METHODOLOGY

2.0 MATERIALS:

Glutathione reduced (GSH), S-hexyl glutathione, 1-chloro-2,4,-
dinitrobenzene (CDNB), dithiothreitol (DTT), epoxy-activated Sepharose 6B,
phenylmethylsulfonyl fluoried (PMSF), Tris, glycine, L-serine, Tween-20,
Triton X-100, sodium chloried, $\Delta^5$-androstene-3,17-dione($\Delta^5$AD),
bromosulfophthalein (BSP), 1,2-dichloro-4-nitrobenzene (DCNB),
Ethacrynic acid (EA), 4-hydroxynonenal (4-HNE). Freund's complete and
incomplete adjuvant were purchased from Sigma Chemicals Company
(St. Louis, USA)

Acrylamide (99.9%), N,N'-methylene-bis-acrylamide,N,N,N',N'-
tetramethyl-ethylenediamine (TEMED), 2-mercaptoethanol, natriumlauryl-
sulfat (SDS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue
tetrazolium (NBT), comassie brilliant blue G-250, coomassie brilliant blue R-
250, bio-lyte ampholines and bromophenol blue were purchased from Bio-
Rad laboratories (Richmond, USA).

Low molecular weight markers for SDS-PAGE were purchased from
Pharmacia Biotech (Uppsala, Sweden). Ultrafiltration units were procured
from Amicon (MA, USA). Nitrocellulose membranes for immunoblotting were
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from Amersham (England, UK). C18 and silica cartridges were purchased from Waters, India.

Class specific antibodies of GSTs were a kind gift from Yogesh C. Awasthi (University of Texas, Galveston). HPLC solvents like acetonitrile, methanol were purchased from Spectrochem India Ltd. All other chemicals procured were from the local companies and were of high quality.

2.1 TISSUE HANDLING:

Human uteri were procured from local hospitals, immediately after hysterectomy. The normal portions of the collected uterus were confirmed by a pathologist at the source facility and were stored in -80° C till further use.

Cancerous and surrounding normal tissues were obtained from Mehidi Nawaz Jung Cancer Hospital (Regional Cancer Center), Hyderabad. All the tissues were collected in liquid nitrogen and stored at -80 ° C till further use. A portion of each tissue was examined by a pathologist at the source facility and confirmed as being cancerous or normal. All the tissues were classified into different stages of cancer (stage I = Tumor cells found only in original site, stage II = Tumor cells found in original site and regional lymph nodes, stage III = Tumor cells found in original site, regional lymph nodes and in distant lymph nodes and stage IV = Metastatic tumor cells found in many body areas) by the pathologist.
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2.2 PROCESSING OF TISSUE FOR GST ACTIVITY:

Normal and cancerous tissues were slightly thawed, minced with scissors and homogenized in 50 mM Tris - HCl buffer, pH 8.0 containing 0.25 M sucrose and 1mM PMSF using a glass homogenizer. Homogenization was done by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheesecloth and the resulting supernatant was centrifuged at 10,000 X g on high speed refrigerated centrifuge (KUBOTA 6700, rotor RA-4) for 30 min. The resulting supernatant was used as the enzyme source.

2.3 GST ACTIVITY ASSAY:

GST activity was assayed by the conventional method of Habig et al (1981). The typical enzyme mixture in a volume of 1 mL consists of 1 mM CDNB (1, chloro, 2,4- dinitrobenzene), 1 mM glutathione (GSH) and 100mM potassium phosphate buffer (pH 6.5). Thioether formation was determined by reading the absorbance at 340 nm and quantification was done using 9.6 \( \text{mM}^{-1}\cdot\text{cm}^{-1} \) as the extinction coefficient.

2.4 AFFINITY CHROMATOGRAPHY OF GSTs:

2.4.1 Preparation of affinity matrix

Affinity matrix was prepared by coupling glutathione (GSH) to epoxy activated Sepharose -6B as per the method of Simmons and Vander Jagt (1977). About 4 g of epoxy - activated Sepharose 6B was washed with 500
Methodology

mL of distilled water followed by 40 mL of 44 mM phosphate buffer pH 7.0. Gel was transferred to another flask and the volume was adjusted to 20 mL with the same buffer and nitrogen gas was passed through for 5 min. To this 4 mL of GSH (400 mg of GSH in 4 mL of distilled water, pH was adjusted to 7.0 with KOH) was added and allowed to stay for 24 hr at 37°C with constant stirring.

The coupled gel was washed with 100 mL of distilled water followed by 100 mL of 0.5 M KCl in 0.1 M sodium acetate, pH 4.0 and 0.5 M KCl in 0.1 M sodium borate buffer, pH 8.0. Finally the gel was transferred to 10 mM potassium phosphate buffer, pH 7.0 containing 150 mM KCl.

2.4.2 Cytosol preparation

Normal and cancerous tissues were homogenized in four volumes of 10 mM potassium phosphate buffer, pH 7.0 containing 0.25 M sucrose, 1 mM EDTA, 2 mM dithiotheritol. Homogenization was done in potter Elvejahm homogenizer by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation and centrifuged at 10,000 X g for 30 min. The resultant supernatant was passed through two layers of cheesecloth to remove the cell debris. The supernatant was again centrifuged at 1,05,000 X g on Hitachi ultracentrifuge with P50AT2 rotor for one hour. The supernatant obtained was referred to as the cytosolic fraction.
2.4.3 Purification of GSTs

Cytosolic fractions after dialysis were loaded on to GSH-Sepharose 6B affinity column previously equilibrated with 10 mM potassium phosphate buffer pH 7.0 containing 0.15 M KCl and then washed with the same buffer till the protein content dropped to zero (by spectrophotometric detection). The affinity bound GSTs were eluted with 50 mM potassium phosphate buffer pH 7.5 containing 10 mM GSH and 1 mL fractions were collected. Active fractions were pooled and concentrated by using centricon concentrators (AMICON).

2.4.4 Protein determination:

Protein content in the chromatographic fractions was determined spectrophotometrically by the procedure of Warburg and Christian (1941) by measuring the absorbance at 280 nm and 260 nm.

Protein content in the samples like crude homogenate and cytosol were assayed by the method of Bradford (Bradford et.al, 1976).

2.5 SUBSTRATE SPECIFICITIES:

In order to screen different GST isozymes for substrate specificities, they were assayed for activity with ethacrynic acid, 1,2 epoxy - 3(-p-nitrophenoxy )- propane, Sulfochromophalein, 4-nitropyridine-N-oxide and 3,4 dichloronitrobenzene in addition to 1-chloro-2,4-dinitrobenzene, the classical substrate for GSTs.
Table 3: Reaction conditions for the assay of GSTs with different substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Mol. Wt</th>
<th>Con. mM</th>
<th>pH</th>
<th>X max. mM</th>
<th>GSH mM</th>
<th>Ac /mM/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chloro-2,4-dinitrobenzene</td>
<td>192.0</td>
<td>1</td>
<td>6.5</td>
<td>340</td>
<td>1</td>
<td>9.6</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>303.1</td>
<td>0.2</td>
<td>6.5</td>
<td>270</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>1,2-Epoxy-3-(p-nitrophenoxy) propane</td>
<td>195.2</td>
<td>5</td>
<td>6.5</td>
<td>360</td>
<td>1</td>
<td>05</td>
</tr>
<tr>
<td>Sulforhodamine</td>
<td>8380</td>
<td>0.03</td>
<td>7.5</td>
<td>330</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>4-Nitropyridine-N-oxide</td>
<td>140.1</td>
<td>0.2</td>
<td>7.0</td>
<td>295</td>
<td>1</td>
<td>7.0</td>
</tr>
<tr>
<td>3,4-Dichloronitrobenzene</td>
<td>192.0</td>
<td>1</td>
<td>75</td>
<td>345</td>
<td>5</td>
<td>8.5</td>
</tr>
</tbody>
</table>
2.6 SDS-PAGE ANALYSIS

Vertical slab gel electrophoresis was performed according to the method of Laemmli (1970). Electrophoresis was carried out in 12% acrylamide gel with 5% stacking gel. Samples were treated with sample buffer containing 2% SDS, 5% \( p \)-mercaptoethanol, 0.01% bromophenol blue for five min. in boiling water bath. Samples were subjected to electrophoresis at constant voltage (100 Volts) till the dye reached the bottom.

2.7 TWO-DIMENSIONAL GEL ELECTROPHORESIS:

Two-dimensional electrophoresis was performed as described by O'Farrell (1975). Isolelectric focusing gels were cast in glass tubes (130X2.5 mm). To setup the pH gradient the ampholyte polyacrylamide gels were prefocused at 200 V for 15 min, at 300V for 30 min and at 400V for 30 min. The samples were loaded and the gels were run at 400 V for 12 h and 800 V for 1 h with 0.01 M \( H_3PO_4 \) as the anolyte and 0.02 M NaOH as the catholyte. The gels were extruded into 5 mL of 0.0625 M Tris- HCl (pH 6.8) buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and equilibrated for 2 h at room temperature with shaking. The gels were stored frozen at -20° C till use. The second dimension separation was carried out using 3.3 % stacking gel and 12% resolving gel. Pharmacia LMW markers were run at the acidic end of the gel. The gels were run at 25 mA until the dye (bromophenol blue) reached the bottom of the gel. The gels were fixed and silver stained. To determine the pH gradient of the IEF gels, parallel
gels were cut into pieces of 0.5 cm length and incubated for 2 h in 0.5 mL degassed distilled water. The pH of the eluant was determined electrometrically and was taken as isoelectric point (pI) of the protein present in the gel.

2.8 SILVER STAINING

Gels were stained with silver nitrate by the method of Blum (Blum et al 1981). Gels were treated with a solution of 50% methanol and 7.5% acetic acid for five minutes followed by washing thrice (5 min. each) with 10% ethanol and 5% acetic acid. Gels were treated with 0.1% potassium dichromate in nitric acid (24 mL /100 mL) for five min and washed thoroughly with distilled water. The gels were treated with 0.2 % silver nitrate solution for 15 min, rinsed with distilled water and color was developed with 3% sodium carbonate solution containing formaldehyde (500 mL /1000 mL). Color development was stopped by 1% acetic acid and gels were preserved in 5% acetic acid.

2.9 MOLECULAR WEIGHT ANALYSIS

Molecular weight of the proteins was determined from the calibration curve generated using Pharmacia low molecular weight (LMW) markers using gel documentation system (UVP, San Gabriel Inc. UK). The LMW markers included phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and a lactalbumin (14.4 kDa).
2.10 PRODUCTION OF ANTISERA

Purified protein from affinity as well as HPLC eluants were used for immunization. Rabbits (New Zealand white male, 2 months old), prior to the injection of sample, were bled a week before the serum was collected and stored as pre-immune sera. The samples were injected subcutaneously which were emulsified in Complete Freund's adjuvant in 1:1 ratio. The booster injections were given with the sample in incomplete Freund's adjuvant after 15 days and continued for 3 months giving booster for every 15 days. Rabbits were bled a week after the final booster injection, serum was collected, Ig G was purified using the protein -A agarose and stored in aliquots at -20° C as immune sera (primary antibody).

2.11 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

ANALYSIS

Affinity purified GSTs were further subjected to RP-HPLC (Shimadzu 6A) using Waters bondapack C-18 (3.9 X 300 mm) column by employing gradient elution. The mobile phase was 20 % acetonitrile with 0.1 % (v/v) trifluoroaceticacid (solvent A) and 65 % acetonitrile with 0.1 % of trifluoroaceticacid (solvent B). Affinity purified sample (100 μg) was injected in 100 % of solvent A and the elution was done with a linear complex gradient from 0 to 100 % of solvent B over a period of 50 min.

The gradient was constructed in the linear increasing concentration of solvent B in four successive steps, (0-10 % in 1st 10 min, 10-40 % from
Methodology

15th to 20th min, 40-50% from 25th to 35th min and 50 - 100% from 40th to 50th min, using SCL-6A system controller. The eluted polypeptides were monitored at 214 nm using SPD-6AV UV-VIS spectrophotometric detector.

2.12 ENZYME LINKED IMMUNOSORBENT ASSAY

Enzyme linked immunosorbent assay (ELISA) was performed with the normal and cancerous samples. The samples were loaded into the wells of microtitre plate (200 μL/well) and the plate was incubated at 37 °C for 90 min in a humid container. The plate was washed thrice with Phosphate Buffer Saline with 0.05% Tween-20 (PBS-T) at three min interval. Primary antibody diluted (a 1:1000, μ1.5:1000, μ2:1.5:1000 & π1:1:1000) in PBST with 2% Polyvinylpyrrolidone - 40,000 and 0.2% Ovalbumin (PBS - TPO) was added (200 μL / well) and incubated at 37 °C for 90 min and washed with PBS - T. Secondary antibody conjugated with alkaline phosphatase (Genie, Bangalore) diluted (1:5000) in PBS - TPO was added (200 μL / well) in to the wells and incubated at 37 °C for 90 min. After incubation, the wells were washed with PBS - T and the substrate, paranitrophenyl phosphate (5mg / 10 mL Diethanolamine buffer pH 9.8) was added (200 μL / well). The plate was incubated at room temperature till the desired color was developed (10 min.) and the color was read at 405 nm.

2.13 WESTERN BLOTTING

Immunoblot analysis was carried out on nitrocellulose membranes according to the published procedures of Towbin et al (1975). The cytosolic
Methodology

GSTs separated on SDS-PAGE were transferred on to the nitrocellulose membrane. The gels were initially soaked in 25 mM Tris, 192 mM glycine & 20 % methanol. The separated peptides were transferred with a current of 0.8 mA/cm² for four hours.

After the transfer process, the membrane was air dried for few seconds. Immediately the membrane was made wet in Tris buffer saline (TBS) & thorough rinsing was done. Then the membrane was transferred in to TBS, which contains 5% nonfat milk for 30 min or more to block the nonspecific binding sites. The membrane was immersed in TBST (Tris buffer saline with 0.05% Tween 20) with nonfat milk containing the primary antibody and incubated for 30 min. The unbound primary antibody was removed by washing with TBST, 3 times (5-10 min each). Membrane was incubated for 30 min in TBST with 5% non-fat milk containing secondary antibody linked to alkaline phosphatase. The membrane was washed with TBST 5 times (5 min each) and was subjected to color development.