



Determination of acrylamide-induced chick embryo brain glutathione S-transferases expression through enzyme activity and western blot

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ABSTRACT

Glutathione S-transferases (GSTs) are major detoxification enzymes which belong to Phase II defense enzymes; they can able to metabolize a variety of toxic chemical agents such as carcinogens, genotoxins, neurotoxins, and pesticides. Usually, GST will express when the living beings are encountered toxic chemical compounds. Acrylamide (ACR) is synthesized industrially and widely used in various industries. Usually, ACR formation occurs when food products prepared at high temperature. So that ACR is an environmental and food contaminant and it is well-proven neurotoxin. Due to highly mobile nature, birds that include poultry birds are main victims to xenobiotics (e.g., ACR) through food, water, and agricultural chemical formulas. In this study, ACR administered chick embryo brain GST activity level was assayed using 1-chloro-2,4-dinitrobenzene, and expression was assessed by western blot studies. The results show that the GST expression levels were increased in response to ACR by 24 and 48 h intervals. However, in 48 h interval, GST expression levels decreased slightly. Western blot studies also show similar pattern of GST expression. Immune blot studies showed similar GST band pattern as purification studies showed (our published work). In this study, enzyme activity and western blot analysis proved that the chick embryo brain GST was expressed more to detoxify ACR.

1. INTRODUCTION

Glutathione S-transferases (GSTs) [E.C.2.5.1.18] are Phase II defense enzymes, and they certainly involved in cellular detoxification and excretion of various xenobiotic molecules [1,2]; this is the versatile defense enzyme that requires GSH for antioxidant function. Usually, toxic agents such as carcinogens and environmental pollutants including oxidative stress products are detoxified by GSTs, which conjugate an electrophilic center with reduced GSH [3]. GSTs can detoxify a wide range of hazardous substances by transferase activity and GST associated peroxidase activity [4]. Cytosolic GSTs are dimeric proteins, those have 199–244 amino acids in length and 23–30 kDa molecular mass [5]. Based on amino acid sequence similarities, mammalian cytosolic GSTs (human, rat and mouse) were classified as alpha (α), mu (μ), pi (π), theta (τ), sigma (σ), zeta (ζ), and omega (ω) [2,6]. The avian GSTs have a complex isoenzyme system, but researchers put little attention on them. A xenobiotic which shown noxious effects

by direct interaction with specific sites on cellular macromolecules (e.g., enzymes), and this interaction is dictated by the chemical nature of the toxicant [7,8]. There are no prominent studies about antioxidant enzymes and biomarkers of oxidative stress that related to various bird species [9,10]. Birds have peculiar molecular mechanisms such as (1) low rates of mitochondrial oxygen radical production and (2) high blood glucose levels compared to other vertebrates, and birds can effectively defend free radicals and oxidative stress by this peculiar feature [11].

Acrylamide (ACR) is an electrophile; it causes cytotoxicity through interaction with nucleophile residues of biological macromolecules such as enzymes and DNA by covalent bonds [12,13]. ACR is formed in carbohydrate-rich food products, those prepared at high temperature in addition to environmental accumulation. Orally administered ACR can rapidly absorbed into the blood circulation, and hence, distributed to vital organs and reacts with nerve cells, enzymes, hemoglobin, and cellular DNA [14]. ACR can suppress metabolism and axonal transport in neurons which leads to a deficiency of nutritional factors [15]. ACR shows its toxic effect on both central nervous system and peripheral nervous system [16]. Both oxidative stress and apoptosis play a key role in ACR-induced toxicity [17-19]. ACR-induced lipid peroxidation is the reason for decrease of antioxidant ability in nervous tissue and sciatic nerve [20]. According to the previous literature, ACR has carcinogenic,

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reproductive, and neurotoxic properties [21,22], induces apoptosis in cerebral cortex [19], and efficiently binds to the brain [23]. In both laboratory animals and humans, ACR neurotoxicity is characterized by ataxia and distal skeletal muscle weakness [24]. That the brain, spinal cord and sciatic nerve affect to the ACR-induced oxidative stress in the nervous system including sensory and motor dysfunction in rats [20]. The present study is aimed to evaluate the expression of chick embryo brain GSTs by ACR administration through enzyme activity and western blot studies.

2. MATERIALS AND METHODS

2.1. Chemicals

ACR (99.9%), GSH (reduced), N,N-methylene-bis-ACR, β -mercaptoethanol, 1-chloro-2,4-dinitrobenzene (CDNB), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), Ponceau-S stain, and Freund's incomplete and complete adjuvants were purchased from Bio-Rad laboratories, Richmond, USA, and Genei, Bengaluru, India. Goat anti-rabbit IgG antibodies (Bio-Rad laboratories, Richmond, USA) and nitrocellulose (NC) membrane (Amersham, UK) were gifted by my friend Reddy Peera, National Brain Research Center, Gurgaon, Haryana, India. Ammonium persulfate, ethylenediaminetetraacetic acid (EDTA), glycine, hydroxymethyl aminomethane (Tris base), phenylmethanesulfonyl fluoride (PMSF), sodium azide, sodium dodecyl sulfate (SDS), sodium chloride (NaCl), potassium chloride, skim milk powder, and all required chemicals were purchased from the local chemical companies with high quality.

2.2. Maintenance of Eggs and Route of Chemical Administration

Fertilized Babcock strain chicken eggs weighing about 50 g were incubated at 37°C, 60% humidity, and were rotated for every 3 h. On the 12th and 15th day of incubation eggs were injected under sterile conditions with ACR in ppm (1 mg/L) in distilled water as vehicle. Control eggs were injected with vehicle only. A hole was made in the shell at the blunt end of the egg, and a microsyringe was inserted through the air sac, onto the inner shell membrane, where 2–6 ppm ACR was deposited. Usually, the inner shell membrane has direct contact with the chorioallantoic membrane. Hence, this route of administration gives a faster uptake of the substances than yolk injections. During this process, the survival embryos were tested using Candler light. The dead eggs were discarded and survived eggs were selected for the experiments.

2.3. Tissue Collection and Sample Preparation

Brain tissue was collected from normal and treated 18th-day embryos by pressing small head with forceps and washed with cold 50 mM Tris HCl buffer (pH 8.0), containing 1 mM EDTA to remove excess blood and body fluids, and instantly collected tissue was preserved at –20°C for further experimentation. At the time of experimentation, the brain tissues were slightly thawed and 20% of brain tissue homogenate was prepared in 50 mM Tris-HCl buffer (pH 8.0) containing 0.25M sucrose and 1 mM PMSF using a potter Elvijhem homogenizer. Homogenization was done by keeping the potter Elvijhem homogenizer in an ice jacket, and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheesecloth to remove floating lipid materials, and the resulting supernatant was centrifuged at 10,000 rpm on high-speed refrigerated centrifuge by 2 times for 45 min at 4°C. The collected supernatant was known as cytosolic fraction, and it was used as the enzyme source for purification and activity assays.

2.4. Assay of GST Activity

Conjugation of reduced GSH to CDNB was stimulated by the addition of cellular protein as described by Habig *et al.* [25]. Briefly, 100 mg of cellular fractions was added to cuvettes containing 30 mM CDNB 100 μ l and 30 mM GSH 100 μ l and with phosphate buffer (pH 6.5) adjust for a final volume of 1.0 ml. Change in absorbance (340 nm) was measured over a span of 3 min to calculate the rate of conjugation of CDNB and expressed as μ mole CDNB-GSH conjugate formed/min/mg protein.

2.5. Estimation of Protein

Protein concentration was determined in both control and treated sample by the method of Lowry *et al.* [26].

2.6. Antisera Production

That the affinity-purified GST protein was used for immunization of rabbits (New Zealand white male, 3 months old) to produce antibodies after SDS PAGE analysis as described by Laemmli [27]. Rabbit was obtained from Department of Physiology, Sri Venkateswara Veterinary University, Tirupati. Blood was collected from rabbit, and serum was stored as control for further experimental analysis. That the rabbit hair was removed at 4–6 sites, and then, 200 mg of affinity-purified GST protein per ml was emulsified with an equal volume of Freund's complete adjuvant and that emulsified mixture was injected subcutaneously to the rabbit at 4–6 sites. The booster doses were given with an interval of a week for about 4–5 times. Titer of antibodies was tested before the fifth dose of immunization. The last dose was given with an incomplete adjuvant. 1 week after the last injection, the rabbit was bled and the serum was centrifuged at 6,000 rpm by Remi refrigerated centrifuge for 30 min at 4°C and that the collected supernatant was considered as antisera of chick embryos brain GSTs.

2.7. Electrophotting

According to Towbin *et al.* [28], electroblotting was carried out using Mini Blot Module (Vertical Gel System-EC 120, USA) and NC membrane. SDS-PAGE gel was placed for 30 min in Towbin buffer, i.e., 25 mM Tris HCL (pH 8.8), 192 mM glycine, and 20% methanol. The NC membrane was washed several times with distilled water and then with Towbin transfer buffer until it was equilibrated and precaution was taken that the membrane should not become dry. The blotting stack was assembled on the top of stainless steel grid cathode located in the trough of the frame stand of the mini blot module, to which transfer buffer was added (about 2.5 Lit). The red lead was connected to the anode and the black lead to the cathode, and the proteins transfer was allowed to move anions to the direction of anode. The transfer process was performed at 4°C overnight using a constant voltage of 35V. The NC membrane was removed and placed in the Ponceau-S stain to check the transferred protein bands.

2.8. Immunostaining of NC Membrane

Immunostaining was carried out according to the instruction manual provided with the goat anti-rabbit IgG secondary antibodies, and all of the incubations were performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

The electroblotted NC membrane was incubated in the blocking solution (5% non-fat dry milk powder) in transfer buffer saline (TBS) for 30 min. After that, the NC membrane was incubated with chick embryo brain anti-GST primary antibodies (1:2000 dilutions) in the blocking solution for 30 min. After hybridization with primary antibodies, NC membrane was then washed 5 times with TBS

(5 min each) and incubated with the secondary antibody (goat anti-rabbit IgG-ALP conjugate) with the 1:5000 dilution in TBS for 5 h. After that, the membrane was washed 3 times (5 min each) with TBS, and then, the ALP conjugate color developing solution (BCIP/NBT) was added. The specific protein bands started to appear after 10-30 min. Finally, the membranes were carefully dried, and the images were obtained using a scanner connected to the computer.

2.9. Statistical Analysis

All the data related to the present study and documented results were calculated from three different experiments, and they presented as the mean ± standard deviation (SD). Student *t*-test was used in this study to identify AC-treated brain samples difference from the mean for respective controls. The difference between experimental groups at the level $0 < P < 0.05$ was considered as statistically significant.

3. RESULTS

3.1. GST Expression Studies

3.1.1. GST activity assay

As shown in Table 1 and Figure 1, in 24 h interval, expressed GST activity was significantly increased to 4.0-fold and 8.5-fold with 2 ppm ACR and 4 ppm ACR, respectively, and decreased to 0.6-fold with 6 ppm of ACR than 8.5-fold by 4 ppm of ACR when compared to control, i.e., 4.0-fold.

As shown in Table 2 and Figure 2, in 48 h interval, expressed GST activity was significantly increased to 4.7-fold and 8.2-fold with 2 ppm ACR and 4 ppm ACR, respectively, and decreased to 0.8-fold with 6 ppm of ACR than 8.2-fold by 4 ppm of ACR when compared to control, i.e., 5.3-fold.

3.1.2. Western blot analysis

As shown in Figure 3, in 24 h interval, GST expression levels were significantly increased in response to 2 ppm ACR and 4 ppm ACR,

but GST expression levels were slightly decreased in response to 6 ppm ACR. Similarly, in 48 h interval, GST expression levels were significantly increased in response to 2 ppm ACR and 4 ppm ACR, but GST expression levels were slightly decreased in response to 6 ppm. Initially, GST expression was activated when ACR entered into cell, but excess infiltration of ACR leads to deactivation of GST expression.

4. DISCUSSION

Either intentionally or unintentionally, environment is continuously loading by toxic chemical compounds (xenobiotic) and metals, which released mainly through industries, in the 20th century, thousands of organic pollutants were produced and released them into the environment [29]. Usually, oxidative damage is associated with lot of environmental factors such as weather, availability of food, competition between intra- and inter-specific species, and risk of predation; those factors are vary in different stages of life such as reproduction, hibernation, moulting, and migration; these factors put the animals long period under pressure [11]. In addition to the normal metabolism, detoxification of toxic compounds can also generate various reactive oxygen species (ROS); they promote the oxidative stress in species which are living in contaminated environment [30].

Table 1: Activity profile of chick embryo brain GSTs in response to ACR by 24 h interval.

Acrylamide	Sample: Chick embryo brain	Substrate: CDNB
Control		0.04±0.02
2 ppm		0.08±0.04 ^a
4 ppm		0.125±0.09 ^a
6 ppm		0.119±0.05 ^a

The values represented in Table 1 are average of three separate experiments of three samples. Mean±SD. Student test ($*P < 0.05$, regarded as significant activity of GST). ACR was administered by doubling the dose 2 ppm per day by 24 h interval. One unit GST activity is expressed as μ mole GSH-CDNB conjugate formed/min/mg protein, GST: Glutathione S-transferases, ACR: Acrylamide

Table 2: Activity profile of chick embryo brain GSTs in response to ACR by 48 h interval.

Acrylamide	Sample: Chick embryo brain	Substrate: CDNB
Control		0.053±0.02
2 ppm		0.1±0.06 ^a
4 ppm		0.135±0.09 ^a
6 ppm		0.127±0.05 ^a

The values represented in Table 2 are average of three separate experiments of three samples. Mean±SD. Student test ($*P < 0.05$, regarded as significant activity of GST). ACR was administered by doubling the dose 2 ppm per day by 48 h interval. One unit GST activity is expressed as μ mole GSH-CDNB conjugate formed/min/mg protein. GSTs: Glutathione S-transferases, ACR: Acrylamide

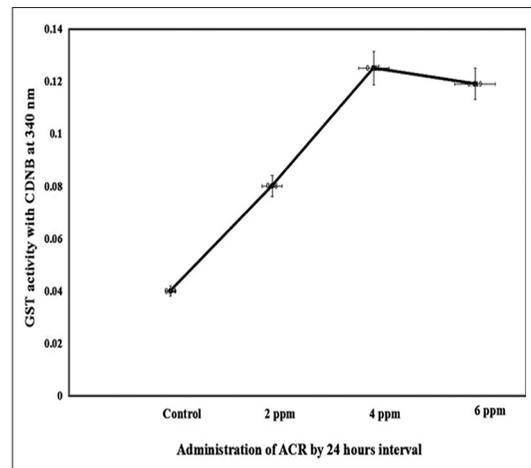


Figure 1: Activity of chick embryo brain glutathione S-transferases by the administration of acrylamide by 24 h interval.

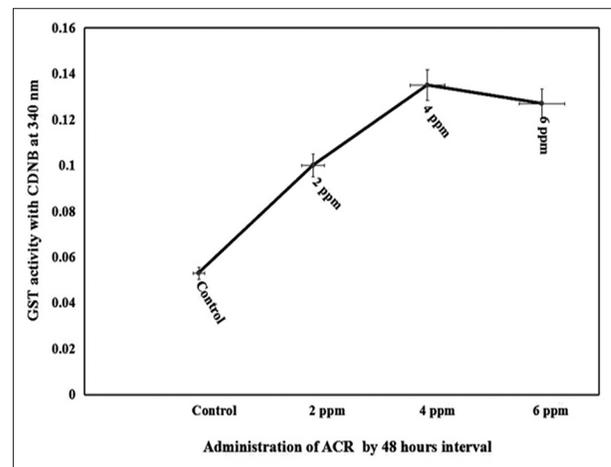


Figure 2: Activity of chick embryo brain glutathione S-transferases by the administration of acrylamide by 48 h interval.

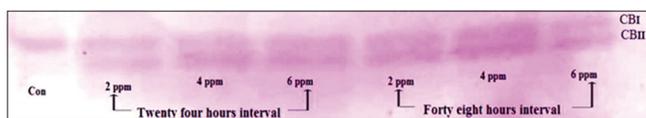


Figure 3: Western blot of chick embryo brain glutathione S-transferases expressed by the administration of acrylamide.

Different strategies have developed by organisms to face the ROS causing physiological imbalance by antioxidant enzymatic system and non-enzymatic antioxidant molecules [11,31]. Biological cells handle the ROS using GSH antioxidant system includes GSTs; it protects cells from oxidative stress [32,33]. In bird species, detoxification efficiency is sometimes associated with the type of diet [34], and sometimes, it is connected to the metabolic rate [35]. ACR is a neurotoxin, and it can be absorbed into the body by several ways such as respiration, digestive system, and skin including muscle. In addition to that, ACR can rapidly spread throughout the body. GSTs are key enzymes to defend cells against cytotoxic and carcinogenic agents [5]. The remarkable change in expression of drug-metabolizing enzymes which may significantly show influence on metabolism and biological effects of drugs, industrial, and environmental contaminants [36].

In the present study, GST activity was evaluated in each dose and interval, using a universal substrate of GST, i.e., CDNB. As shown in Table 1 and 2 and Figures 1 and 2, with the substrate CDNB, GST activities were significantly increased to 4.0-fold and 8.5-fold by 24 h interval, 4.7-fold and 8.2-fold by 48 h interval, in response to 2 ppm ACR and 4 ppm ACR, respectively, and decreased to 0.6-fold by 24 h interval, 0.8-fold by 48 h interval, in response to 6 ppm ACR than 4 ppm (8.5-fold and 9.5-fold) of ACR in both intervals, when compared to control, i.e., 4.0-fold and 5.3-fold, respectively, in both intervals.

As shown in Figure 3, chick embryo brain GST expression levels were significantly increased from 2 ppm ACR to 4 ppm ACR in both intervals, respectively, and slightly decreased by 6 ppm ACR, in both intervals.

As shown in Tables 1 and 2 and Figures 1 and 2, it was observed that the GST activity was increased to 4.0-fold and 8.5-fold by 24 h interval, and 4.7-fold and 8.2-fold by 48 h interval, in response to 2 ppm ACR and 4 ppm ACR, respectively, when compared to 4.0-fold of control in both intervals. Here, elevation of GST activity in response to ACR was observed in this study, and this may be due to increased process of detoxification. As in Figure 3, immunoblot with the primary antibody that is specific to chick embryo brain GSTs showed a band pattern similar to purified GST band pattern (CB_I and CB_{II}, our published work), and it was observed that the increased levels of GST expression in response to 2 ppm ACR and 4 ppm ACR. Here, elevation of GST expression in response to ACR was observed by immunoblot analysis; it may be due to increased process of detoxification. As shown in Tables 1 and 2, and Figure 1 and 2, it was observed that the GST activity decreased to 0.6-fold and 0.8-fold in response to 6 ppm ACR than 8.5-fold and 9.5-fold by 4 ppm ACR, respectively, in both intervals, when compared to control of both intervals, i.e., 4.0-fold and 5.3-fold, respectively. Here, a decrease of GST activity in response to ACR was observed, and this may be due to deactivation of GST by excess ACR. Similarly as shown in Figure 3, it was observed that the slightly decreased levels in GST expression in response to 6 ppm ACR in both intervals. Here, decrease of GST expression was observed by immunoblot analysis, and this may be due to suppression of GST expression by excess ACR.

A potent inducer may involve in transcriptional upregulation of Phase II drug metabolism enzyme genes [37]. That the biotransformation enzymes which can protect organisms from potentially toxic chemicals and ROS. Usually, ROS can damage macromolecules under oxidative stress conditions, while antioxidant enzymes of the body are expressed more [38]. It was reported that the increased activities of GST may protect rats from xenobiotics, and they alter drug metabolism that is, especially, GST substrates [39]. ACR is a potent neurotoxin [15,40] and can induce neurotoxicity in both prenatal and perinatal rodents [41,42]. Purification studies and substrate-specific reaction studies show that CB_I and CB_{II} GSTs of chick embryo brain have similarity with alpha (α) and mu (μ) class GSTs [43]. Chick embryo brain GST protein biomarkers can protect the developing brain from environmental toxic chemical agents [44]. Therefore, the present study was agreed with the study of 37–39, [15], 40–44.

5. CONCLUSION

Chick embryo brain cytosolic GST activity was elevated in response to 2 ppm and 4 ppm ACR, in 24 h and 48 h intervals, but that GST activity was decreased in response to 6 ppm of ACR, in both intervals with the substrate CDNB. Western blot revealed expression of cytosolic GSTs was elevated from 2 ppm to 4 ppm of ACR by 24 h and 48 h intervals, but decreased by 6 ppm of ACR, in both intervals. Increased GST activity was due to an increase of GST expression, and this condition may occur when detoxification process increases. Decrease of GST activity was due to suppression of GST expression, and this may be due to excess accumulation of ACR. Key conclusion, through western blot studies and purification studies that includes biochemical characterization, under normal physiological condition as well as under neurotoxic chemicals, same GSTs were expressed in chick embryo, i.e., CB_I and CB_{II}, and these GSTs expression may elevate to detoxify neurotoxins.

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