natural resources. The recurrence of viral epidemics in tropical and subtropical countries is a common phenomenon where high mortalities were recorded in the past few years. At present, the control of various viral diseases has become a matter of great concern. However, the main constraint in developing an effective drug has been the unique characteristics of antigenic variation of virus resulting in the emergence of new variant virus strains[14]. There are a number of antiviral drugs introduced in the market such as tricyclic symmetric amine, amantadine hydrochloride and its analogue rimantadine, which are effective drugs used for influenza type-A infection[14]. However, though these drugs have been proved non-toxic, sometimes they cause dizziness and insomnia, particularly in elderly persons.

The present investigations showed that marine animals have great potential for developing useful drugs. Extraction of important biologically-active compounds from marine resources will certainly be helpful in protecting and treating various viral diseases in human beings.

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ACKNOWLEDGEMENTS. We are grateful to Dr E. Desa, Director, National Institute of Oceanography, Goa for his valuable comments and suggestions to improve the manuscript; Dr Igor Shaboneev, Ministry of Science and Technology of the Russian Federation, Moscow, Russia for moral support and the Council of Scientific and Industrial Research, New Delhi and Ministry of Science and Technology of the Russian Federation, Moscow, Russia for providing financial assistance. Thanks are also due to Dr G. C. Mishra, Dr A. H. Parulekar and Dr R. Bhonde for critically reviewing the manuscript. This is contribution number 3728 from the National Institute of Oceanography, Goa.

Received 2 June 2001; revised accepted 4 February 2002

Effect of β-methylcholanthrene on glutathione S-transferases of rat testis

K. N. Devi[†], C. C. Reddy[#], A. Raveendra[†] and K. Thyagaraju[†,*]

[†]Department of Biochemistry, Sri Venkateswara University, Tirupati 517 502, India
[#]Department of Veterinary Science, The Pennsylvania State University, University Park, PA 16802, USA

The glutathione S-transferases (GSTs) of rat testis, purified using S-hexylglutathione linked agarose 4B affinity matrix were fractionated into four cationic and six anionic isoenzymes using the polybuffer exchangers 118 and 94, respectively. Their pI values ranged from 5.4 to 9.2. On electrophoresis, the testis GSTs showed identical subunit pattern to that of brain, i.e. Yc, Yb, Yβ and Yδ To study the induction of these proteins, rat testis treated with 24 mg of β-methylcholanthrene on dot and transblot analyses with antibodies prepared against affinity purified GSTs revealed induction of α-class (Yc subunit) and μ-class (Yβ subunit) GSTs. This observation was further confirmed by using substrate specificity. The β-methylcholanthrene-induced samples have shown more activity with peroxides and 1,2 epoxy 3-(*p*-nitrophenoxy) propane indicated the elevation of Yc and Yβ subunits, respectively, in rat testis. Therefore the Yc and Yβ subunits may be used as marker proteins for chemical toxicity in the testis.

GLUTATHIONE S-transferases (GST.EC 2.5.1.18) are a family of multigene and multifunctional dimeric enzymes that catalyse the nucleophilic attack of the thiol moiety (conjugation) of the glutathione (GSH) on the electrophilic centre of various carcinogens, mutagens and other xenobiotic compounds[1]. The primary step of this reaction is involved in the formation of mercapturic acids, a pathway through which hydrophobic xenobiotics are inactivated and excreted from the body[2]. These enzymes are ubiquitously distributed in a variety of biological systems and in various organs like liver, kidney, intestine, brain and testis. They occur in the cytoplasm, membranes of mitochondria, microsomes and nuclei[3].

Each organ possesses a unique profile of GSTs, the liver and testis having the high GST activity in rodents[4]. The GST enzyme system consists of several isozymes. In rat liver, the GST isozymes are mainly made up of three major subunits, the Ya (Mw 25,600), Yb (Mw 27,000) and Yc (Mw 27,500). Each subunit has a specific function; Yc and Ya catalyse the reduction of hydro peroxides, Ya, Yc and Yδ catalyse the isomerization of prostaglandin, and Yb and Yβ are involved in the formation of leukotrienes and conjugation of GSH to xenobiotics. Yδ subunit is structurally related to the

*For correspondence. (e-mail: thyagarajuk_1999@yahoo.com)

Yp gene family of GST, which is a marker for hepatocarcinogenesis. This GST subunit is also present in rat brain, seminal vesicles and testis[5].

The structural diversity of the compounds that induce the expression of GSTs suggests that several distinct mechanisms may be prevalent for their induction. β-methylcholanthrene (MC), one of the tumour promoting steroidal agents, has been extensively studied as an inducer of GST in rats. During the treatment of MC, tumour promotion may be organ-specific and dose-dependent[6]. The present work was aimed to study the effect of MC on the induction of GSTs in rat testis and their characterization.

The GST affinity matrix, S-hexylglutathione-linked agarose-4B, polybuffer exchangers (PBE) 94 and 118, polybuffers 74 and 96, ampholytes pH 3–10.5, GST substrates and SDS–PAGE chemicals and the toxicant, MC were purchased from Sigma Chemical Co, St. Louis, USA.

Male Wistar rats, procured from Venkateswara Enterprises, Bangalore, weighing 200–250 g were treated with 4 mg of MC in coconut oil with an interval of 24 h per injection, to a total of 24 mg. The control animals were treated with coconut oil. MC was given to rats by intraperitoneal (ip) administration.

The following buffers were used for purification of GSTs. Buffer A: 25 mM Tris (hydroxymethyl) amino methane hydrochloric acid (Tris-HCl) pH 8; buffer B: 0.3 M phosphate buffer, pH 6.5; buffer C: 25 mM imidazole HCl, pH 7.0, buffer D: 25 mM triethanolamine–HCl, pH 10.5, buffer E: polybuffer 74 (1 : 8), pH 4.0; buffer F: polybuffer 96 (1 : 13); ampholine (1 : 45), 1 : 1 v/v, pH 7.0.

The Wistar strain rats were killed by decapitation and the testis (160 g) were collected from them. The testis were washed with normal saline to remove blood and fat debris and stored at –20°C until further use. The 20% homogenates of tissues were prepared in buffer A containing 0.2 M sucrose in Potter Elvejham homogenizers with a Teflon pestle and centrifuged at 20,000 rpm for 60 min in a Himac centrifuge at 4°C. The supernatant was dialysed for 24 h against 10 volumes of buffer A with five changes to remove the endogenous GSH. After centrifugation, this dialysate was loaded onto S-hexylglutathione-linked agarose-4B affinity matrix chromatography column (2 cm × 7 cm) which was equilibrated with 100 ml of buffer A to pH 8.0. Then the flow rate was adjusted to 60 ml/h. The eluant was collected in 3 ml fractions and the column was washed with buffer A containing 0.2 M potassium chloride (KCl) until absorbance of protein in eluant was less than 0.005 OD at 280 nm. The bound protein was eluted with buffer A containing 5 mM S-hexylglutathione, 2.5 mM GSH and 0.2 M KCl. The active fractions with maximum protein absorbance at 280 nm and high enzyme activity with CDNB at 340 nm were pooled and dialysed against buffer A for 24 h with four changes to remove S-hexylglutathione, GSH and KCl. The dialysed protein was concentrated by freeze-drying in a lyophilizer. The isoenzymes of testis affinity GSTs were separated on chromatofocusing columns, PBE 94 for anionic proteins and PBE 118 for cationic proteins, according to the instruction manual given by the manufacturer.

GST activity in rat tissue extracts was measured spectrophotometrically at 340 nm by the method of Habig *et al.*[7], with CDNB as substrate. The blank value was measured without the enzyme and it was subtracted from the experimental value. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one micromole of 2,4-dinitrophenyl GSH product per minute. Specific activity was expressed as micromoles of GSH conjugate formed per milligram of protein per minute.

The activity levels of affinity-purified GSTs with several substrates such as bromosulphophthalein

Table 1. Assay conditions for spectrophotometric measurement of GST activity levels with different substrates

Substrate	Content of reaction mixture	Molar extinction coefficient (cm^{-1})	Absorbance at λ(in nm)
p-Nitrophenyl acetate (*p*-NPA)	125 mM PO_4 buffer pH 7.0 0.3 mM *p*-NPA 0.5 mM GSH	8.79×10^3	400
p-Nitrobenzyl chloride (*p*-NBC)	100 mM PO_4 buffer pH 6.5 1 mM *p*-NBC 5 mM GSH	1.9×10^3	310
1,2-Epoxy-3-*p*-nitrophenoxy propane (EPNP)	125 mM PO_4 buffer pH 6.5 1 mM *p*-EPNP 5 mM GSH	0.5×10^3	360
Bromosulphophthalein (BSP)	125 mM PO_4 buffer pH 7.5 0.003 mM BSP 5 mM GSH	4.5×10^3	330

Table 2. Purification profile of rat testis GSTs and their isozymes

Sample	pI	Total protein (in mg)	Total activity	Specific activity	Yield (%)
Crude	–	1351	300	0.222	100
Affinity-purified GSTs	–	18	117	6.5	39
PBE-94 anionic isozyme					
GST t-1	6.20	0.33	1.718	5.17	0.57
GST t-2	6.14	0.52	3.906	7.54	1.30
GST t-3	6.0	0.61	0.594	0.96	0.20
GST t-4	5.80	0.74	0.104	0.14	0.03
GST t-5	5.60	0.74	0.031	0.04	0.01
GST t-6	5.40	0.35	0.312	0.90	0.10
PBE-118 cationic isozyme					
GST t-7	9.2	0.30	3.125	10.50	1.04
GST t-8	9.0	0.18	1.078	5.90	0.36
GST t-9	8.6	0.40	6.375	15.90	2.12
GST t-10	7.9	0.05	1.624	29.85	0.54

Enzyme activity expressed in µmol of GSH conjugate formed per mg protein per min.

(BSP), 1,2-epoxy-3-*p*-nitrophenoxy propane (EPNP), *p*-nitrophenyl acetate (*p*-NPA) and *p*-nitrobenzyl chloride (*p*-NBC) were determined for the purified proteins. (Table 1). The reactions were carried out using different substrates, buffers and initiators in a total volume of 3 ml reaction mixture. The blank reaction without enzyme was subtracted from the test values and the activities were calculated using molar extinction coefficients as mentioned in Table 1. All the enzyme assays were carried out at room temperature and the specific activities were expressed as mentioned above. Glutathione peroxidase (GP_x) assay was carried out by monitoring the oxidation of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) in a recycling assay as described by Wendel[8]. Total GP_x activity was determined using CHP as substrate. Selenium-dependent GP_x activity was measured using H_2O_2 as substrate.

Protein content in all samples was determined by the method of Lowry *et al.*[9], using bovine serum albumin (BSA) as the standard and also by measuring the change in absorbance at 260 nm and 280 nm, spectrophotometrically[10]. Polyacrylamide gel electrophoresis was conducted according to the method of Laemmli[11].

Antiserum was raised against affinity-purified GSTs of rat testis isozymes by following the published procedure of Tu *et al.*[12]. The Ouchterlony[13] double immunodiffusion method was followed for the cross reactivity determination of antibody and antigen. Western blotting technique was performed according to the procedure by Towbin *et al.*[14].

The cytosolic GSTs of rat testis were purified by using S-hexylglutathione-linked agarose-4B affinity chromatography column. The affinity-bound protein was eluted as a single sharp peak. The pooled fractions were assayed for GST activity using CDNB as substrate and the specific activity was found to be 6.5-µmol mg^{-1} protein min^{-1}. The overall yield of purified testis GST protein was about 39%. These affinity GST proteins were resolved into six peaks on PBE-94 chromatofocusing column in a pH gradient of 7 to 4 in buffer E. They were designated as anionic GSTs, t-1 to t-6, based on their elution order. The unbound protein peak eluted from PBE-94 was resolved into four peaks on PBE-118 chromatofocusing column in a pH gradient of 10.5 to 7. They were designated as cationic GSTs t-7 to t-10, based on their order of elution.

The pI values, subunit composition and specific activities of the above isozymes were determined (Table 2). The pI values of rat testis GSTs were determined by separating individual isozymes on chromatofocusing columns, PBE-94 and PBE-118. The pI values of cationic proteins ranged from 7.9 to 9.2 and the anionic proteins from 5.40 to 6.20.

SDS–PAGE analysis of the GST isozymes was conducted and the data are presented in Table 3. As indicated in Table 3, the affinity-purified proteins of rat testis GSTs contained four subunits Yc – 27.5 kDa, Yb – 26.3 kDa, Yβ – 26.0 kDa and Yδ – 24.8 kDa. Six anionic isozymes and four cationic isozymes were observed: the cationic forms were abundant in activity.

The specific activities of testis GST isozymes with CDNB, *p*-NPA and CHP as substrates and both anionic and cationic isozymes are tabulated in Table 4. The rat testis GSTs exhibited varying substrate specificities towards substrates like CDNB, *p*-NPA, *p*-NBC, EPNP, BSP and CHP. Among the anionic isozymes, maximum specific activity was observed in GST t-2 and least in

GST t-5. Almost all the testis cationic isozymes showed higher specific activity towards CDNB, ranging from 6 to 30. The isozymes, GSTs t-4 and t-6 containing Yβ subunit with Yb as minor component, respectively, showed identical activity towards *p*-NPA. The GSTs t-1, t-7 and t-9 containing Yc and Yb subunits showed activities of 0.005, 0.026 and 0.048 µmol of GSH conjugate per min per mg protein respectively, towards CHP. Both the anionic and cationic GSTs of rat testis did not show any activity towards the substrates *p*-NBC, BSP and EPNP.

The effect of MC administration to rats resulted in an increase in GST and GP_x activities, when compared to control (Table 5). Because no profound effect was observed in GST and GP_x activities of the rat testis on single dose treatment with MC, multiple dose treatment was done. Nearly 1.36- and 2.14-fold increased activity of GST with CDNB and GP_x with H_2O_2, respectively was observed in MC-treated rats compared to control.

The specific activity of testicular GSTs of control and MC treated rats with the substrates CDNB, *p*-NPA, *p*-NBC, EPNP and BSP, and GP_x activity with CHP and H_2O_2 was determined (Table 5). In MC-treated testis samples, higher levels of activity were observed with CDNB, *p*-NPA, *p*-NBC, BSP and EPNP substrates. The maximum activity variation was found towards BSP and EPNP at 24 mg dose of MC. The GP_x levels were decreased with CHP and H_2O_2 at 24 mg of MC in testis.

The antisera raised against affinity-purified GSTs of rat testis, liver and brain Yδ GSTs, on dot blot analysis showed immunoprecipitin spots with affinity GST proteins of rat testis, liver and brain isozyme b-4 (Yc and Yβ). The immuno cross-reactivity of transblotted protein of control, MC-induced and GST proteins of testis with the antisera of affinity-purified GSTs of testis are given in Table 6. Both dot and protein transblot immunoprecipitation analysis showed identical results in MC-treated sample as well as in control. The MC-treated sample with Yδ subunit antibody was similar to the control.

Table 3. Subunit composition of rat testis GSTs

Isozyme	Subunit composition
GST t-1	Yc, Yb
GST t-2	Yc, Yb, Yβ
GST t-3	Yb, Yβ
GST t-4	Yb, Yβ
GST t-5	Yβ, Yδ
GST t-6	Yβ
GST t-7	Yc, Yb
GST t-8	Yb, Yδ
GST t-9	Yc, Yb
GST t-10	Yb

Subunit composition was determined using SDS–PAGE.

GSTs are encoded by a multigene family and are differentially regulated in a tissue-specific manner to meet the special detoxication needs of various organs. The isoforms may be distinguished on the basis of differ-

Table 4. Substrate specificities of rat testicular GSTs

Sample	CDNB*	*p*-NPA*	CHP*
Affinity-purified GST	7.6	0.013	0.010
Anionic isozyme			
GST t-1	5.17	ND	0.005
GST t-2	7.54	ND	ND
GST t-3	0.96	ND	ND
GST t-4	0.14	0.018	ND
GST t-5	0.04	ND	ND
GST t-6	0.90	0.019	ND
Cationic isozyme			
GST t-7	10.50	ND	0.026
GST t-8	5.90	ND	ND
GST t-9	15.90	ND	0.048
GST t-10	29.85	ND	ND

ND, No detectable activity; *Activity levels are expressed in terms of µmol of GSH conjugate formed per minute per mg protein.

Table 5. Effect of MC treatment on the levels of GSTs and GP_x of rat testis

	Control	MC treated
Substrate	Units	
1-Chloro-2,4-di-nitrobenzene*	253 ± 15	343^b ± 26
p-Nitrophenyl acetate*	139 ± 40	265^b ± 20
p-Nitrobenzyl chloride*	551 ± 10	661^b ± 20
1,2-Epoxy-3-(*p*-nitrophenoxy) propane*	18 ± 9.0	36^a ± 3.0
Bromosulphophthalein*	12 ± 5.0	18.6 ± 2.0
Hydrogen peroxide**	4.93 ± 1.0	10.52^c ± 2.2
Cumene hydroperoxide**	15.43 ± 2.8	33.75^b ± 3.0

*One unit is defined as one nmol of GSH conjugate/min/mg protein; **One unit is defined as one nmol of NADPH oxidized/min/mg protein; Values are average of three separate experiments, mean ± SD, [a], ≤ 0.001; [b], ≤ 0.01; [c], ≤ 0.02.

Table 6. Subunit composition of GSTs of rat testis cytosols of control and MC-treated rats as evidenced by the immunoprecipitation with rat liver (A) and testis (B) GST antibodies

	Subunit composition	
Sample	A	B
Control testis	Yc, Yb, Yβ, Yδ	Yc, Yb, Yβ
Control liver	Yc, Yb, Ya	Yc, Yb
MC testis	**Yc**, Yb, **Yβ**	**Yc**, Yb, **Yβ**
Brain GST b-4 isozyme	Yc, Yβ	Yc, Yβ

Data are taken from the transblot analysis from nitrocellulose membrane. Bold letter indicates dominance of the band.

ences in binding specificities and catalytic properties toward various molecules[15]. The highly protected extra hepatic organs include the kidney, heart, brain and testis. Among these tissues, the testis and brain cytosols have high GST activity, but their isozyme patterns differed with respect to species as well as organ[16,17]. A survey of GST in various tissues of rat has revealed that testis has high activity and isozyme pattern differs significantly from that of liver[3].

The affinity-purified GST proteins of rat tissues on 12% SDS–PAGE are resolved into various bands with molecular weights ranging from 27.5 to 24.8 kDa. The testis GST proteins were resolved into four subunits like those of the brain, designated as Yc, Yb, Yβ and Yδ respectively, based on their molecular weights. This band pattern is identical to the reports of Li *et al.*[5] in brain and Anuradha *et al.*[18] in the testis.

Ten isozymes were separated from the affinity-purified rat testis GSTs. They were six anionic and four cationic isozymes. In contrast to the brain and similar to the liver, the testis GSTs showed 65% cationic isozymes and 35% anionic isozymes. These results elucidate that the cationic isozymes in testis and liver, and anionic isozymes in brain are abundant. In rat, testis, basic GSTs showed very high activity towards CHP. In rat testis, isozymes t-7, t-9 and t-1, which are heterodimers of Yc and Yb subunits, showed more nonselenium-dependent GP_x activity. These results are in agreement with the earlier reports of Chang *et al.*[3] and Thyagaraju *et al.*[16]. This peroxidase activity associated with GSTs in testis might offer protection from oxidative stress. The testis cationic GSTs have more of GP_x activity than those in the brain, as reported earlier[16]. In rat testis, isozymes GSTs t-1, t-7 and t-9 contained heterodimers of Yc and Yb subunits, t-2 contained heterotrimers of Yc, Yb and Yβ subunits, t-3 and t-4 contained heterodimers of Yb and Yβ subunits, t-5 contained heterodimers of Yβ and Yδ subunits, t-6 contained homodimer of Yβ subunit, t-8 is a heterodimer of Yb and Yδ subunits, whereas t-10 is a homodimer of Yb subunit. The existence of cytosolic GSTs as homo, hetero, di and trimers was reported in rat soft tissues. This type of combination of isozymes in GST may occur due to the exchange of gene products at the level of transcription and translation[19].

The detoxication of reactive intermediates in biological systems plays a major role in prevention of chemical lesions such as mutagenesis, carcinogenesis, and tissue necrosis. The enzymatic mechanisms of detoxication are involved in the protection of tissues from various toxicants[1]. Many detoxication enzymes exist as isozymes with multiple genes and exhibit differential expression with a wide variety of xenobiotics. In the present communication, the effect of MC on GSTs was studied to identify the specific GST involvement in the protection of testis of rats.

The GSTs exist in several isomeric forms, which are again tissue-specific and relevant to its function[20]. The quantitation of each subunit in tissue extracts of control and experimental animals was analysed either by immunological methods or by the separation of GST isozymes followed by enzymatic assays[20,21].

Dose selection was an important issue to examine the effect of MC on testis GST isozyme expression. The induction of GSTs was studied by the administration of MC in a dose-dependent manner and observed maximum effect at 24 mg/kg body weight. In testis, the GST and GP_x levels were markedly elevated just like in the brain[22] with various concentrations of MC, except with the substrate CDNB. These results of elevation in activities of GST and GP_x in testis are in agreement with the earlier studies of Kaplowitz *et al.*[23].

A number of workers have studied the GSTs in rat testis. Rat testis cytosol GST has approximately the same specific activity as that of rat liver cytosol, when assayed with CDNB as the substrate. However, the isozyme distribution is distinctly different in both cases. The most characteristic feature of the testicular GSTs is the association of approximately 50% of the total cytosolic activity with proteins having isoelectric points below pH 7. The quantitative analysis of the tissue distribution of subunits showed that it occurs in greatest amounts in testis and brain, but has low affinity for CDNB and GSH[24].

Western blot analysis of MC-treated testis cytosol with rat liver GST antibodies showed the induction of Yc and Yβ subunits. The immunoblot analysis of testicular GSTs with its GST antibodies, however, showed the induction of Yc and Yβ The immunoprecipitation forms and the specificity of subunit induction with MC were found to be inconsistent with the earlier reports of Pickett *et al.*[25]. The induction of specific subunits in rat testis was also confirmed by dot blot analysis using the GST subunit-specific antiserum. This report supports the fact that the Yc and Yb subunits of rat testis GST may thus have sequence homology with the rat liver Yc and Yb GSTs, as evidenced by immunoprecipitation studies with rat liver antisera.

The tissue degeneracy when compared with enzyme activities suggests that induction of GST is mainly to protect the tissues from MC and other toxicants. The effect of inducers on the expression of phase II enzymes revealed that the animal systems on exposure to chemical carcinogens have evolved various defence mechanisms to protect themselves from the oxidative damage. The α (Yc) and μ (Yb, Yβ) classes induced by MC might inhibit the initiation of chemical toxicities and thereby act as chemo protectors.

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ACKNOWLEDGEMENTS. We acknowledge the financial support from CSIR and DST, New Delhi.

Received 4 October 2001; revised accepted 26 March 2002