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Isolation and purification of Glutathione-S-transferase (GST) and its role on oxidative stress in hepatic tissues of Marek's disease affected chicken

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Abstract
A study was conducted to isolate and purify Glutathione-S-transferase (GST) and to determine its role on oxidative stress in liver tissues of Marek's disease (MD) affected chicken. Ten healthy and MD suspected layer birds of 35-40 weeks age for each group were procured from poultry farms in Chittoor district of A.P. The postmortem examination of MD affected birds revealed enlarged liver with discrete grayish nodules of various sizes. Histopathological examination of liver revealed extensive proliferation and infiltration of pleomorphic cells. The purified DNA samples from liver tissue of MD suspected birds were further used for PCR analysis which was found to be positive yielding a 314 bp product indicating the presence of two copies of 132 bp tandem repeat and a 50 bp primer sequence. The liver samples positive for MD were used for further analysis. The GST enzyme was isolated, purified and characterized from liver tissue of both healthy and MD affected layer chicken using glutathione agarose affinity chromatography. The enzyme was purified to 2.38 fold with a yield of 32.27% in healthy chicken whereas in MD affected chicken it was purified to 4.54 fold with a yield of 41.98%. The analysis of active fractions using SDS-PAGE showed GST-α expression with a molecular weight of 25.6kDa in both healthy and MD affected liver. In addition GST-π was expressed in MD affected chicken with a MW of 27 kDa. These results confirm that Marek's disease in chicken induces oxidative stress which in turn leads to alterations in antioxidant enzymes. The oxidative stress induced in liver is responsible for GST π expression as a defense mechanism in MD affected chicken.

Keywords: Glutahione-S-transferase, Affinity column chromatography, SDS- PAGE, PCR

1. Introduction
Marek's disease is a lymphoproliferative and neuropathic disease of domestic chickens, and less common in turkeys and quails. It was found to be caused by a highly contagious, cell-associated, oncogenic herpes virus [1]. Marek's Disease is a commercially significant poultry disease, a recognized biomedical lymphoma model [2] and also a model for the vaccine-derived evolution of pathogen virulence [3].

Most of the electrophilic molecules, such as endogenous oxidative stress products, carcinogens, drugs, pesticides and herbicides, pass through the liver before they reach and accumulate in target tissues. Hence, it is essential to study the role of antioxidant enzymes and their characterization in liver tissues of birds.

GST is a family of phase II enzymes that can conjugate GSH with various environmental etiological factors, and it is ubiquitously expressed in normal and malignant tissues [4]. Glutathione-S-transferases are evolutionarily conserved enzymes that are important in the detoxification of many xenobiotic compounds. This enzyme catalyzes the conjugation of glutathione to electrophilic substrates, producing compounds that are generally less reactive and more soluble. This facilitates their removal from the cell via membrane – based glutathione conjugate pumps. The broad substrate specificity of GSTs allows them to protect cells against a range of toxic chemicals and pathogens [5]. Glutathione-S-transferases inactivate hydroperoxides formed as secondary metabolites during oxidative stress [6]. It catalyzes the conjugation of reduced glutathione via a sulfhydryl group to electrophilic centers on a wide variety of substrates [7]. It causes detoxification of endogenous compounds such as peroxidized lipids and breakdown of xenobiotics [8].

Glutathione-S-transferases are of interest to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies [9] and metabolize cancer chemotherapeutic agents, insecticides, herbicides, carcinogens, and by-products of oxidative stress [10]. Furthermore, elevated levels of GST have been associated with tolerance of

insecticides and with herbicide selectivity [11]. All these findings collectively indicate that induction of GST is an evolutionarily conserved response of cells to oxidative stress. In most animal models of hepatocarcinogenesis there is uniform *de novo* expression of pi class GST by neoplastic hepatocytes. Hence pi class GST has been regarded as a marker of preneoplasia, and it's over expression is thought to be involved in the acquisition of drug resistance [12]. Even though recent investigations suggest that hepatic GST protect cells from oxidative stress in various cancers, studies pertaining to the GST expression in MD affected chicken are little understood. The present study was therefore aimed to evaluate the potential association between GST expression and oxidative stress in MD affected chicken liver with objective to isolate and purify the enzyme GST from hepatic tissues of normal and Marek's disease affected chicken and to determine the molecular weight of GST enzyme in hepatic tissues.

Material and methods

Chemicals: All the chemicals used in the present study were of analytical grade (AR) and were obtained from Sisco research laboratories Pvt. Ltd., (Mumbai, India) and Himedia laboratories Pvt. Ltd., (Mumbai, India). The GST affinity column was obtained from Genie.

Source of poultry birds: The present study was conducted in the Department of Veterinary Biochemistry, College of Veterinary Science, Tirupati. All the birds were layers with 35 to 40 weeks of age and were procured from various poultry farms in Chittoor district. The birds showing clinical signs of MD and control birds of the same age group were obtained from the farms.

Collection of tissues: For each group ten layer birds of 35 to 40 weeks age were collected and sacrificed for collection of liver samples. These samples after collection were preserved at -20ºC for further analysis of anti-oxidant enzymes, lipid peroxidation, PCR and purification studies.

Histopathological studies: Liver tissues collected for histopathological studies were fixed in 10% neutral buffered formalin (pH 7.2) until further analysis. The samples were processed and sections of 4-7 µm were cut and stained with Haematoxylin and Eosin stain (H&E). The specimens were examined under light microscope [13].

DNA Isolation and Purification: For this experiment kit method (Himedia) was used.

Polymerase chain reaction (PCR): Marek's disease virus positive DNA sample obtained from Department of Biotechnology, TANUVAS, Chennai was used as a positive control for all PCR reactions. A known positive and negative control comprising of nuclease free water were included in the test and amplification was performed in Thermo cycler [14]. The specificity of PCR products was confirmed by the appearance of the fragments of predicted size on the agarose gel.

Purification of Glutathione-S-transferases

Processing of tissue

The liver tissue was thawed and homogenised at 4^0C in 50 mM Tris-HCl buffer (pH 8.0) containing 0.25M sucrose and 1 mM PMSF 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 10,000g in high-speed refrigerated centrifuge for 30min. The supernatant was passed through glass wool to remove fat and the pellet was discarded. The filtrate was centrifuged at 1,05,000g for 45 min using ultra centrifuge and the 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.

Affinity purification of GSTs: Cytosolic fractions after dialysis were loaded onto a glutathione-CL-agarose affinity column (Genie) 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. Elutions were collected each in 2ml fractions. Active fractions were pooled, dialysed and concentrated by using lyophilizer and further subjected to electrophoresis.

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins with relative molecular mass not less than 10 KD. The procedure was done according the method of Laemmli [15].

Statistical analysis: All the data obtained were subjected to analysis by using statistical package for social sciences (SPSS) Version 20.0.

Results and Discussion

Gross lesions: Liver enlargement with multifocal grayish white nodules of 2-5 mm in diameter was observed in all cases (Figure 1 and 2). Presence of lymphomas in different visceral organs of the MD affected birds was also reported by [16] and [17].

Histopathology: In MD affected chicken histopathological examination of liver tissues was done using H &E stain and observed severe infiltration of pleomorphic lymphoid cells which was the characteristic feature of liver cells affected with MD (plates 1, 2). These findings are in accordance with the observations of [18] who also reported infiltration of pleomorphic lymphoid cells in different tissues of poultry tumors.

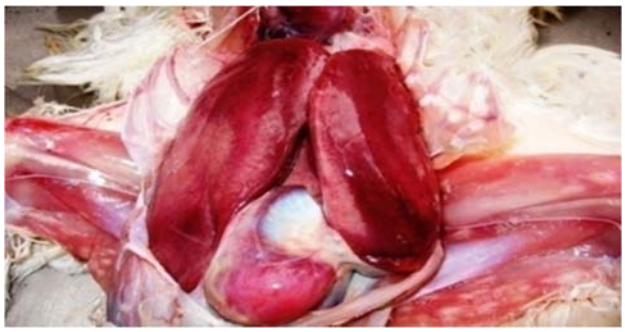

Fig 1: MD- liver: diffuse enlargement of liver occupying the entire abdominal cavity

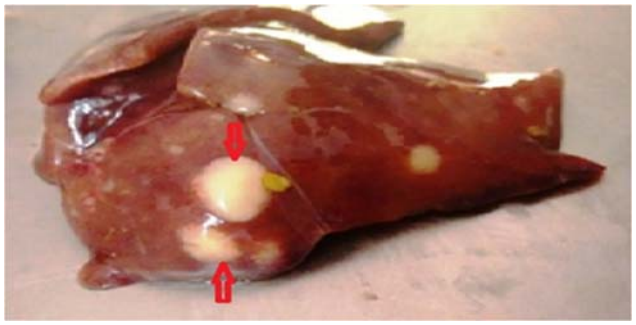

Fig 2: MD liver: Enlarged liver with multiple nodular growth and pale appearance

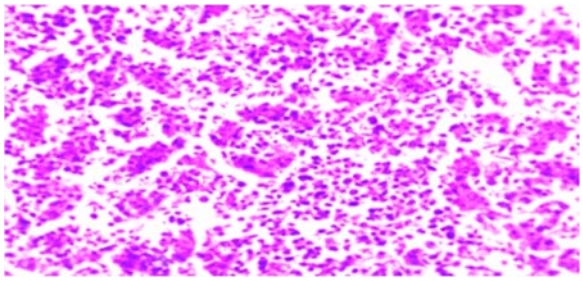

Plate 1: MD Liver: Severe infiltration of pleomorphic lymphoid cells. H&E x100

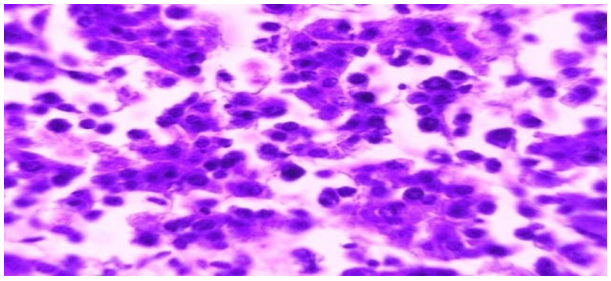

Plate 2: MD Liver: Severe infiltration of pleomorphic lymphoid cells. H&E x400

PCR analysis: The purity of DNA isolated from normal and MD affected birds was found to be optimum for PCR analysis. Primers were used to amplify the 132 bp repeat region in MD suspected cases using PCR. All the samples were positive for serotype-1 specific MDV yielding a 314 bp product in PCR amplification and showed 100 % positivity. This showed that the 314 bp product consisted of two copies of 132 bp tandem repeats (264 bases) along with 50 bases of primers. All the samples of MD showed two copies of 132 bp tandem repeats and results were shown in Figure 3 which was in accordance with previous findings [19]. The liver samples that were positive for MD were used for further analysis.

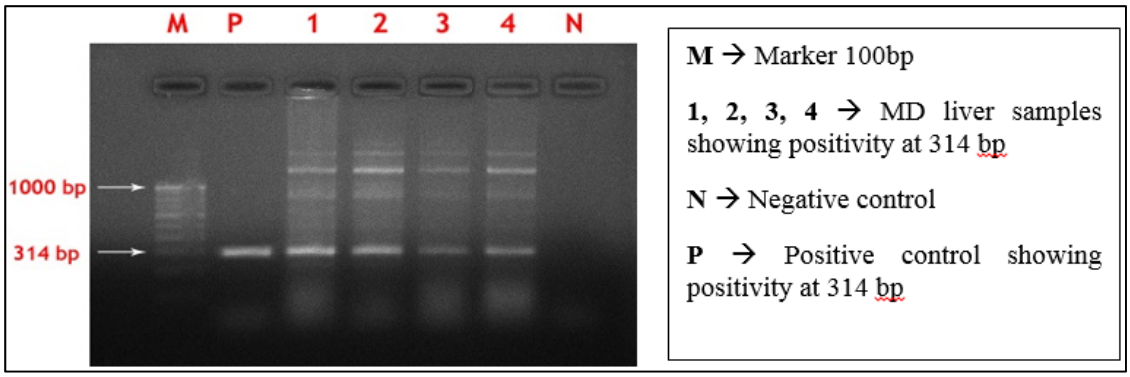

Fig 3: Agar Gel Electrophoresis of PCR product

PCR amplified samples showing positivity at 314 bp repeat region in Agar Gel electrophoresis

Isolation and purification of GST from liver tissue

Protein elution profile with washing buffer

After isolating GST from liver tissues, purification was carried out using Glutathione-agarose (Genie) affinity chromatography. The protein elution profile from liver of control and MD affected chicken with washing buffer were represented in Table 1. The column was washed until the protein concentration reduced to zero.

Table 1: Protein elution profile with washing buffer in control and MD affected chicken liver

Fraction	Protein At 280nm	
	Control	MD
1.	3.94	4.00
2.	3.64	3.71
3.	3.28	3.14
4.	3.01	2.45
5.	2.64	2.04
6.	2.14	1.68
7.	1.04	0.97
8.	0.48	0.21
9.	0.05	0.04
10.	0.00	0.00

Protein elution profile and GST activity with elution buffer in Control chicken:

The protein elution profile and GST activity with elution buffer in liver of control chicken were represented in Table 2 and figure 4. The maximum absorbance at 280 nm and GST activity was observed in fraction 6.

Table 2: Protein elution profile and GST activity with elution buffer in live tissues of control chicken

Fractions	Protein at 280nm	GST activity (U/mg protein)
1.	0.0419	0.096
2.	0.0521	0.215
3.	0.0589	0.368
4.	0.0639	0.427
5.	0.0686	0.563
6.	0.121	1.023
7.	0.0756	0.673
8.	0.0546	0.363
9.	0.0486	0.166
10.	0.0325	0.091

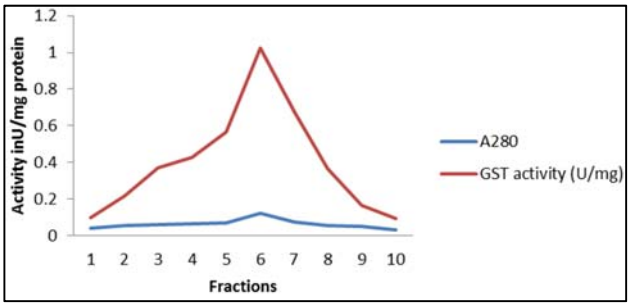

Fig 4: Protein elution profile and GST activity with elution buffer in liver tissues of control chicken

Protein elution profile and GST activity with elution buffer in MD affected chicken:

The protein elution profile and GST activity with elution buffer in liver of MD affected chicken were represented in Table 3 and figure 5. The maximum absorbance at 280 nm was observed as 0.124 and 0.142 whereas GST activity was 1.015 and 1.363 in fractions 3 and 6 respectively.

Table 3: Protein elution profile and GST activity with elution buffer in liver tissues of MD affected chicken

Fractions	Protein at 280nm	GST activity (U/mg protein)
1.	0.0179	0.129
2.	0.0617	0.563
3.	0.124	1.015
4.	0.0458	0.459
5.	0.0689	0.625
6.	0.142	1.363
7.	0.0541	0.527
8.	0.0325	0.297
9.	0.0198	0.138
10.	0.0101	0.0578

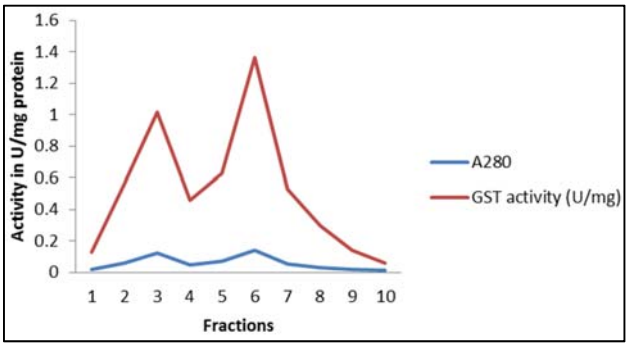

Fig 5: Protein elution profile and GST activity with elution buffer in liver tissues of MD affected chicken

Purification profile of GST from liver tissues of control and MD affected chicken:

The purification profile of GST in liver of control and MD affected chicken were represented in Tables 4 and 5. The GST eluted from liver of MD affected chicken was purified to 4.54 fold with an overall yield of 41.98% as compared to control chicken which was purified to 2.38 fold with an overall yield of 32.27%. Our findings are in accordance with [20] who purified and characterized major GST from adult toad liver with a yield of 65% by using glutathione-agarose affinity chromatography. Similar findings were observed by [21] where purification of GST was carried out in acrylamide induced chick embryonic liver using affinity chromatography with an overall yield of 31%. Purification of GST enzyme was also done from rainbow trout erythrocytes by affinity chromatography using glutathione-agarose column [22].

Table 4: Purification profile of GST in liver of control chicken

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (Ua)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	10	12.08	120.77	616	5.10	1	100
Affinity purified sample	10	1.634	16.34	198.8	12.16	2.38	32.27

[a]One unit is defined as micromoles of GSH conjugate formed per minute

Table 5: Purification profile of GST in liver of MD affected chicken

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	10	9.93	99.26	324.42	3.26	1	100
Affinity purified sample	10	0.92	9.18	136.36	14.85	4.54	41.98

[a]One unit is defined as micromoles of GSH conjugate formed per minute

SDS-PAGE of GST purified from liver samples of control and MD affected chicken

The SDS-PAGE of the GST purified from liver samples of control and MD affected chicken was showed in the Figure 6. The purified sample from control liver showed only a single band with MW of 25.6 kDa whereas, from MD affected liver two bands with MW of 25.6 kDa and 27 kDa were observed. The 25.6 kDa band indicated the presence of GST α and 27

kDa band showed the presence of GST π. It was observed that GST-α was present both in control and MD affected chicken. In addition in MD affected chicken GST-π was induced due to oxidative stress. The results showed that the MD affected birds expressed GST π in addition to GST-α indicating the role of GST π expression to oxidative stress in MD affected chicken liver. These results are in accordance with [23] where they have reported GST-π in hepatocellular carcinoma which was absent in normal liver tissues. Therefore increased GST-pi in cancer cells may protect these cells against oxidative stress. The presence of GST-Pi interferes with cell's defense [24]. GST-Pi seems to be involved in the regulation of cell proliferation by inhibiting the effects of ROS on cell division [25]. GST-π was also reported in HBV infected hepatocellular carcinoma of human patients [26].

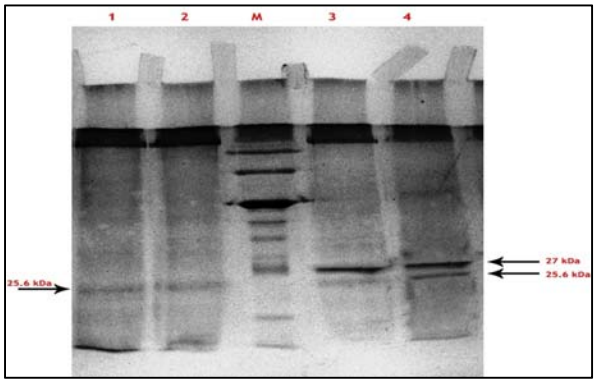

Fig 6: SDS-PAGE of GST purified from liver of control and MD affected chicken

M → Protein Marker **1, 2** → Control liver GST **(**MW 25.6 kDa)
3, 4 → MD liver GST (MW of 25.6 kDa and 27 kDa)

Conclusion

The purified samples in SDS-PAGE analysis showed the expression of GST-α with a MW of 25.6 kDa in both normal and MD affected chicken liver, whereas in addition, GST-π was expressed in MD affected chicken with MW of 27 kDa. The results confirm that oxidative stress is responsible for GST-π expression as a defense mechanism in liver of MD affected chicken. A better understanding of the role of oxidative damage in viral infections may lead to improved therapeutic strategies that will reduce the extent of tissue damage during viral infections.

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