CHARACTERIZATION OF GLUTATHIONE-S-TRANSFERASES-SUPPRESSION OF ANTIOXIDANT ENZYMES BY ACRYLAMIDE IN DEVELOPING CHICK EMBRYONIC BRAIN

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ABSTRACT

Glutathione-S-transferases (GSTs) constitute a multifunctional family of dimeric and cytosolic enzymes which play an important role in biotransformation of tissues from oxidative stress. They catalyze the conjugation of intracellular glutathione with chemicals, that possess an electrophilic centre to facilitate their metabolism and elimination through mercapturic acid pathway. Acrylamide, a known neurotoxicant, causes damage to almost all organs including brain, liver, testis, and kidney. In the present study, effect of acrylamide was evaluated on GST activities, total-specific activity using substrates and purified from 11th old day developing chick embryonic brain tissue by a glutathione affinity matrix chromatography column, and the GST protein pattern was investigated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The results on activities of glutathione-S-transferases (GSTs) have been shown an increase up to 0.4mg of acrylamide and decline from 0.5 to 0.6mg of acrylamide due to dose dependant acrylamide treatment. The GSTs were purified to 37.36-fold with an yield of 62%, with a specific activity of 25.18 U/mg protein from chick embryonic brain using glutathionyl linked Agarose affinity chromatography. The SDS-PAGE analysis of chick embryonic brain purified GSTs were resolved in to three bands, CB1 (α), CB2 (µ) and CB3 (π), with relative molecular weights of 29.0, 26.0 and 24.0 kDa, respectively. The present data conclude that acrylamide as neurotoxicant induces oxidative stress in developing chick embryo brain due to suppression of the antioxidant enzymes. Further GST proteins were purified to electrophoretic homogeneity with an overall yield of 37.36% showed α, µ and π class GSTs protein bands.

KEYWORDS: Acrylamide, Chick Embryo Brain, Glutathione- CL-agarose affinity column, Glutathione-S-transferases, Substrate specificity, Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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INTRODUCTION

The glutathione-S-transferases (GSTs) are a multigene super gene family enzymes which catalyzes the nucleophilic addition of thiol of reduced glutathione to varieties of electrophiles\(^1\). These GSTs are phase-II defense soluble proteins composed of two subunits with molecular masses of 22–27 kDa. These are classified into several classes (alpha, mu, pi, theta, sigma, zeta and omega) based on their primary structure, immunological and kinetic properties\(^3\). Different classes of GSTs vary in their functional role of protecting the cell against exo and endogenous toxic compounds, including secondary metabolites of lipid peroxidation. However, Gallagher et al\(^4\) have reported that GST detoxifies a number of environmental carcinogens and epoxide intermediates due to its multifunctional properties. Acrylamide (AC) is an α, β-unsaturated carbonyl compound with a significantly high chemical activity, used as an intermediate monomer in the synthesis of polyacrylamide polymers, in numerous applications as soil conditioners, in water treatment, cosmetic, paper and textile industries, and in the research laboratories\(^4\). Recently, the discovery of acrylamide in a variety of human foods was reported by Swedish researchers\(^5\) and similar findings were confirmed later in numerous other countries. Acrylamide is not only genotoxic but also considered to be a potential carcinogen. The predominant chemistry involves a Millard reaction, a non enzymatic browning reaction that occurs by a condensation of the amino group of the amino acid, asparagine, and the carbonyl group of reducing sugars (fructose and glucose) during high-temperature heating\(^6,7\). Acrylamide induced central-peripheral neuropathy and symptoms were primarily associated with occupational exposure potential via both inhalation and dermal contact\(^8\). Information in humans is available from numerous case reports in which acrylamide exposure has been associated with signs of impaired neurological performance in central and peripheral nervous systems that include impaired motor function and muscle weakness. Evidence of degenerative lesions in peripheral nerve fibers, as observed by light and electron microscopy, have been detected at oral doses lower than those eliciting clinical signs and other overt indications of functional deficit. Acrylamide is a carcinogen with the potential to cause nervous system damage\(^9,10\). Knowledge of the type of GSTs in normal tissues is a prerequisite for a sound understanding of their variations in abnormal conditions, as seen in many diseased states. For example a specific class of GST expression and induction will depend upon tissue type and compound that influences the tissue. Therefore considering all these facts. The aim of the present study selected is an analysis as the effect of Acrylamide on GSTs and purification of GSTs from 11- day-old chick embryonic brain using affinity chromatography.

MATERIALS AND METHODS

Acrylamide (99.9%), a monomer form, purchased from Bio-Rad laboratories (Richmond, USA), The GST affinity matrix (glutathione-CL agarose) was purchased from Genei Bangalore were purchased. The substrates 1-chloro-2,4- dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), p-nitrophenylacetate (p-NPA), 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), reduced glutathione (GSH), were purchased from Sigma Chemical Co. (St. Louis, USA). The electrophoresis chemicals, N, N1- methylene-bis-acrylamide, 2-mercaptoethanol, N, N1, N1-tetramethylethylenediamine (TEMED), sodium lauryl sulphate (SDS), nitro blue tetrazolium (NBT), 5-bromo- 4-chloro-3-indolyl phosphate (BCIP), Coomassie brilliant blue R-250 and bromophenol blue were purchased from Bio-Rad laboratories (Richmond, USA). Low molecular- weight markers for SDS-PAGE were from procured Amersham (England, UK).
Source of fertilized eggs and incubation conditions
Freshly laid Bobcock strain zero day old fertilized eggs were purchased from Sri Venkateswara Veterinary University, Tirupati and Sri Balaji hatcheries, Chittoor, Andhra Pradesh. The eggs were incubated horizontally at 37.5±0.5˚C with a relative humidity of 65% in an egg incubator, we consider day1 (d1) as an incubation period of 24h.

Treatment
Acrylamide treatment and collection of brain tissue
A group of six eggs (n=6) were maintained for each time point and dose, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6mg, of acrylamide (10µl) in saline was administered as single dose separately to fertilized chick embryos on day8 (d8),day9 (d9) and day10 (d10) of incubation. On 11th day the embryo’s brain was collected and stored for further use at -20°C.

Tissue processing for assay and purification of glutathione s-transferases
The control and treated chick embryonic brain tissue was thawed and homogenised in 50 mM Tris-HCl buffer pH 8.0 containing 0.25 M sucrose and 1 mM PMSF using a glass homogeniser. Homogenisation was done by keeping the glass homogeniser in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth to remove fat and the resulting supernatant was centrifuged at 10,000rpm for high-speed refrigerated centrifuge for 30 min. The supernatant was passed through glass wool to remove fat and the pellet was discarded. The filtrate was centrifuged at 105,000rpm for 45 min and the cytosolic fraction was passed through glass wool to remove floating lipid materials. The resulting filtrate was used as the enzyme source and was dialysed against four volumes of 50 mM Tris-HCl buffer overnight with four changes of buffer. All the purification procedures were conducted at 4°C unless otherwise stated.

Protein determination
Protein content of control, treated and affinity-purified samples was determined by the method of Folin-Lowry colorimetric method using bovine serum albumin (BSA) as standard and also by measuring the change in absorbance at 260 nm and 280 nm spectrophotometrically.

Glutathione- S- Transferases (GSTs)
Glutathione-S-transferases activity using CDNB as substrate was assayed spectrophotometrically essentially as described by. The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5) for assaying chlorodinitrobenzene, 1 mM GSH and 1 mM of either substrate or suitable aliquots (usually 20 µl) of appropriately diluted enzyme from the different sources. Change in absorbance at 340 nm was followed against a blank containing all reactants excepting enzyme protein, specific activity was expressed as µmol conjugate formed/ min/mg protein using a molar extinction coefficient of 9.6 for CDNB.

Glutathione Peroxidase (GPx)
Glutathione Peroxidase (EC.1.11.1.9) activity was assayed by the method of Rotruck et al. To 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of homogenate were added. To this mixture, 0.2 ml of glutathione followed by 0.1 ml of hydrogen peroxide was added.

Affinity purification of GSTs
Cytosolic fractions of above supernatant after dialysis were loaded onto a glutathione- CL-agarose affinity column previously equilibrated with 50 mM Tris-HCl pH 8.0 and then washed with 50 mM Tris-HCl pH 7.4 containing 0.2 M potassium chloride till the protein content dropped to zero (by spectrophotometric detection). The affinity-bound GSTs were eluted with 50 mM Tris, pH 8.0, containing 5 Mm GSH and 0.2 M KCl. One-millilitre fractions of the elutions were collected. Active fractions were pooled, dialysed and concentrated by using centri concentrators.
**SDS-PAGE analysis**

For analytical SDS-PAGE, samples were diluted to 1:4 with a solubilizer (1% SDS; 0.02% bromophenol; 1% mercaptoethanol in running buffer) and boiled for 3 min before electrophoresis. Separation gels were 12% acrylamide 0.1% SDS in 0.5 M Tris-HCl pH 8.8. Stacking gels were 3.5% of acrylamide in 1.5 M Tris-HCl pH 6.8. The Laemmli\textsuperscript{17} discontinuous buffer system was used; the running buffer is 2 M glycine 0.1% SDS, 0.4 M Tris pH 8.3. Electrophoresis was carried out at 45 V and 20 mA overnight in a Hoeffer vertical electrophoresis. Gels were stained with Coomassie Brilliant Blue and after destining the bands were observed on illumination light and photos were taken.

**Substrate specificities**

In order to screen different isozymes for substrate specificities, the purified GST protein were assayed for activity with DCNB, pNPA, pNBC, EPNP, Δ5-androstene-3, 17- dione, and H$_2$O$_2$ in addition to cDNB, the classical substrate for GSTs, by the method of Habig and Jakoby\textsuperscript{12}.

**Statistical Analysis**

Results obtained for enzymatic activities were expressed as the means ± standard deviation (SD). Differences between groups were evaluated by using one-way ANOVA, followed by Duncan’s test. All statistical analyses were performed using the statistical software SPSS 11.0 (SPSS Ltd., Surrey, UK). The p-value of less than 0.05 was considered as statistically significant.

**RESULTS**

The Figure 1 represents the levels of glutathione s-transferases (GSTs), respectively, in brain tissue of normal and all six AC-treated developing chick embryos. The embryos administered with acrylamide, showed a significant increase in the levels of GST activity as compared to control; the biggest activities were noted in 72h of the 0.4 mg AC dose group when compared to controls and 0.5 and 0.6mg AC-treated brain tissue of developing chick embryos at 48 and 72h time of interval were significantly decreased compared with the control group. Among the changes in GSTs activities, 1.703 fold biggest declines were noted in 72h of the high dose of AC (0.6mg) group compared with controls. The Figure 2 represents the levels of glutathione peroxidase (GPx) in the brain of normal and 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6mg of acrylamide treated developing chick embryos. The activity of GPx was 0.1351 ± 0.03, 0.1785 ± 0.04, 0.1702 ± 0.03, 0.1621 ± 0.0, 0.1619 ± 0.03, 0.1573 ± 0.02, 0.1476 ± 0.01 at 24h, 0.1871 ± 0.01, 0.1661 ± 0.01, 0.1580 ± 0.01, 0.1499 ± 0.01, 0.1380 ± 0.01, 0.1361 ± 0.02, and at 48h, 0.1910 ± 0.01, 0.1548 ± 0.01, 0.1531 ± 0.01, 0.1358 ± 0.01, 0.1318 ± 0.01, 0.1234 ± 0.03 at 72h. The GPx activities were decreased with dose and time dependent manner in the developing chick embryonic brain.
Figure 1
Effect of acrylamide on the glutathione s-transferases activity of d (11) developing chick embryo brain. Brain of control, acrylamide of increasing quantities of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg per 100 µl respectively, were assessed for a period of 24, 48 and 72h. The enzyme activity was expressed as µmoles of CDNB-GSH conjugate formed per minute per mg protein. The values not sharing a common superscript in a row differ significantly p<0.05, according to Duncan’s Multiple Range Test (DMRT).

Figure 2
Effect of acrylamide on the glutathione peroxidase activity of d (11) developing chick embryo brain. Brain of control, acrylamide of increasing quantities of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg per 100 µl respectively, were assessed for a period of 24, 48 and 72h. The enzyme activity was expressed as nanomoles of NADPH oxidized /minute/ milligram protein. The values not sharing a common superscript in a row differ significantly p<0.05, according to Duncan’s Multiple Range Test (DMRT).
Affinity purification
The typical elution profile of GSTs from the glutathione affinity column is represented in Figure 3. The GSTs were eluted in a single sharp peak and all the active fractions were pooled and concentrated to 5 ml by ultrafiltration using Amicon concentrators with a 20-kDa cutoff. Purification was achieved with an overall yield of 37.36% (Table 1).

![Figure 3](image)

**The typical elution profiles of 11-day-old chick embryonic brain GSTs.**

The purification yield of the affinity chromatography in the developing chick embryonic brain tissue was 37.36% of the total CDNB-GST activity in the crude homogenate was desorbed with 15 mM GSH. The GSH-affinity chromatography eluates were used without for SDS-PAGE as mentioned. Studying the substrate specificity using a 0.5 mM final concentration of CDNB, of the GST affinity bound fraction, the specific activities of control crude extract and affinity purified sample of developing chick embryonic brain 0.674 and 25.18 mol/min per mg was shown in Table 1. The Table 2 represents developing chick embryonic of brain control crude extract and affinity purified samples GST activities corresponding to CDNB, pNPA, DCNB, Δ5 Androstene 3,17 dione, EPNP and H₂O₂ were 4.850±0.26, 1.213±0.26, 0.606±0.21, 0.693±0.26, 0.520±0.00, 30.176±2.54, 8.324±1.61, 4.161±1.61, 5.202±1.61, 3.641±1.01, and 0.282± 0.03 mol/min per mg, respectively. The electrophoretograms related to developing chick embryonic control brain revealed the presence of three bands in the Lane-2 and 3, i.e., CB-I, CB-2 and CB-3 indicates isoenzymes of GSTs respectively shown in Figure 4. The molecular sizes of the bands are estimated at approximately 29, 26 and 25 kDa when compared to the molecular marker which was used in the gel (Lane-1).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Sample</th>
<th>Total Protein (mg)</th>
<th>Total activity (Units)</th>
<th>Specific activity (Units/mg protein)</th>
<th>Fold purification</th>
<th>Yield (%) activity</th>
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<tr>
<td>1</td>
<td>Crude extract</td>
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<td>0.674</td>
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<td>100</td>
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<td>25.18</td>
<td>37.36</td>
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*One Unit is defined as micromoles of GSH conjugate formed per minute*
Table 2

<table>
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<tr>
<th>S. No</th>
<th>Sample</th>
<th>CDNB</th>
<th>pNPA</th>
<th>DCNB</th>
<th>∆5 Androstene 3,17 dione</th>
<th>EPNP</th>
<th>H2O2</th>
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<tbody>
<tr>
<td>1</td>
<td>Crude extract</td>
<td>4.850±0.26</td>
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<td>0.606±0.21</td>
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<td>0.520±0.00</td>
<td>0.1351±0.03</td>
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<tr>
<td>2</td>
<td>Affinity purified GSTs</td>
<td>30.176±2.54</td>
<td>8.324±1.61</td>
<td>4.161±1.61</td>
<td>5.202±1.61</td>
<td>3.641±1.01</td>
<td>0.282± 0.03</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as micromoles of GSH conjugate formed/min/mg protein (CDNB, pNPA, DCNB, ∆5 Androstene 3, 17 dione, and EPNP). One unit is defined as micromoles of NADPH oxidized/min/mg protein (H2O2). Values are average of three separate experiments of three samples. Mean ±SD significant (t-test).

Figure 4

**SDS-PAGE analysis of affinity-purified samples of GSTs from 11th day developing chick embryonic control brain (CB) tissue.** Lane 1, molecular mass markers (Bio-Rad) with the sizes shown on the right in kilodaltons; Lane 2 and 3 are affinity-purified samples.

**DISCUSSION**

GSTs are encoded by a multigene family and are differentially regulated in a tissue-specific manner\(^{19}\) to meet the special detoxification needs of various organs\(^{20}\). The glutathione plays a critical role in protecting cells from oxidative stress and xenobiotics, in the central nervous system (CNS)\(^{21}\) with a particular emphasis on the mechanism by which neuronal GSH synthesis is regulated. Alpha GST is mainly found in kidney, brain and other tissues. Mu GSTs are restricted to liver, brain and few other tissues. Pi GSTs has a widespread distribution in most tumor cells and tissues but it is absent in liver however expressed in the presence of phenobarbitol. As the detoxifying enzymes, GSTs play an important protective role in embryonic tissues. Therefore, the present study investigated expression patterns...
of GSTs in brains of 11th day embryonic chick brain.

Acrylamide alone the conjugation of AC with GSH catalyzed by GST to form mercapturic acid was considered as a major pathway for AC metabolism in chick embryos. However, Ruxana and Thyaga Raju22 have suggested that AC inhibits hepatic GST, which may result in increased metabolism of AC to GA via the cytochrome P-450 pathway. Chick embryos have been used in the past for several years to investigate the effect of environmental chemicals and radiations on developmental effects, morphogenesis, etc. During development the brain differs from other tissues in being a highly aerobic and totally oxygen-dependent tissue and may be especially at risk from free radical attack, because this tissue is characterized by a low content of natural antioxidants and generates a greater amount of free radicals per gram of tissue than any other organ23. Acrylamide may pose more than a neurotoxic health hazard to exposed ones which shall have signs of impaired neurological performance in central and peripheral nervous systems that include impaired motor function and muscle weakness. Acrylamide and its principal metabolite, glycidamide (epoxide), react with various biologically significant targets. AC enhances the production of reactive oxygen species and potentially affects brain.

In the present study, examination of total GST activity in the AC- treated chick embryo showed that total GST activity was significantly higher up to 0.4 mg treatment following decrease from 0.5-0.6 AC exposure, compared to controls (Figure 1) as reported earlier by Vasundhara et al24 in rats and in the present study 11-day-old chick embryonic brain cytosolic GSTs were purified and individual subunits were characterized. The molecular masses of CB1, CB2 and CB3 subunits are estimated to be 29, 26 and 24 kDa respectively and are within the range of molecular mass reported for mammalian brain cytosolic GSTs of alpha, mu and pi classes26. The epoxide formed from acrylamide is a good substrate for mu and the alpha GSTs, which are present in brain and liver of chick embryo. It is largely responsible for the carcinogenic effect of AC. High levels of expressions of also GSTs are associated with increased tolerance of cells to noxious chemicals and failure to express GSTs is associated with increased risk of disease. The substrate specificity study of affinity-purified GSTs showed increased activity with CDNB, Δ5A, EPNP, pNPA, DCNB and H2O2(Table 2), and indicating the presence of alpha, mu and pi isoforms.

The GST enzyme was purified from 11th day chick embryonic brain. The crude extracts of chick brain was loaded onto S-hexyl- GSH linked-4B affinity chromatography column and on elution the bound protein was eluted as indicated by a single sharp peak which was similar in all groups as shown in Figure 3. The pooled active fractions were assayed for GST activity using cDNB as substrate. The pooled active fractions were assayed for GST activity using cDNB as substrate. The specific activity in normal developing chick embryonic brain was found to be 25.18µmol/min/ml. In the of 11th day chick embryonic brain GST purification, the GSH agarose matrix of affinity chromatographic column retained about 60-63% of the GST activity from brain crude extract and about37-40% was not fixed to the column matrix this may be attributed to that not all GST isozymes can bind to the affinity column matrix. The SDS-PAGE related to developing chick embryonic control brain revealed the presence of three bands in the Lane-2 and 3, i.e., CB-I, CB-2 and CB-3 indicates α, μ and π classes of GSTs respectively shown in Figure 4. The molecular sizes of the bands are estimated at approximately 29, 26.3 and 25 kDa when compared to the molecular marker which was used in the gel (Lane-1).

CONCLUSION

The treatment of the acrylamide to developing chick embryo can reduce the enzymatic activity of GSTs, GPx and antioxidant content in brain. These effects on the degeneration of central nervous system by damaging the neurons by the influence of acrylamide. The SDS-PAGE
indicated protein the presence of three bands $\alpha$ (CB1), $\mu$ (CB2) and $\pi$ (CB3), with relative molecular weights of 29.0, 26.0 and 24.0 kDa, respectively, and overall yield of 37.36% purified form.

ACKNOWLEDGMENT

We are grateful to University Grants Commission (UGC) for providing financial support under Rajiv Gandhi National Fellowship (RGNF) and DRDO to carry out the research work. Prof. Thyaga Raju is recipient of UGC BSR OTG (2013).

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