Int J Pharm Bio Sci 2013 July; 4(3): (B) 341 - 348

Research Article **BioChemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EXPRESSION OF II CLASS GLUTATHIONE-S-TRANSFERASES IN MICE LIVER UNDER THE INFLUENCE OF PARACETAMOL TREATMENT AND THE RECOVERY OF II GSTS TO μ CLASS GLUTATHIONE-S-TRANSFERASES WITH *HYBANTHUS ENNEASPERMUS* (L.) F. MUELL. TREATMENT

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ABSTRACT

The present study was designed to investigate the expression of GSTs under the influence of paracetamol and *Hybanthus enneaspermus.* Study with different substrates of glutathione-s-transferases were done to know the expression of GSTs. Along with this purification and isolation of GSTs were performed to know the expression of subunits of liver GSTs.Three subunits were found. Newly π GSTs were expressed upon treatment with paracetamol.In control liver μ GSTs are generally expressed. Hence the selected plant *Hybanthus enneaspermus* showed the recovery of π into μ GSTs in the case of liver.

KEYWORDS: ***Hybanthus enneaspermus*, Hepatoprotective effect, Expression of GSTs, Paracetamol.**

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INTRODUCTION

The cytosolic glutathione S-transferases (GSTs) form a family of dimeric enzymes which exhibit a number of catalytic activities, including the conjugation of glutathione to lipophilic electrophiles[1] (Coles and Ketterer, 1990). On the basis of nucleotide and amino acid sequence similarities, mammalian cytosolic GSTs can be grouped into four subfamilies designated Alpha, Mu, Pi and Theta[2,3,4] (Mannervik et al., 1985, 1992; Meyer et al., 1991). The Pi subfamily has attracted considerable attention following the observation by Sato and co-workers that the Pi-class GST enzyme is markedly elevated in rat liver tumour development[5,6] (Sato et al., 1984a, b). In the early stages of rat liver carcinogenesis, single cells and foci appear that exhibit a different phenotype from that of normal hepatocytes; these foci are believed to represent precursors of tumors. One of the most reliable markers for the identification of such (pre) neoplastic foci is the Pi-class enzyme,GST-P[7] (Sato, 1989). This protein is essentially absent from normal hepatocytes, whereas it is abundant in foci- or nodule bearing livers. GST-P is expressed not only in foci or nodules induced by chemicals but also in spontaneously occurring hepatic (pre-) neoplastic lesions, without administration of any exogenous carcinogens[8] (Sawaki et al., 1990).

In the human, there are several reports demonstrating a significant increase in the expression of the Pi-class enzyme, GST-ir (GSTP1-1), in a number of tumors including oral, colon, stomach and lung[7,9,10,11,12] (Sato, 1989; Howie et al., 1990; Black and Wolf, 1991; Volm et al., 1991; Hirata et al., 1992). However, GST-r levels are not increased in hepatocellular carcinomas[13] (Hayes et al., 1991). In addition, Pi-class GST is expressed at elevated levels in cell lines made resistant to anticancer drugs and to chemical toxins in culture[14,15,16] (Batist et al., 1986; Cowan et al 1986; Wolf et al., 1990; Black and Wolf, 1991). Therefore, studying the function and regulation of Pi-class GSTs will lead to a better understanding of the mechanisms underlying carcinogenesis and drug resistance. Studies in the mouse are of particular importance, as this species is genetically well characterized, and isolation and purification technology provide powerful tools for investigating gene expression and characterizaton.

MATERIALS AND METHODS

Plant material

The plant materials (*Hybanthus enneapermus*) leaves were collected from the surroundings of Sri Venkateswara university campus, Tirupati, Andhra Pradesh, India and identified by comparison with a voucher specimen deposited in the herbarium of the department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Preparation of the extracts

The collected plant leaves were air-dried in the shade and powdered. About 100g of leaf powder was soxhlated with 500ml of ethanol for more than six hours and the extracts were then concentrated invacuo to yield dense residues. Experiments were carried out in albino male mice weighing 25±5.0g. They were obtained from Sri Venkateswara Enterprises, Bangalore and maintained in standard laboratory conditions, fed with ad libitum and water.

Paracetamol-induced hepatotoxicity in mice(Acute treatment)

The male mice divided into control, paracetamol and plant product treatments were allowed for seven days of treatment and the experiments were made as follows for each group. Group I was control mice treated with saline 1ml /kg bw, ip, Group II were toxic control treated with paracetamol alone (300mg/kgbw in saline, ip), Group III and IV were treated with ethanolic *Hybanthus enneaspermus* extract alone, 100mg /kgbw and 200mg/ kg bw, in cmc, respectively, and Group V and VI were treated with ethanolic *Hybanthus enneaspermus* extract, 100mg /kgbw along with Paracetamol (300mg /kgbw), and 200mg /kgbw and Paracetamol 300mg /kgbw, respectively. Group VII mice were treated with *Active fraction* (50mg /kgbw) and

Paracetamol (300mg /kgbw). Paracetamol administration was observed for every 48 hrs intervals. Remaining treatments duration was about 7 days. On the eighth day mice were sacrificed by decapitation.

Preparation of the tissue homogenate

The liver of mice were homogenized in 50 mM Tris-HCl buffer (pH 8.0), and then centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for assaying antioxidant enzymes, lipid peroxidation and liver marker enzymes. For lipid peroxidation, 10% tissue homogenate was prepared in 1.15% KCl and for estimating total sulfhydryl content the tissue was homogenized in 0.2 M EDTA.

Protein Estimation

Protein content of all samples was determined by the method of Lowry[17], using bovine serum albumin (BSA) as the standard, and also by measuring the change in absorbance at 260 and 280nm spectrophotometrically[18] (Warburg and Christian ,1941).

Isolation and Purification of GST

The purification of GSTs was carried out by affinity chromatography[19,20] (Frangioi et al., 1993; Simons et al., 1977). The following buffers were used for purification of GSTs and prepared in double distilled water.

Buffer A: 50mM Tris-HCl pH8.0,

Buffer B: Buffer A+ 0.2M KCl

Buffer C: Buffer B + 5mM GSH All purification steps were carried out at 4°C. The tissue for purification was cut in to small pieces and homogenized with buffer A containing 0.25 M sucrose and PMSF proteinase inhibitor in a glass homogenizer. The homogenate was centrifuged at 36,000Xg for 20 min. The supernatant fraction was collected and the pellet was discarded. The filtrate was centrifuged at 105,000 x g for 45 min. and the cytosolic fraction was passed through glass wool to remove floating lipid material. The filtrate was dialyzed overnight against buffer A with four changes[21] (Reddy et al, 1983).

Affinity column chromatography

The Glutathione S- transferase affinity column has cross-linked with 4%beaded agarose as the matrix to which Glutathione is coupled by epoxy method and hexyl chain as a spacer arm. The ten ml affinity column was equilibrated with ten volumes of buffer A and the flow rate was adjusted to 10 ml/hr .The crude extract was applied on to the column and the elutes were collected in 5 ml fractions after the application of buffer A. The fraction, which was unbound, was collected as flow through. To remove the non-specifically bound proteins buffer B was applied to the column until A280 for the fraction reaches 0.0005.The bound protein was eluted by using buffer C. The fractions were collected until the absorbance reached to 0.0005. The active fractions with maximum protein absorbance at 280 nm and high enzyme activity with CDNB at 340 nm were pooled and dialyzed against buffer A for 24 hrs with four changes to remove glutathione and KCl. The dialyzed protein was concentrated by freeze drying in a lyophilizer.

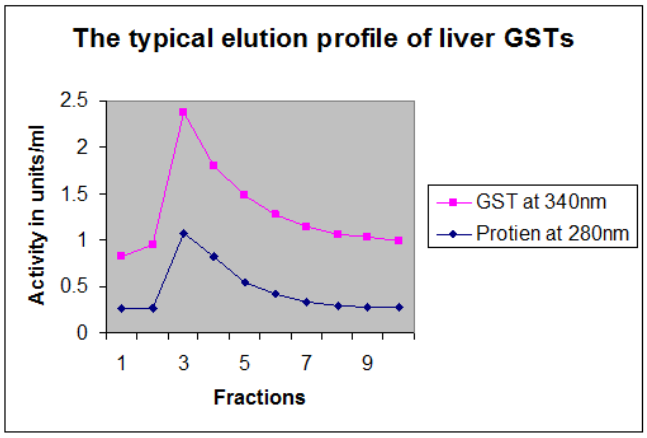
Table 1
Typical elution of GSTs

Fraction Numbers	Protein at 280nm	Gst activity at 340nm
1	0.26	0.568
2	0.27	0.681
3	1.071	1.309
4	0.818	0.99
5	0.544	0.943
6	0.421	0.85
7	0.34	0.809
8	0.293	0.768
9	0.282	0.764
10	0.274	0.715

Table 2
Affinity purification profile of mice liver GSTs

Organ	Total activity (units)	Total protein (mg)	Specific activity	Yield (%)	Purification fold	Subunit composition on electrophoresis
Liver (crude)	1217	256	4.75	100	1	
Affinity purified	855	12.8	66.7	70.2	14.02	Yc- 26Da Yb- 24.5Da Ya- 23KDa

Figure 1
Affinity chromatography elution profile of mice liver glutathione S- transferases

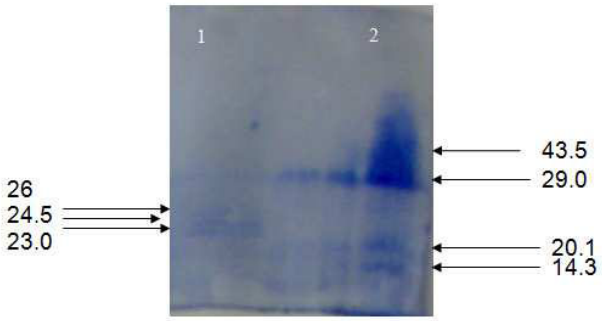


SDS-PAGE:
SDS-PAGE was conducted to observe the GST subunit profile of Purified proteins.
Solutions

1. ***Acryl amide and bisacrylamide in a ratio of 29:1***
2. ***1.5 M Tris-HCl, pH 8.8***
3. ***0.5 M Tris-HCl, pH 6.5***
4. ***10%SDS***
5. ***10%APS (Ammonium persulphate)***
6. ***TEMED***
7. ***Tank buffer:25 mM Tris base, 192 mm glycine and 0.1%SDS, pH 8.3***
8. ***Staining solution: Methanol: acetic acid: water in a ratio of 40:10:50(v/v) Containing 100 mg% coomassie brilliant blue R-250.***
9. ***Destaining solution: Methanol: acetic acid: water in a ratio of 40:10: 50(v/v).***

Polyacrylamide gel electrophoresis (PAGE) was conducted according to the method of Laemmli 1970. The denaturing gel electrophoresis was carried out with 12% resolving gels and 5% stacking gels containing 0.1% SDS. Samples with 20µgms protein concentration was boiled at 100°C for 5 min in the presence of 1x loading dye and was loaded into the wells. The separation of protein bands will be carried out at a voltage of 50 for stacking gel and 80 for resolving gel. The separated proteins on the gel were stained with coomassie brilliant blue solution, for overnight and destained in destaining solution till the background completely reaches transparent with good visibility of protein bands.

Figure 2
SDS-PAGE analysis of GSTs of mice liver

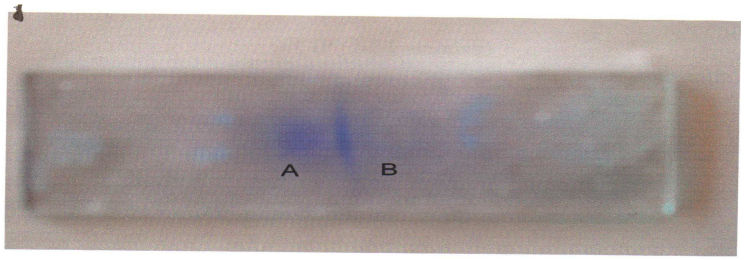


LANE - 1 : Mice liver Affinity Purified GSTs.
LANE - 2 : Marker Proteins

Preparation of Antisera against affinity purified GSTs

In male New Zealand white rabbits antibodies were produced to 100µgms of affinity purified GST protein per ml emulsified with an equal volume of Freund's complete adjuvant. The emulsified mixture injected subcutaneously to the rabbit at 6-10 sites. The booster doses were given with incomplete adjuvant with an interval of a week for four times. The titer of the antibodies was tested after booster dose. After the fourth week of booster dose the immune response was high and the animals were bled and the serum was collected after centrifugation at 6000Xg.

Figure 3
Immuno diffusion analysis of Affinity purified mice liver GST proteins



A: Purified Liver GST B: Antiserum

Enzyme activity and substrate specificity assays

Glutathione S-transferase activity in rat tissue extracts was measured spectrophotometrically at 340nM by the method of Habig[22] et al., (1974) with CDNB as substrate. The reaction mixture containing 1ml of 0.3 M PO_4 buffer, pH6.5, 100µl of enzyme, 30mM CDNB and 30mM GSH were added and the total volume was made up to 3ml with distilled water and the increase in absorbance was read at 340nm. The blank value will be measured without the enzyme and it will be subtracted from the experimental value. The activities were calculated using molar extinction coefficient of 9.6. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one micromole of 2, 4 Dinitrophenol GSH adduct formed per minute. Specific activity was expressed as micromoles of GSH conjugate formed per milligram of protein. The GST activity with several substrates such as BSP, EPNP, P-NPA, P-NBC and DCNB was determined to the purified protein by the method of Habig and Jakoby(1974). The reactions were carried out using different substrates, buffers and initiators in a total volume of 3ml reaction mixture, the blank reaction without enzyme were subtracted from the test value and the activities were calculated using molar extinction coefficient as mentioned in Table. All the enzyme assays

were carried out at room temperature and the specific activities will be expressed as micromoles or nmoles conjugate formed/min/milligram protein.

Assay conditions for spectrophotometric analysis of GSTs.

Substrate	Contents of reaction mixture	Molar extinction coefficient	λ_{max}
P-nitrophenyl acetate	125mMpo_4 Buffer-P_H7.0 0.3mM P-NPA 5mM GSH	8.79x10 ^{3} cm^{-1}	400
P-nitrobenyl acetate	100mMpo_4 Buffer-P_H6.5 1.0Mm P-NBC 5mM GSH	1.9x10 ^{3} cm^{-1}	310
1,2epoxy-3 (P-nitrophenoxy) propane	125mMpo_4 Buffer-P_H6.5 1.0Mm EPNP 5mM GSH	0.5x10 ^{3} cm^{-1}	360
Bromosulfopthalein	125mMpo_4 Buffer-P_H7.5 0.03mM BSP 5Mm GSH	4.5x10 ^{3} cm^{-1}	330
DCNB	125mMpo_4 Buffer-P_H7.5 0.03Mm DCNB 5mM GSH	8.5x10 ^{3} cm^{-1}	344

STATISTICAL ANALYSIS

The experimental results were expressed as the Mean ± SE for six animals in each group. The biochemical parameters were analysed statistically using one-way analysis of variance ANOVA, followed by Dunnett's mutiple range test (DMRT). P value of < 0.01 was considered as statistically significant for the analyzed data.

RESULTS

Albino male mice liver GSTs were purified to electrophoretic homogeneity by GSH-affinity column chromatography.On SDS-PAGE analysis affinity purified cytosolic liver GSTs were resolved into three bands with relative molecular weights of Y_c-26KDa, Y_b- 24.5Da, Y_a- 23KDa(Fig:2). This indicates the presence of, μ and α GSTs. πGSTs are formed in paracetamol treated mice liver. These are the indicators of carinigenesis. Polyclonal antibodies were raised in rabbits against purified liver GSTs. A precipitin band was found by the cross reactivity of purified GST liver and antiserum. The group I results of control were compared with the results of all six groups. The group II mice Paracetamol (300mg /kgbw) treated showed GST substrate specific activities with purification folds of DCNB, EPNP, and pNPA found to have an increase by 3.4, 6.4 and 2.5 folds. The EPNP was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 99.58 moles/min/mg protien 40.21 moles/min/mgprotien compared to other substrates of GSTs. Extract of EEHE alone (100mg) on treated to mice showed GST substrate specific activities with purification folds of CDNB, DCNB, pNPA and Δ^5 androsten 3,17 dione was increased by 1.47, 1.49, 5.2 and 2.6 folds. The pNPA was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and pNPA showed highest activity 118.96moles/min/mg protien and 70.42moles/min/mgprotien compared to other substrates of GSTs. Extract of EEHE alone (200mg) on treated to mice showed GST substrate specific activities with purification folds of CDNB, pNPA, EPNP, Δ^5, found to be increased by 1.3, 4.5, 4.2 and 6.1 folds. The Δ^5 was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 120.54moles/min/mgprotien and 87.56moles/min/mgprotien compared to other substrates of GSTs.

The combinational studies of EEHE (100mg/ kgbw) and Paracetamol (300mg/ kgbw) showed GST substrate specific activities with purification folds of DCNB, pNPA, EPNP,

Δ^5, found to be increased by 1.28, 5.1, 1.6 and 5.1 folds. The pNPA and Δ^5 were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 112.69moles/min/mgprotien and 59.96moles/min/mgprotien compared to other substrates of GSTs. The combinational studies of EEHE (200mg/ kgbw) and Paracetamol (300mg/ kgbw) were showed GST substrate specific activities with purification folds of DCNB, EPNP, Δ^5, found to be increased by 3.04, 1.0 and 3.9 folds. The Δ^5 were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 117.11moles/min/mgprotien and 38.86moles/min/mgprotien compared to other substrates of GSTs. The combinational studies of active fraction (50mg/ kgbw) and Paracetamol (300mg/ kgbw) showed GST substrate specific activities with purification folds of CDNB, DCNB, PNPA, Δ^5, found to be increased by 1.47, 1.3, 5.2, and 6.1 folds, respectively. The $_P$NPA and Δ^5 were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB showed highest activity 125.28moles/min/mgprotien and compared to other substrates of GSTs.

Table 3
Effect of Paracetamal and EEHE on activity levels of GSTs of mice liver on different Substrates.

GROUPS[G1-G7]	CDNB	DCNB	pNBC	pNPA	EPNP	Δ5-Androstene	BSP
Control	121.7±0.40[a]	11.6±0.42[c]	33.68±0.43[c]	38.55±0.40[e]	23.43±0.40[a]	35.6±0.35[b]	38.5±0.34[d]
Paracetamal[300mg]	99.58±0.40[d]	9.32±0.28[d]	29.1±0.34[d]	32.6±0.37[f]	20.19±0.40[b]	31.54±0.41[c]	40.21±0.39[c]
EEHE[100mg]	118.96±0.39[b]	37.1±0.42[a]	2.93±0.37[g]	70.42±0.33[b]	9.7±0.35[c]	40.6±0.33[a]	41.38±0.35[c]
EEHE[200mg]	120.54±0.34[a]	5.43±0.42[d]	37.36±0.39[b]	76.41±0.33[a]	19.59±0.30[b]	16.34±0.37[d]	87.56±0.41[a]
P+EEHE[100mg]	112.69±0.42[c]	3.4±0.40[e]	59.51±0.39[a]	55.88±0.37[c]	4.25±0.37[e]	16.5±0.36[d]	59.96±0.36[b]
P+EEHE[200mg]	117.11±0.30[b]	10.4±0.37[c]	18.11±0.35[f]	33.83±0.33[f]	4.8±0.36[e]	15.6±0.34[d]	38.86±0.36[d]
Active fraction+EEHE[200mg]	125.28±0.30[a]	17.32±0.22[b]	25.0±0.32[e]	40.04±0.30[d]	8.99±0.33[d]	14.22±0.39[d]	42.53±0.26[c]
F value	**467.329**	**996.305**	**2472.001**	**2938.034**	**517.319**	**902.517**	**2591.067**

Values are expressed as Mean±SE by Duncan's multiple range test (DMRT). Means having same subscripts in eachcoulm donot differ significantly at 0.01 level by Duncan's Multiple range Test (DMRT).p<0.01
Note: Specific activity observed moles/min /mg of protein.

DISCUSSION

The GST isozymes differed in their specificity toward xenobiotic or endogenous substrates such as paracetamol shows variation in the expression of GSTs. The CDNB is the main substrate, which undergo nucleophilic displacement of the chloride moiety by GSH. All classes (α, μ, π) of GSTs except θ catalyze this reaction.Control showed highest activity with CDNB and pNPA, this shows the induction of π, α & μ GSTs subunits'. Paracetamol alone showed highest activity with CDNB. This shows the induction of π GST subunit, which indicates the toxic effect. Extract alone (100mg /kgbw) showed highest activity with CDNB and pNPA. The result indicates the presence of ' π, α & μ GSTs subunits'. Extract alone (200mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of 'π & μ GST subunits'. EEHE (100mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of 'π & μ GST subunits'. EEHE (200mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of π & μ GST Subunits'. Active fraction MeOHHE (50mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB. The result indicates the induction of 'π & μ GST subunits'. These results indicate with the above expressed subunits that plant extract is having protective activity as it regained 'μ -GST subunit', which deals with detoxification by conjugation reactions, it combacts oxidative stress by thiolysis. There fore the effect of toxicity has been removed.

CONCLUSION

The present investigation indicates that EEHE exert significant protection against paracetamol-induced toxicity. Paracetamol express pi GSTs where as in control liver α and μ GSTs. Pi GSTs are the indicators for carcinogenesis. μ GSTs are expressed by plant extract.These GSTs deals with detoxification of oxidativestress.As there are different substrates for GSTs they express different types of subunits. Especially on oxidativestress the pi GSTs formed in paracetamol treated mice were recoverd in the treatments of plant extract.

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