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Purification and Characterization of Mice Liver Cytosolic GSTs

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ABSTRACT

Mice GSTs encoded by a multigene family are differentially regulated expression in a tissue for special detoxification needs of various organs. Mice liver GSTs were purified, individual subunits were located and then characterized. The affinity purification studies revealed that the GSTs can be purified in a single step process to the purity level of 1.4 fold with the GST protein yield of 70%. The major isoenzymes which accounted for detoxification function are approximately 60% of the cytosolic GSTs such as the μ class. The α- class GST are involved in isomerization of molecules and π GST shall be considered as marker molecule of hepatotoxicity and carcinogenesity. the purification studies revealed that the existence of three subunits Yc, Yb and Ya in mice liver must play a critical role to have multifunctional GST proteins. The immunological studies revealed that the mice liver GSTs have homological sequences with the proteins of rat liver and not with sequence of chick liver GSTs.

Key words: *Mice liver, Immunodiffusion, SDS-PAGE, Protien purification.*

INTRODUCTION

GSTs in normal tissues is a prerequisite for a sound understanding of their variations in abnormal conditions, as seen in many diseased states. Hence in the present study GSTs were purified from mice liver by affinity chromatography. Antisera raised against purified GSTs of mice liver in rabbits were employed for probing the variations in GST subunits in treated tissues. These are generally categorized as the kidney type and liver type glutaminases and both types have been purified and characterized (Svenneby et al., 1973; Curthoys et al., 1976; Heini et al., 1987).

In mice each organ possesses a unique profile of GSTs, the liver and testis having highest activity in rodents (Baars et al., 1981). The mice liver comprises two major classes of three subunits, Yc, Yb and Ya with molecular weights of 26, 24.5, 23KDa, respectively. Ya belongs to alpha class, Yb belongs to mu class of GSTs. Different homo and heterodimeric combinations of various subunits constitute the GST isozymes, they have different but overlapping substrate specificities and a wide array of substrates like CDNB, EPNP, pNPA, pNBC, BSP, Δ^5 Androstene 3,17 dione (Habig et al., 1974). The characterization of individual GSTs in each organ requires a battery of tests involving specific activities with several substrates, sensitivities to several inhibitors, molecular weight of subunits, and precipitation with antibodies. Therefore the present study was conducted on liver GSTs for their purification and characterization.

MATERIALS & METHODS

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.

Protein Estimation:

Protein content of all samples was determined by the method of Lowry et al., 1951**,** using bovine serum albumin (BSA) as the standard, and also by measuring the change in absorbance at 260 and 280nm spectrophotometrically **(**Warburg and Christian, 1941).

Isolation and Purification of GST:

The purification of GSTs was carried out by affinity chromatography **(**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 (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 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.

SDS-PAGE:

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

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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.

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.

RESULTS & DISCUSSION

Purification of GST by affinity chromatography:

Glutathione-S-transferases have been purified to electrophoretic homogeneity from mice liver by affinity chromatography. The cytosolic fraction of mice liver was analyzed for GST activity and was purified using GSH linked agarose-4b affinity chromatography column. The bound protein of the column upon elution with buffer showed a typical elution profile for 10 fractions mentioned in **Table 1**. Mice GSTs are encoded by a multigene family are differentially regulated expression in a tissue specific manner to meet the special detoxification needs of various organs. The GST isoforms that are existing in mice may be distinguished based on the differences in binding specificities and catalytic properties toward various molecules (Jokoby, 1978; Mannervik et al., 1985, Tahir and Mannervik, 1986).

Table No. 1: Typical elution of GST

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

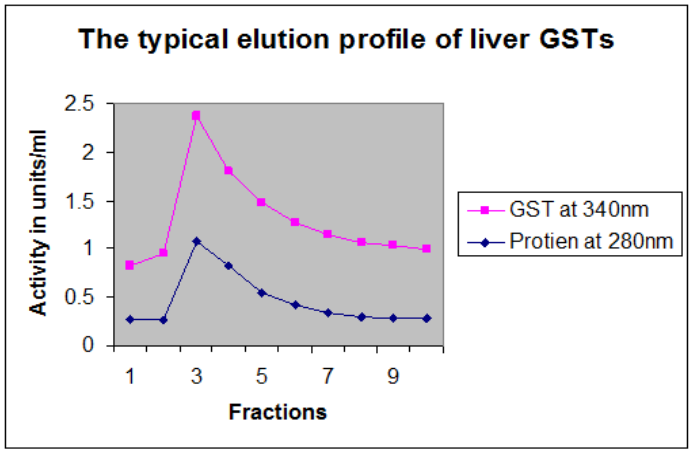


Fig. 1: Affinity chromatography elution profile of mice liver glutathione S-Transferases

This is the elution profile graph of mice liver GSTs. This profile up on drawn as a graph which showed a single sharp protein and activity peak. (**Fig. 1**).

Table No. 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**

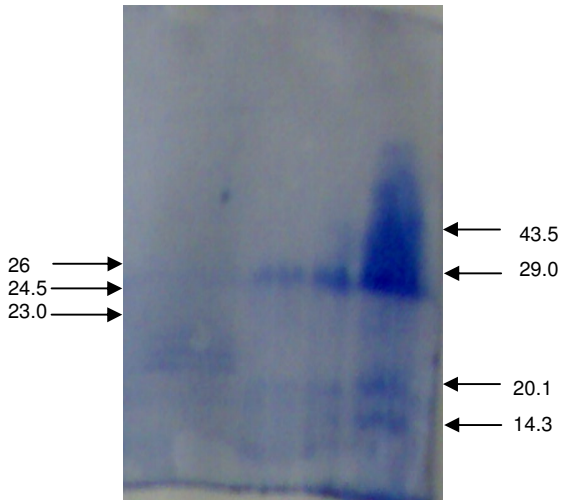
The active fractions GSTs were pooled, to determine the activity and protein content. The specific activity of liver GSTs was determined and found as 66.7 µ moles/min/mg proteins. The purification was achieved with an overall yield of 70.2% (**Table 2**).

In the present study mice liver GSTs were purified, individual subunits were located and then characterized. The affinity purification studies revealed that the GSTs can be purified in a single

step process to the purity level of 1.4 fold with the GST protein yield of 70% (**Table 2**).

SDS-PAGE analysis of affinity purified mice liver GSTs:

The affinity purified GSTs were subjected to electrophoresis to determine the purity of the preparation of proteins and analyzed the molecular weights of the GSTs based on movement of proteins on denatured gel. Calibration of the relative molecular weights was done by loading the molecular weight marker proteins along with the affinity purified GSTs side by side in a single gel (**Fig. 2**).As shown in the fig 2, the affinity purified GSTs (Lane-II) were resolved into three bands with relative molecular weights of 26, 24.5, 23KDa as Y_c, Y_b and Y_a, respectively. This indicates the presence of μ, α GSTs.



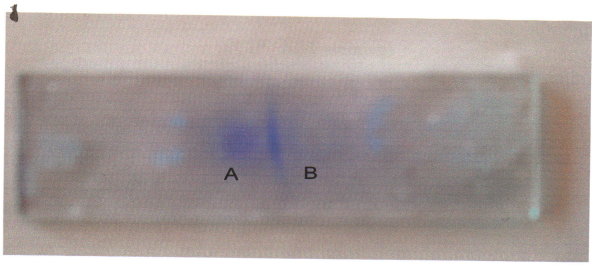
LANE - 1 : Mice liver Affinity Purified GSTs.
LANE - 2 : Marker Proteins

Fig. 2: SDS-PAGE analysis of GSTs of mice liver

This procedure is most useful to purify the GSTs and the purified proteins on electrophoresis found to contain only specific GST proteins (**Fig. 2**). The present study revealed structural multiplicity of GST subunits in mice liver. In view of the detoxification role of the GST against various electrophilic compounds, the presence of three GST isoenzymes in liver tissues might contribute to the protection of liver tissues from all sorts of insects of nature. The major isoenzymes which accounted for detoxification function are approximately 60% of the cytosolic GSTs such as the μ class. The α-class GST are involved in isomerization of molecules and π GST shall be considered as marker molecule of hepatotoxicity and carcinogenesity. In conclusion the purification studies revealed that the existence of three subunits Yc, Yb and Ya in mice liver must play a critical role to have multifunctional GST proteins.

Analysis of mice liver GSTs using immunodiffusion:

Antisera prepared against affinity purified GSTs proteins of mice liver by following the published procedure (Tu et al., 1985) used for the analysis of mice liver GSTs. The antisera showed specific cross reactivity with the GSTs of mice liver (**Fig. 3**).



A: Purified Liver GST B: Antiserum

Fig. 3: Immuno diffusion analysis of Affinity purified mice liver GST Proteins

The immunological studies revealed that the mice liver GSTs have homological sequences with the proteins of rat liver and not with sequence of chick liver GSTs. The N-terminal sequence data of frey et. al(1983) for the rat Yb subunits and deduced sequence of cloned cDNA coding mouse GST (pearson et.al 1983) support this hypothesis.

CONCLUSION

Glutathione –S-transferases, a selective adaptation, by the living being in biological evolution can deal with a wide spectrum of exogenous and endogenous reactive molecules in phase II drug detoxication mechanisms and catalyses the attack on electrophilic reactive intermediates. They are followed by their end product excretion from the cell mainly cytosolic and microsomal proteins distributed in almost all the organs of the living organisms. And have several subunits which combine in several combinations to give rise to dimeric protein that can acts as on the electrophilic centers of the substrates and reduces their toxicity. 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. This indicates the presence of μ, α GSTs. Polyclonal antibodies were raised in rabbits against purified liver GSTs.

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