategy and partial restriction map for the cDNA insert of pHT108 is shown in Fig. 1. The nucleotide sequence was determined in both directions with a Sequenase kit (United States Biochemical Co.). The data were compiled and analyzed with programs from GENETYX(Software Development Co.) and IDEAS(SEQFP).

Results and Discussion

Nucleotide sequence of GST Y-2 cDNA and the deduced amino acid sequence.

The only possible frame was found in which 15 and 26 amino acid sequences completely agreed with those of the N-terminal and lysilendopeptidation fragments respectively from the GST Y-2 subunit (28)(Fig. 2). The deduced amino acid sequence indicates a protein comprising 190 amino acids with a molecular weight 21,520 (Fig. 2). The initiation codon is located at nucleotide position 12 and the termination codon at 585. Another termination codon was found in the 5'-noncoding region upstream from the initiation codon. The initiation codon lies in a consensus sequence for translational start in yeast Saccharomyces cerevisiae (nucleotide position 8-17) (4). The consensus sequence is (A/Y)A(A/T)AATGTCT (where Y is a pyrimidine). Although the underlined nucleotides A

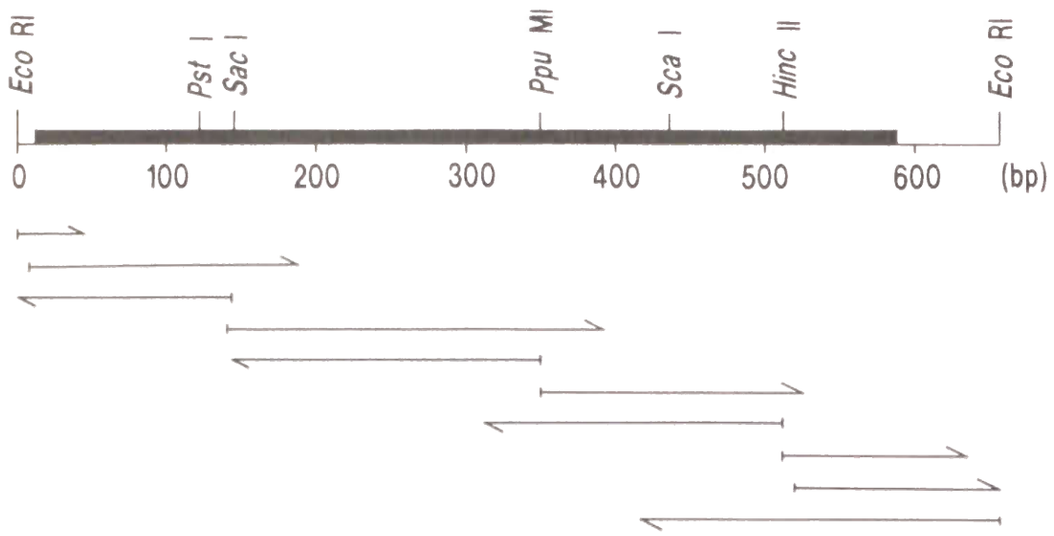

Fig. 1. Restriction map of the 650 bp cDNA insert of pHT108 coding for the glutathione S-transferase Y-2 and summary of the sequencing strategy. The arrows indicate the direction of sequencing and the extent of the sequence obtained from individual clones.

and T in this sequence are proposed to be important in the initiation by yeast ribosomes, nucleotide A was substituted for T at nucleotide position 15 in I. orientalis. Zaret and Sherman had proposed that the termination signals of yeast are characterized by a three-part signal as TAG...TAGT...TTT or TAG...TATGT...TTT (33). Henikoff and Cohen proposed that the sequence TTTTTATA in the 3'-noncoding region is responsible for transcription termination in yeast Saccharomyces cerevisiae (10). The 3'-noncoding region of the GST Y-2 cDNA is 61 bp long excluding the poly A sequence, but both these consensus sequences of transcription termination were not observed.


```
                    10                  20                  30                  40                  50                  60
AATAAATTAAAATGACTTTCGCAACTGTTTATATCAAGCCACACACCCCAAGAGGTGACT
  ***          MetThrPheAlaThrValTyrIleLysProHisThrProArgGlyAspTrp
  Stop           1                                                 10

          70                  80                  90                  100                 110                 120
GGTTAGCATCTCTAGGTCAATATGTTGGTCTTGAAATCAAGACTGTTGATTACAAGTCTG
  LeuAlaSerLeuGlyGlnTyrValGlyLeuGluIleLysThrValAspTyrLysSerAla
            20                                                  30
Pst I                                   Sac I
          130                 140                 150                 160                 170                 180
CAGAGGCATCCAAGTTCGAAGAGCTCTTCCCATTGAAGAGAGTCCCTGCACTTGTCACCC
  GluAlaSerLysPheGluGluLeuProLeuLysArgValProAlaLeuValThrPro
            40                                                  50

          190                 200                 210                 220                 230                 240
CCAACGGGTTCCAACTAACTGAACTCATTGCTATTGTTGAGTACATTGTTGCAAAGGGTT
  AsnGlyPheGlnLeuThrGluLeuIleAlaIleValGluTyrIleValAlaLysGlySer
            60                                                  70

          250                 260                 270                 280                 290                 300
CAAAGCCCGAGTTGTCTGGAAAGACTACCGAGGAAAGGGCAACAAACACCAGATGGTTAT
  LysProGluLeuSerGlyLysThrThrGluGluArgAlaThrAsnThrArgTrpLeuSer
            80                                                  90
                                                                                Ppu MI
          310                 320                 330                 340                 350                 360
CATTCTTTAACTCCGACTTTGTTCAAGCAGCCGGTGGTTACTTCATGGGTCCTAACGACG
  PhePheAsnSerAspPheValGlnAlaAlaGlyGlyTyrPheMetGlyProAsnAspGlu
            100                                                 110

          370                 380                 390                 400                 410                 420
AAATCAAGCAACAAAGTCTTCAAACCATGTTGAGCTTACTCGAATATATCGACAAACACT
  IleLysGlnGlnSerLeuGlnThrMetLeuSerLeuLeuGluTyrIleAspLysHisLeu
            120                                                 130
                Sca I
          430                 440                 450                 460                 470                 480
TATCCCAATCCAAGTACTTCACCAATAATACCATCTTAACTGCCGACATCTTTGCCTTCC
  SerGlnSerLysTyrPheThrAsnAsnThrIleLeuThrAlaAspIlePheAlaPheGln
            140                                                 150
                                        Hinc II
          490                 500                 510                 520                 530                 540
AAATCTTCGCAATGGCAAAACAATTCGGTGTTGACTTCACCCACTATCCGAACGTTGAGA
  IlePheAlaMetAlaLysGlnPheGlyValAspPheThrHisTyrProAsnValGluArg
            160                                                 170

          550                 560                 570                 580                 590                 600
GGTTTACTGGTGAGGTCTCCCAGCATCCAATTATCAAGAACATGTAAACAGGTGAGTCTC
  PheThrGlyGluValSerGlnHisProIleIleLysAsnMet***
            180                                                 190   Stop

          610                 620                 630                 640                 650
TGTATTTGCCTACATTGTATAACTCAATTTATCTAACTTATTTAGTTTAAAAAAAAAA
```

Fig. 2. Nucleotide sequence of yeast GST Y-2 cDNA and the deduced amino acid sequence. Nucleotide residues are numbered in the 5'-to-3' direction, begining with the 5'-end residues. The deduced amino acid sequence is given below the nucleotide sequence. The lines below the amino acid sequences in the N-terminus and near the C-terminus indicate the amino acid sequences matched with that obtained by protein sequencing. A possible consensus sequence for translational start in yeast Saccharomyces cerevisiae is shown by a wavy line above the nucleotide sequence. The lines above the nucleotide sequence indicate the recognition sites for the restriction enzyme noted.

Sequence alignment for similarity between I. orientalis, rat and maize GSTs.

The amino acid sequence of yeast GST Y-2 was compared with those of rat GST Yb_2 (8) and maize GST I, (25) which showed the highest homology score in mammals and plants respectively(Fig 3). The identities of the amino acid sequence between yeast GST Y-2 and maize GST I and between GST Y-2 and rat GST Yb_2 were 25.0% and 21.1% compared with 177 and 151 amino acid residues, respectively. Although the identity of amino acid sequences between the yeast GST Y-2 and rat GST Yb_2 or maize GST I was low, much of the proteins were conserved through conservative substitutions of amino acids (6). The stretches showing high similarity are surrounded by boxes(Fig. 3). The amino acid residues Pro-52, Ala-67, Ile-68, Tyr-71, Lys-78, Leu-81 and Glu-88 within the boxes were relatively conserved through many amino acid sequences in mammals and plants. Especially, Pro-52 and Ile-68 were completely conserved through all

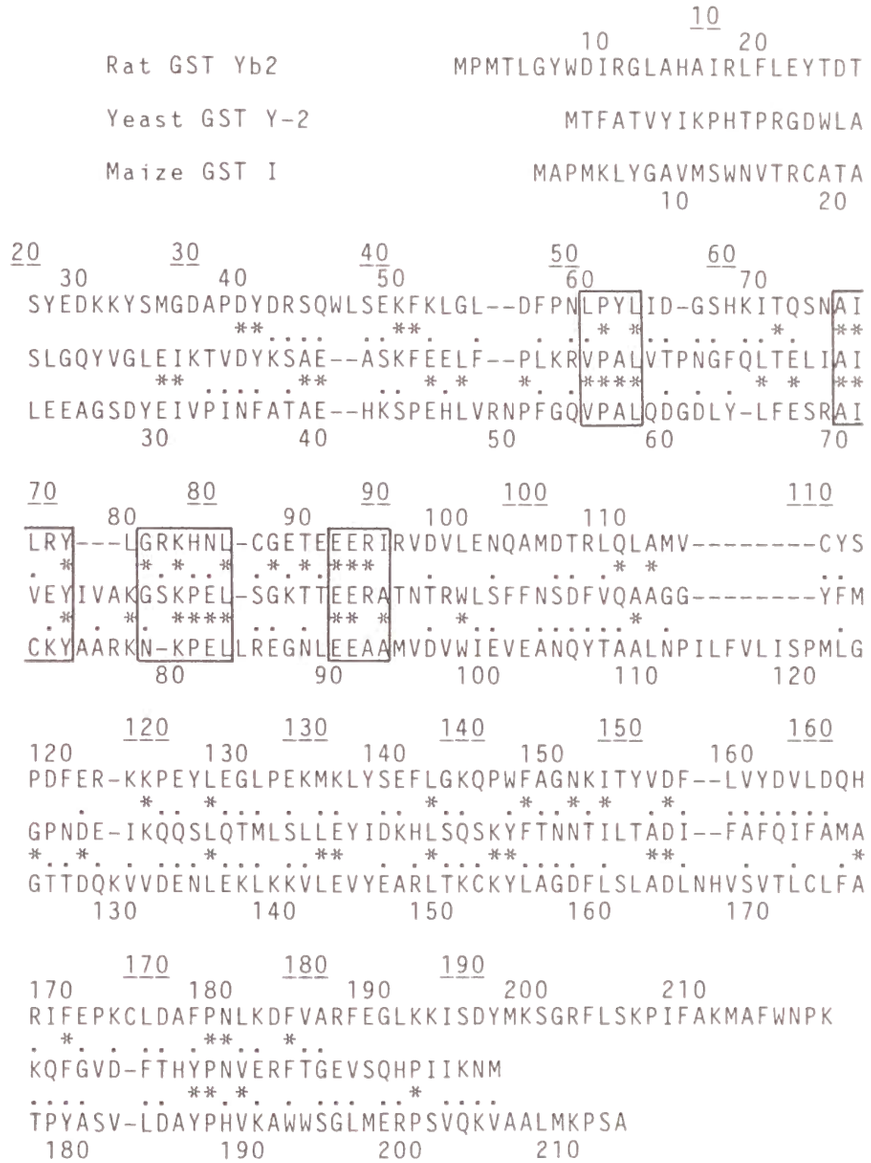

Fig. 3. Sequence alignment for similarity between yeast GST Y-2, rat GSTYb2 and maize GST I. The amino acid sequences of rat GST Yb2 and maize GST I were from Ding et al.[8] and Shah et al.[25], respectively. Highly similar regions between yeast, maize and rat GSTs are boxed. Asterisks (*) denote amino acid identity and periods (.) denote conservative amino acid replacements. The amino acid numbers of yeast GST Y-2 were underlined.

amino acid sequences analyzed except for rat microsomal GST (7,20). Except for the boxed area, several other amino acids are also highly conserved such as Leu-137, Asp-152 and Pro-173.

Recently, La Roche and Lesinger reported that the dichloromethane dehalogenase structural gene from Methylobacterium sp. strain DM4 had a region of amino acid sequences that exhibit high similarity to eucaryotic GSTs (14). This enzyme also showed similarity to yeast GST Y-2 and the identity of this amino acid sequence was 25.3% with 83 amino acid sequences compared. Yeast GST Y-2 and dichloromethane dehalogenase streches which showed high similarity coincided with the regions boxed in Fig. 3 except for one amino acid (aa.) region 76-81. Highly conserved amino acids Pro-52 and Ile-68 in GST Y-2 were also conserved in this enzyme.

There have been a few reports about the active site of GST. On the basis of results from chemical modification studies, Ketterer et al. indicated that one thiol residue is involved in the active site of GST B (3). Schasteen et al. reported that arginyl residues may function as a recognition site for glutathione on rat GST B (24). Awasthi et al. reported that a functional histidyl residue is essential for the catalytic activity of human GST ψ (1). However, information concerning the structure and function of the active site of GST is still lacking. For GST Y-2, the regions of amino acid sequences with high similarity to rat GST Yb_2 and maize GST I in Fig. 3 were observed as two hydrophobic sites(aa. 51-54, 67-71) and two adjacent hydrophilic sites (aa. 76-

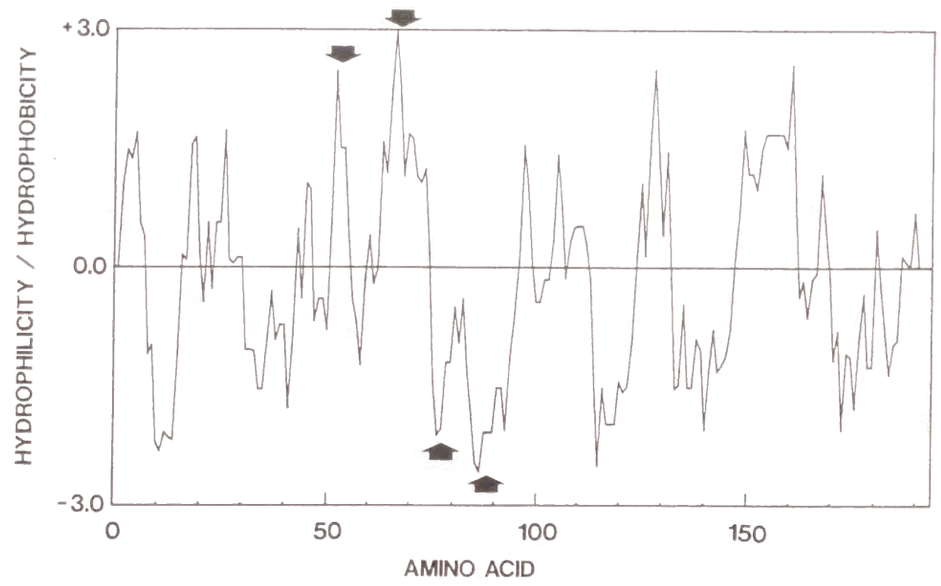

Fig. 4. Hydropathic profile of GST Y-2 from *I. orientalis*. Hydrophilicity is designated by - (below the 0 line), while hydrophobicity is designated by + (above the 0 line). Arrows indicate the regions with high similarity to the rat GST Yb_2 and maize GST I in Fig.3.

81, 87-90) in a hydropathy profile (13)(Fig.4). Mannervik et al. proposed that there is a binding site for glutathione (G-site) and an adjacent, partly hydrophobic binding site for electrophilic substrates (H-site) in each subunit (17). One hydrophobic and one hydrophilic site may correspond to the G- and H-sites, respectively. More chemical modification and site-directed mutagenesis studies are needed to elucidate structure and function of GST.

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Conclusions

The author studied on glutathione S-transferase (EC 2.5.1.18) of yeast Issatchenkia orientalis to elucidate the physiological role of the enzyme and glutathione in yeast cells.

The findings in each chapter are summarized as follows:

Chapter I.

The distribution of glutathione S-transferase (GST)(EC 2. 5. 1. 18) in yeasts was investigated. High enzyme activity was found in some strains of Issatchenkia and Candida. Of 168 strains tested, I. orientalis showed the highest activity. The enzyme activity exists constitutively in the yeast cells but it increased with the addition of an enzyme substrate, o-dinitrobenzene, to the culture medium. Moreover, the addition of L-cysteine and glycine to the medium also increased the enzyme activity. This enzyme was so unstable that it lost almost all its activity on ammonium sulfate precipitation and 93 % of its activity was lost when it was stored at 4°C for two weeks in a soluble state. The author found that it was stabilized considerably in a solution containing 20 % glycerol, 1 mM EDTA, 2 mM DTT and 10 mM sodium sulfite.

Chapter II.

Glutathione S-transferase (GST)(EC 2. 5. 1. 18) was purified from a cell-free extract of Issatchenkia orientalis and two GST

isoenzymes were isolated. They had a molecular weight of 37,500 and 40,000, and were designated GST Y-1 and GST Y-2, respectively. GST Y-1 and Y-2 gave one band with a molecular weight of 22,000 and 23,500 on sodium dodecyl sulfate-polyacrylamide gelelectrophoresis. GST Y-1 and Y-2 were immunologically distinguished from each other. GST Y-1 showed 10.4 times and 6.0 times higher specific activity than GST Y-2, when 1-chloro-2,4-dinitrobenzene and o-dinitrobenzene were used as a substrate, respectively. GST activity was not detected for either isoenzyme when other substrates such as bromosulfophthalein and trans-4-phenyl-3-buten-2-one were used. GST Y-1 and Y-2 had Km values of 0.51 and 0.75 mM for glutathione, respectively, and of 0.16 and 4.01 mM for 1-chloro-2,4-dinitrobenzene. GST Y-1 was significantly inhibited by Cibacron Blue 3G-A and GST Y-2 by bromosulfophthalein.

Chapter III.

Culture medium containing 200μM o-dinitrobenzene suppressed cell-growth of I. orientalis for about 24 hr and after a lag phase, the cells began to increase with concomitant metabolism of o-dinitrobenzene by glutathione conjugation. The resulting glutathione conjugate, S-(2-nitrophenyl)glutathione, was further enzymatically metabolized and released into the growth medium. The final metabolite was identified as S-(2-nitrophenyl)cysteine by comparison with the authentic compound on HPLC. In the presence of o-dinitrobenzene, intracellular glutathione was not detected in the

early log phase, but it began to accumulate after o-dinitrobenzene was metabolized. S-(2-nitrophenyl)glutathione and S-(2-nitrophenyl)cysteine, both chemically synthesized, did not suppress cell growth. These results suggest that there is a glutathione-related detoxification system in yeast I. orientalis.

Chapter IV.

Glutathione S-transferase Y-2 mRNA synthesis was induced in yeast Issatchenkia orientalis approximately 37-fold by cultivation with o-dinitrobenzene. A cDNA library complementary to poly $(A)^+$ RNA of I. orientalis grown with o-dinitrobenzene was screened by colony hybridization. Twenty positive clones were obtained from 6,000 clones and seven of twenty positive clones expressed glutathione S-transferase activity in E. coli. One of the expressing clones harboring plasmid pHT108 had 28 times more glutathione S-transferase activity induced by Isopropyl-β-D-thio-galactopyranoside than a strain harboring plasmid pUC118. Expressed glutathione S-transferase Y-2 protein comigrated with yeast glutathione S-transferase Y-2 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as detected by immunoblot analysis.

Chapter V.

The nucleotide sequence of the cDNA coding for glutathione S-transferase Y-2 of yeast Issatchenkia orientalis was obtained. The

cDNA clone, pHT108, which expressed glutathione S-transferase activity in Escherichia coli has a cDNA insert of approximately 650 bp. The cDNA clone contains an open reading frame of 570 nucleotides encoding a polypeptide comprising 190 amino acids with a molecular weight of 21,520. The start codon ATG lies in the consensus sequence for translational start in yeast Saccharomyces cerevisiae. The amino acid sequence of the N-terminal and lysilendopeptidation fragment from yeast glutathione S-transferase Y-2 was found in the deduced amino acid sequence. The primary amino acid sequence of the enzyme exhibits only 25.0% and 21.1% identity with 177 and 151 amino acid recidues of maize glutathione S-transferase I and rat glutathione S-transferase Yb_2 , respectively. Four regions of amino acid sequence of yeast glutathione S-tranferase Y-2 showed high similarity to rat and maize glutathione S-transferases. These regions were observed as two hydrophobic sites and two hydrophilic sites in the hydropathy profile.

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List of Publications

(1) Hidehiko Kumagai, Hisanori Tamaki, Yoshiko Koshino, Hideyuki Suzuki and Tatsurokuro Tochikura. 1988. Distribution, formation and stabilization of yeast glutathione S-transferase. Agric. Biol. Chem. 52:1377-1382.

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