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Biochemical Characterization of Glutathione Transferase of *Cyprinus carpio* Gonads

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Abstract: Common Carp (*Cyprinus carpio*) is considered as a potential candidate for polyculture ponds beside its important commercial values. Fish gonads experience changes in glutathione (GSH) and its related enzymes at different metabolic activities. Alteration in glutathione transferase (GST) activity has been demonstrated during development and after exposing to environmental insult. Thus, the aim of the current research is to characterize gonadal GST of *Cyprinus carpio.* GST activity of detoxication organs, adrenal gland and gonads were assessed. lipid peroxidation,GSH and specific activities of glutathione transferase (GST), peroxidase (GPx), reductase (GR) and catalase (CAT) were determined in gonads of *C. carpio*. Gonadal GST enzymes were purified to homogeneity by affinity chromatography. k_m and k_{cat} and other biochemical parameters were measured. Ovarian (GSH), lipid peroxidation and specific activities of GST and GPx showed higher values compared to testis. GR and CAT activities were much higher in testis homogenate than in ovary. K_m^{GSH} value of testis GST was nearly doubled that of ovary. V_{max} values of ovary GST were higher compared to testis. Organic isothiocyanate substrates showed the highest GST gonadal activity compared to the other electrophilic substrates. Highest inhibition effect on gonadal GST activity was obtained using organotin compounds. *Conclusion*: GSH content and GST K_m^{GSH} in *C. carpio* gonads are supporting the role of GSH in protecting against elevated ROS. *C. carpio* fish is very sensitive to Tin pollution. Isothiocyanates are efficient substrates for gonadal GST which may indicate GST role in detoxification of biodegrading products.

Key words: Glutathione transferase · Detoxification enzymes · *C. carpio* gonads substrate selectivity · Kinetic parameters

INTRODUCTION

Common Carp (*Cyprinus carpio*) is considered as the most important freshwater fish introduced to almost entire world [1]. The teleost *Cyprinus carpio* (*C. carpio*) was first introduced to Egypt in 1930 for research purposes. Started from 1960, common carp was raised commercially in modern semi-intensive farms in Egypt. Carp used in national rice-cum-fish programs supported by Egyptian government since 1984 [2]. Almost 100 thousand tons of common carp was produced in Egypt during 2010. Its contribution to world aquaculture production has increased from almost 31 in 2005 to 45 million tons by 2012 according to FAO report [3]. Due to its ability to adapt to laboratory conditions, common carp is used in evaluation of environmental pollutants [4, 5].

Although common carp has great commercial values in Asia, it considered as a cause of ecological problems in some western countries. Disturbances in both aquatic and ecological systems have been reported in USA and other European countries. Thus many studies for control density population of *C. carpio* in wetlands and shallow lakes have been conducted [6, 7].

A superfamily of detoxification enzymes that catalyze both exogenous and endogenous alkylating agents have been found in mammalian cells [8]. The superfamily contains glutathione transferases (GSTs, EC 2.5.11.18) which considered as multifunctional phase II detoxification enzymes in the cell [9]. Hormones biosynthesis, tyrosine degradation and peroxide breakdown are other functions of GSTs [10]. They are divided into four groups including cytosolic,

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mitochondrial, microsomal and fosfomycin resistance proteins identified in bacteria [11]. According to DNA sequence, cytosolic mammalian GST can be classified into Alpha, Beta, Mu, Pi, Theta, Omega and Zeta, Sigma and Kappa [12, 13]. GST of fish also contains the major classes of GSTs such Alpha, Mu, Pi and Theta according to cDNA sequences [14, 15]. Another classes of fish GST such as Omega, Kappa and Rho (only in teleost) have been identified [16-18]. GSTs classes also found in other organisms. However classes such as Phi and Tau appeared only in plants [19] while Delta and Epsion classes appeared in arthropods [20]. It is observed that fish GST isoforms are expressed in all tissues including gonadal tissues of both male and female [21, 22]. Fish GST detoxification enzymes activity may vary in both sex in some species [23]. Evidence indicated that changes in GST activities in specific organs of fish are directly related to metabolic alterations and cellular damage [24].

Aquatic organisms exposed to oxidative damage generated by reactive oxygen species (ROS) and other prooxidants may trigger their antioxidant defense mechanism to combat damage. This mechanism system in fish basically constitutes of antioxidant enzymes: superoxide-dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and reduced glutathione (GSH) as non-enzymatic antioxidants [25, 26]. Cellular damage occurs when antioxidant protective system is unable to remove excess of ROS. Fish gonads experience changes in GST and related enzymes during development and aging. Alteration in their activities has also been demonstrated after exposing to environmental insult [27]. ROS are naturally generated by aerobic organisms through oxidative metabolism such as mitochondrial respiration, detoxification process of insecticides [28]. Elevated ROS can cause DNA damage, lipid peroxidation, alternations in gene expression and changes in cell-redox-status [29].

GSH and its related enzymes have been studied in detoxification organs such as kidney, liver and gills [30]. However GST in gonads and its role in freshwater fish are scarce. Therefore, the purpose of this study is to illustrate the difference in GSH and its related enzymes activities in testis and ovary of *C carpio*. More addition, gonadal GST purification and characterization have been performed.

MATERIALS and METHODS

Chemicals: Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), was purchased from Park Company. Oxidized glutathione (GSSG) and hydrogen peroxide (H_2O_2) were obtained from Fluka Company. Sodium dodecyl sulfate (SDS), molecular weight standard protein kit was products of Pharmacia Company. Bovine serum albumin, 2, 4-dithiotheritol (DTT), reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) androstenedione (AD), 4-nitrophenethyl bromide (NPB), 1,2-epoxy-3-(4-nitro[henoxy) propan (EPNP), paranitrophenyl acetate (pNPA), 7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole (NAD-Cl), bromo-sulfophthalein (BSP), phenethylisothiocyanate (phenethyl-ITC), allylisothiocyanate (allyl-ITC) and benzyl isothiocyanate (benzyl-ITC) were obtained from Sigma-Aldrich Company. Epoxy-activated Sepharose 6B was purchased from Pharmacia Biotechnology Company. All other chemicals were of the highest purity commercially available.

Fish: A total of 10 mature *C. carpio* fish, of both sexes (5 males and 5 females) were collected during April to June, 2015. Fish were supplied by NRC fish Hatchery near Alexandria, Egypt. Fish total length was ranging from 35 to 40 cm and total weight from 2 to 2.5 Kg. The fish were dissected to determine sex and maturity stages. Gonads, liver, kidney and adrenal gland, were weighed to the nearest gram. Gonadosomatic (GSI) and hepatosomatic (HSI) indices were recorded for both male and female. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Preparation of Crude Homogenates: Known weights of the *C. carpio* ovaries (20 g) and testes (20 g) were homogenized individually using a glass homogenizer in 50% (w/v) (1:2 volume) of 25 mM Tris–HCl buffer, pH 8.0 containing 1 mM EDTA and 1 mM DTT (buffer A). The homogenates were then centrifuged at 10,000g for 15 min. The supernatants (cytosol) were filtered through a plug of glass wool and the filtrates (crude homogenates) were saved at -20°C for further analyses.

Purification of GST from C. Carpio Gonads: The crude homogenate prepared from *C. carpio* testes and ovaries were coupled individually on DEAE-Sepharose matrix previously equilibrated with buffer A and washed with the same buffer. The unbound proteins were collected by filtration. Unbound proteins were monitored for protein determination at 280 nm and for GST activity at 340 nm using CDNB as a substrate. Reduced glutathione (GSH) was coupled to epoxy-activated Sepharose 6B according to Simons and Vander-Jagt [31]. Unbound proteins from DEAE-Sepharose were mixed with 15 mL of GSH-

Sepharose matrix previously equilibrated with buffer A and allowed to couple for 30 min at 4°C with gentle shaking. The matrix with GST was collected by filtration through centered glass funnel and extensively washed with the same buffer. The matrix with bound proteins was packed to a column (15 cm X 1 cm i.d.) and the bound GST was eluted with 50 mM Tris– HCl buffer, pH 8.0 containing 10 mM GSH at a flow rate of 1 mL/min. Three-milliliter column fractions were collected and monitored for protein at 280 nm and for GST activity at 340 nm using CDNB as a substrate. The homogeneity of the pooled material was analyzed by native PAGE (7%) according to the method of Davis [32]. The SDS-PAGE was performed using 12% (w/v) polyacrylamide gel [33]. Protein bands were then visualized using Coomassie brilliant blue (R-250) stain. The purified enzyme was stored at -20°C for further analyses.

Protein Determination: Protein concentration was measured by the Bio-Rad [34] assay using bovine serum albumin as a standard. Measurements were done on Shimadzu UV spectrophotometer at 595 nm.

GSH Determination: Total GSH was measured colorimetrically according to the method described by [35]. Crude homogenates of *C. carpio* testes and ovaries were mixed individually with equal volume of 13% trichloroacetic acid (TCA). The precipitated proteins were removed by centrifugation at 2000g for 10 min and the supernatant was used in the assay and the absorbance was measured at 535 nm.

Enzyme Assays

Glutathione Peroxidase (Gpx): The activity of GPx was determined according to the method described by [36]. Concomitant oxidation of NADPH is the measure of GPx activity. The assay reaction mixture contained in 1-mL volume, 50 mM potassium phosphate buffer, pH 7.0, 5 mM EDTA, 0.075 mM H_2O_2, 5 mM GSH, 0.28 mM NADPH, 1 IU GR and a suitable crude enzyme homogenate volume. One unit is equivalent to the oxidation of 1 μmol of NADPH in 1 min, at 30°C. The extinction coefficient of NADPH was taken to be 6.22 $mM^{-1}cm^{-1}$. The decrease in absorbance at 340 nm was monitored against control containing buffer instead of the enzyme and treated similarly.

Glutathione Reductase (GR): The activity of GR was determined spectrophotometrically at 30°C following the decrease in absorbance at 340 nm according to the method described by [37]. The assay reaction mixture contained in a total volume of 1 mL, 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1 mM NADPH, 0.5 mM oxidized glutathione and the enzyme solution. The control was routinely included and treated under the same conditions of the enzyme assay. One unit of GR activity is defined as the amount of enzyme which oxidizes 1 μmol of NADPH per min.

Glutathione Transferase (GST): Glutathione transferase activity was determined by measuring the increase in the concentration of the conjugation product of GSH and CDNB at 340 nm over 3 min at 30°C. Unless otherwise stated, the assay mixture contained in a total volume of 1 ml, 0.1 M potassium phosphate buffer, pH 6.5, 1 mM CDNB in ethanol (final concentration of ethanol less than 4%), 1 mM GSH [38]. The EPNP, NPB, pNPA and NAD-Cl were also determined according to Habig *et al*., [38]. GST activity toward phenethyl-ITC, allyl-ITC and benzyl-ITC were determined as described by Kolm *et al*., [39]. The AD was measured as shown by Johansson and Mannervik [40]. One unit of GST activity is defined as the formation of 1 μmol product per min at 30°C.

Catalase (CAT): Catalase activity determination was carried out according to the method described by Aebi [41]. The method is based on monitoring the rate of decomposition of H_2O_2 at 25 °C. For CAT activity determination, suitable volume of crude enzyme was added to 1 mL of substrate mixture, which consisted of 20 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0. The decomposition of H_2O_2 was followed as a decline in absorbance at 240 nm for 1 min. One unit of activity was defined as the calculated consumption of 1 μmol of H_2O_2/min at 30°C. The extinction coefficient of H_2O_2 was taken to be 43.6 M^{-1} cm^{-1}.

Lipid Peroxidation: Lipid peroxides content was estimated by measuring the formed malondialdehyde (MDA) using the method of Ohkawa *et al.,* [42]. The principle is based on the fact that MDA produced from the peroxidiation of membrane fatty acid reacts with 2-thiobarbituric acid (TBA) to yield a pink coloured complex measured spectrophotometrically at 532nm. Lipid peroxides were expressed as nmol/g tissue.

Determination of Kinetic Parameters: The apparent K_m and V_{max} values for GST were determined at pH 6.5 using a GSH range from 0.1 to 2.0 mM at constant concentration of CDNB at 2.0 mM. The apparent K_m and V_{max} values for CDNB were determined using a CDNB range from 0.1 to 2.0 mM at constant GSH concentration of 2.0 mM. Data were plotted as double-reciprocal Lineweaver-Burk plots to determine the apparent K_m values.

Inhibition Studies: Under the standard assay conditions, the effect of hematin, bromosulfophthalein, Fentin chlorid, bromide triethylin, tributylin chloride and cibacron blue was tested for their ability to inhibit GSH-conjugating activity of *C.carpio* purified GST. IC_{50} values were determined by measuring the activity of the enzyme in the presence of varying concentrations of the inhibitor and plotting the percentage activity values versus log inhibitor concentration.

Statistical Analysis: All data are reported as mean ± SE for n = 3-5 independent experiments. The Student's t test was performed to examine the difference between means.

RESULTS

Some biological parameters in *C. carpio*: Significant difference in body weight of female and male *C. carpio* was detected (Table 1). Female showed higher body weight compared to male. Gonadosomatic (GSI) and hepatosomatic (HSI) indexes were calculated for male and female *C. carpio*. The observed differences between male and female concerning GSI and HIS were statistically insignificant. GSI in female was higher than in male while male showed higher HIS than female.

Glutathione and its Related Enzymes in *C. carpio*: GST activity was measured in gonads, adrenal gland and detoxification organs of male and female *C. carpio* fish. GST activity in ovary, adrenal gland, kidney and gills were much higher compared with testis and other corresponding organs of male *C. carpio* (Table 2). However, liver of male showed higher values compared to female (P<0.01). Kidney followed by adrenal, liver, then gills showed highest values of GST in male. Testis activity of GST appeared to have the lowest values (1.06 unit). In female *C. carpio* GST activity of adrenal, kidney, gills and gonads showed higher values compared to the liver.

Table 1: Biological parameters of *C. carpio* fish

Parameter	Male	Female
Weight of fish body (g)	2223.85±26.9^b	2406.92±41.43^a
Weight of liver (g)	6.42±1.41^a	4.51 ± 0.80^a
Hepatosomatic index	0.29±0.06^a	0.18±0.032^a
Weight of gonad (g)	160.71±24.74^a	246.65±54.76^a
Gonadosomatic index	7.20±1.03^a	10.14±2.12^a

Means at the same raw with different superscripts are significantly different at P<0.01

Table 2: Determination of GST (units/g tissue) in gonads, adrenal gland and detoxification organs of *C. carpio* fish

Tissues	Male	Female
Gonads	1.06±0.32^b	11.33±0.89^a
Liver	7.18±0.65^a	5.31±0.11^b
Kidney	14.82±1.31^b	20.18±0.88^a
Gill	4.09±0.19^b	12.77±0.44^a
Adrenal gland	13.17±0.37^b	28.38±0.43^a

Means at the same raw with different superscripts are significantly different at P<0.01

GSH concentration (nmol/g tissue) and specific activities of GST, GPx, GR and CAT of male and female *C.carpio* were determined (Table 3). GSH concentration and specific activities of GST, GPx, in ovary exhibited higher values compared with their corresponding values in testis. Ovarian lipid peroxidation (nmol/mg protein) was also higher compared to testis ((P<0.001). GR and CAT activities were much higher in testis homogenate than in ovary. Protein in ovary was 8 times higher than in testis (P<0.001).

Gonadal GSTs Purification: Table 4 represented a typical two purification step procedure of GSTs from *C. carpio* gonads. Protein retained on the DEAE-Sepharose matrix represented 60% and 54% for total proteins of testis and ovary, respectively. Application of the unbound fraction on GSH-sepharose column resulted in a single activity and protein peak for both testis and ovary (Table 4). The purification fold was increased to 276 for testis and 341 for ovary GST. Recovery of GST was 47% for testis and 41.6% for ovary. The specific activity for the purified GST enzyme was 16.82 and 27.62 (unit/mg protein) in testis and ovary, respectively.

Table 3: Glutathione and its related enzymes, protein concentrations and catalase (CAT) activity in *C. carpio* gonads

Biochemical parameters/g protein	Testis	Ovary	Ratio ovary/testis
GSH concentration (nmol)	3.200±0.208^b	6.900±0.513^a	2.156
Protein concentration (mg/g tissue)	11.79±0.820^b	54.10±0.818^a	4.589
GST activity (units)	0.054±0.012^a	0.093±0.012^a	1.722
GPx activity (units)	0.018±0.002^b	0.051±0.004^a	2.833
GR activity (units)	0.019±0.002^a	0.004±0.001^b	0.210
CAT activity (units)	3.230±0.124^a	0.740±0.015^b	0.229
Lipid peroxidation (nmol/mg protein)	3.250±0.128^b	5.943±0.124^a	1.829

5Values are expressed as means ±SE for n = 3-5

6Means in the same row with different superscripts are statistically different at P<0.001

Table 4: Purification scheme of GST from *C.carpio* gonads

Organ	Purification step	Activity [units]	Protein [mg]	Specific activity [unit/mg protein]	%Recovery	Fold
Testis	Crude homogenate [20g]	16..12	264.8	0.061	100	1
	DEAE- Sepharose unbound fraction	12.50	106.1	0.120	77	2
	GSH-Sepharose purified fraction	7.570	0.450	16.82	47	276
Ovary	Crude homogenate [20g]	104.0	1285	0.081	100	1
	DEAE- Sepharose unbound fraction	62.95	591	0.110	61	1.3
	GSH-Sepharose purified fraction	33.26	1.204	27.62	32.0	341

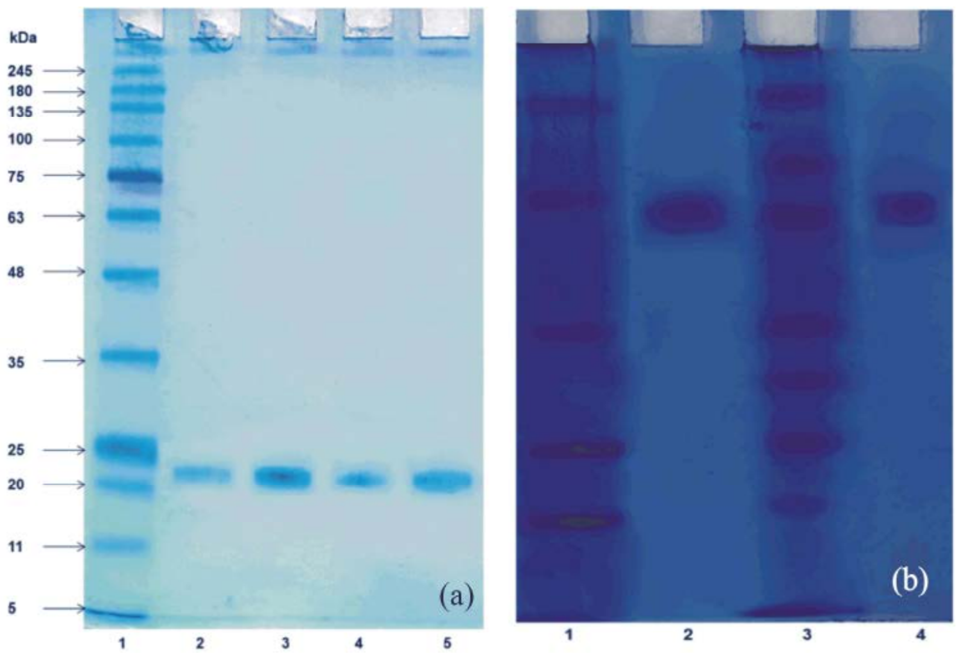

Fig. 1: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of gonadal *C. carpio* GST. SDS-PAGE (a), native PAGE (b) analysis of elution fractions eluted from purification of goand *C. carpio* GST (testis and ovary). Figure 1.a, Lane 1, SDS marker; lane 2, 3, purified ovary *C. carpio* GST; lane 4, 5, purified testis *C. carpio* GST. Figure 1.b, native PAGE stained with comassie lane 1, crude ovary *C. carpio* GST; lane 2, purified ovary *C. carpio* GST; lane 3, crude testis *C. carpio* GST; lane 4, purified testis *C. carpio* GST

Homogeneity: The purity and subunit molecular weight of the purified GSTs were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as well as native PAGE. Gels were stained with 0.1% coomassie brilliant blue R-250 (Figure 1). The purified GST peaks from testis and ovary were proved to be homogenous as judged by native PAGE as well as SDS-PAGE. The molecular weight was approximately 24 KDa for the purified GST from testis and of ovary.

pH Profile: The effect of pH on GST activity was evaluated using CDNB as a substrate. In female carp fish the highest activity of ovary GST was observed at pH 8. The enzymatic activity decreased by almost 40% at pH 9 (Figure 2). A narrow peak (8.5-9) was seen at the alkaline side of the pH for testis of *C. carpio* fish (Figure 3).

Steady State Kinetics of Gonads GST: Enzyme kinetic constants are summarized in Table 5. The effect of substrate on GSH-CDNB conjugation activity was investigated at 25°C for K_m determination. The K_m value of testis GST for GSH (0.72 mM) was higher than that in ovary GST (0.31 mM). V_{max} values of ovary GST for both studied substrates were higher compared to those found for testis GST.

The plots of the initial velocity versus [GSH] and versus [CDNB] in the range of 0.1-2.0 mM for both testis and ovary of *C. carpio* were performed (Table 5). The gonads plots displayed a typical hyperbolic saturation curve, a Michaelis-Menten kinetics. A linear relationship was obtained when 1/v was plotted against 1/s in both organs (Figures 4 and 5).

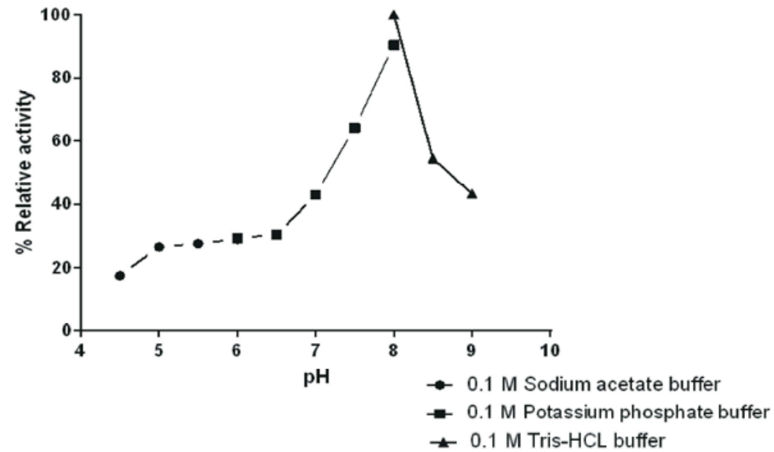

Fig. 2: Effect of pH on the enzymatic activity of the purified *C. carpio* GST from ovary.

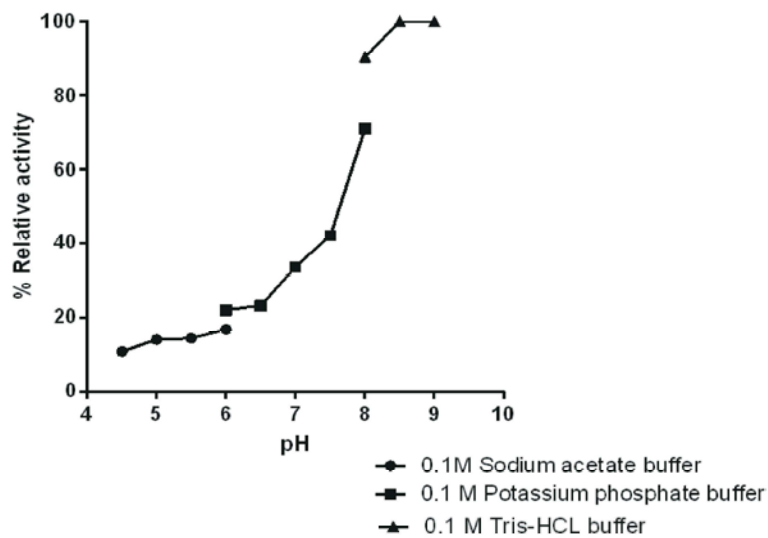

Fig. 3: Effect of pH on the enzymatic activity of the purified *C. carpio* GST from testis.

Table 5: The kinetic parameters of the purified GST from *C.carpio* gonads

		Gonadal GST	
Substrate	Parameters	Testis	Ovary
CDNB	V_{max}	100	500
	K_m	0.173	1.66
	k_{cat}	0.55	2.57
	$k_{cat/km}$	3.18	1.55
GSH	V_{max}	167	222
	K_m	0.72	0.31
	k_{cat}	0.91	1.14
	$k_{cat/km}$	1.26	3.68

Table 6: Specific activities of gonadal *C.carpio* GST using different substrates

Substrate	Testis	Ovary
CDNB	0.595±0.03	2.34±0.07
Phenethyl ITC	6.74±0.22	125±2.79
Allyl ITC	3.78±0.16	15.8±0.86
Benzyl ITC	4.23±0.20	24.5±0.49
NAD-Cl	0.1±0.003	0.24±0.005

The results represent means ± S.E for triplicates.

CDNB, 1-chloro, 2,4-dinitrobenzene; Phenethyl ITC, phenethylisothiocyanat; Allyl ITC, allylisothiocyanat; Benzyl ITC, benzyl isothiocyanate;NAD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AD androstenedione (not detected);NPB, 4-nitrophenethyl bromide (not detected) ;EPNP, 1,2-epoxy-3-(4-nitrophenoxy) propane (not detected)

Substrate Selectivity: The specific activities measured for both testis and ovary GST towards some substrates are shown in Table 6. The highest activity of ovary was obtained toward the organic isothiocyanate substrate phenethyl ITC (125±2.79). Testis GST had a peroxidase activities less than that of ovary GST toward all of the substrates.No GST activity could be detected for *C.carpio* gonads on androstendione, 4-nitrophene thyl bromide and 1,2-epoxy-3-(4-nitrophenoxy) propane.

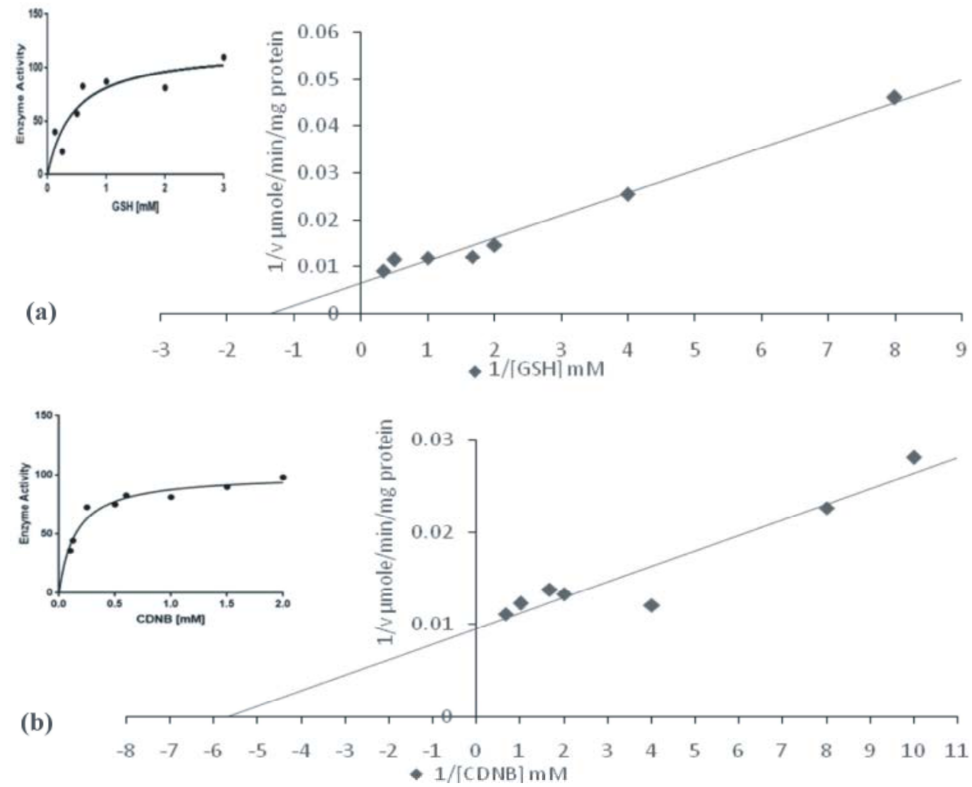

Fig. 4: Lineweaver-Burk plot relating the GST activity purified from *C. carpio* testis to (a) GSH and (b) CDNB concentrations.

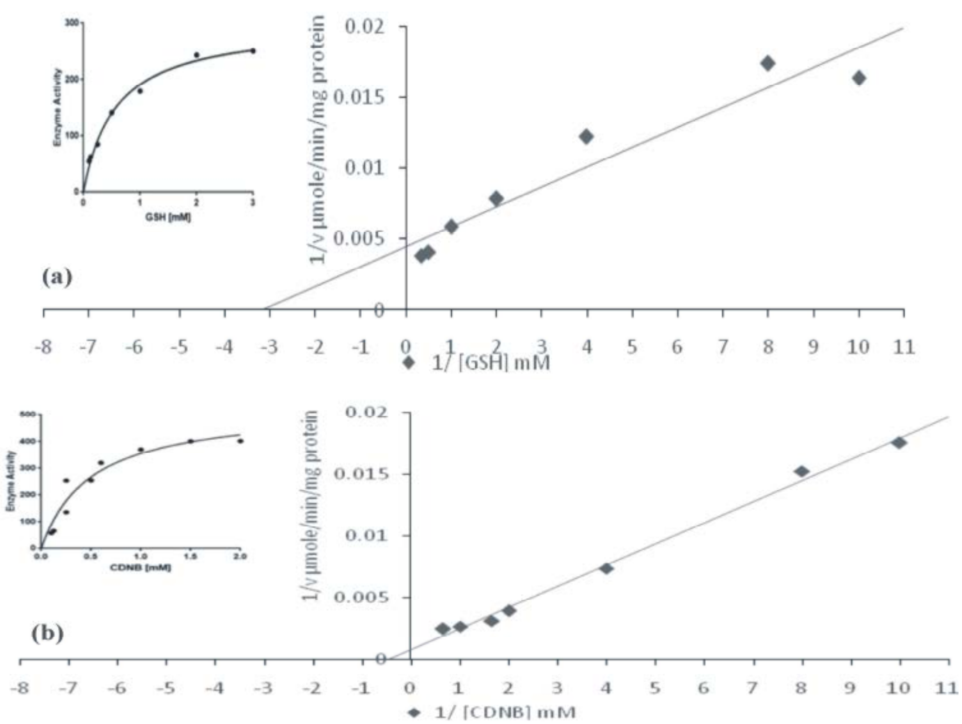

Fig. 5: Lineweaver-Burk plot relating the GST activity purified from *C. carpio* ovary to (a) GSH and (b) CDNB concentrations.

Table 7: IC_{50} values for GST of *C.carpio* gonads measured with selected inhibitors

	IC_{50} (μM)	
Inhibitor	Testis	Ovary
Bromosulfophthalein (BSP)	0.5650	0.4290
Cibacron blue (CB)	0.3810	0.5800
Hematin	11.8710	5.7380
Fentin chloride	0.0125	0.0190
Bromide triethyltin	0.0100	0.0130
Tributylin chloride	0.0100	0.0058

The glutathione transferase activity was measured at pH 6.5 with 1 mM 1-chloro-2, 4-dinitrobenezene (CDNB) and 1 mM glutathione as substrate

Effect of Inhibitors: Under the standard assay condition, gonadal GSTs showed different patterns of sensitivity to the selected inhibitors (Table 7). The organotin compounds (fentin chloride, triethyltin bromide and tributyltin chloride) showed the highest inhibition effect on GST of both testis and ovary. The GST sensitivity to hematin and tributyltin of ovary was almost double that of testis GST. The inhibition by hematin exhibited the lowest values for gonadal GST (IC_{50} 11.87 and 5.74 for testis and ovary, respectively).

DISCUSSION

Glutathione as a natural defense system provides protection against oxidative stress in both male and female gametes. Evidence from mammalian studies suggested a significant role of GSH in gonadal development and maturation [43]. Reactive oxygen species (ROS) in sperm cells could lead to abnormal or damaged spermatozoa, lipid peroxidation of its plasma membrane, and injury of chromatin [44, 45]. Glutathione system has been shown to remove H_2O_2 which consider as the most toxic ROS in human spermatozoa [46]. In the teleost *C. carpio* fish excess ROS caused damage of sperm DNA and subsequently impaired reproductive success [47].

Gonadosomatic index (GSI) often used to monitor the reproductive state of fish during development and seasonal variation [48]. Increase in gonadal weight during stages of maturation which depend on body weight was observed in most fish species [49]. As a start, in the present study, female *C. carpio* showed higher GSI than male, although the difference is statistically insignificant (P>0.11). This is in accordance with GSI of *Oreochromis. nilotica* [48] (Hamed *et al*., 2014). Also, Mahboob and Sheri [50] observed that female *C. carpio* and *Ctenopharyngodon idella* exhibited higher values of GSI compared to male. This may indicate that ovary contributes more towards GSI than testis in male fish.

During oocyte development phospholipids and protein which synthesized in liver and transported to ovary through blood circulation are accumulated in oocyte. Therefore liver may be involved in the process of oocyte maturation and its size is a good indicator for oocyte development. Values of HSI for *C. carpio* reared under different fertilization protocols ranged from 0.98 to 1.41 [50]. In our study the increase value of HSI in male *C. carpio* (0.29) compared to female (0.18), was statistically insignificant (P>0.1).

In our investigation total GSH concentration in ovary is nearly double its concentration in testis. However previous studies on Catfish [51] and Tilapia [52] showed that testis contents of GSH were higher than those of ovary. Fish species may exhibit different pattern of GSH and its metabolizing enzymes due to seasonal, environmental and feeding changes [53].

In mammalian spermatozoa GSH levels showed marked species-specific differences. Rapid decline in intracellular GSH concentrations was observed during incubation of mammalian spermatozoa in aerobic conditions. This decline is not associated with an increase in GSSG concentration [43]. It is well known that proteins bind with GSH (tripeptide thiol) forming mixed disulfides and occurring as hidden glutathione. These disulphides can protect proteins against oxidative stress besides storing low molecular weight thiol in the cell [54].

ROS generation may inhibit cell division in the formed oocyte. Synthesis of intracellular GSH is important for oocyte cytoplasmic maturation [55]. The basic role of GSH in oocyte is to protect cells from oxidative stress. High levels of GSH have been observed in matured oocyte of some mammals. These high levels of GSH are required for forming male pronucleus after fertilization and promoting the early embryo development [56]. In the carp *Catla catla* (*C. catla*) generation of large amount of free radicals during oocyte maturation and ovulation is accompanied by increasing oxidative stress [57].

In our study GPx and GR are almost at the same level in testis.This was not the same in ovary where GPx is almost 12 times that of GR (0.051± 0.004, 0.004±0.001). This means that oxidation of GSH is higher than its reduction however this is associated with increase in total GSH content, The level of SOD, CAT, GPx, GR and GST were studied in the ovarian follicle of the carp *C. catla*. Different redox indices in the ovary were reported [57]. GPx, GR and GST exhibited almost the same level (between 12-15 unit/mg protein). Increase of GST and its antioxidant enzymes in carp *C. catla* indicated their physiological role in oocyte protection from ROS.

GST of kidney in both male and female *C. carpio* exhibits the highest activity in the detoxification organs tested (Table 2). However Hamed *et al*. [58] reported that hepatic GST (units /g tissue) exhibited significantly high values compared to kidney and gills in some Nile fish. This may indicate that kidney is contributed with detoxification in polyculture ponds fish. Beside detoxification of toxic electrophiles GST has an alternative function in steroidogenesis. In this current work GST activity of adrenal gland exhibited nearly the highest activity in both male and female *C. carpio*. In human and higher animals, Alpha class GST type 3 (GSTA3-3) is prominently expressed in hormone producing organs such as gonads and adrenal gland. GST role as an efficient catalyst in the biosynthesis of testosterone and progesterone is supported by cell line experiments [59].

Ovarian GST activity was higher than testis (Table 2). This higher value is comparable to the observed value of GSH in ovary as GSH is the substrate for GST. Ovary of *C. carpio* might experience higher activity of GST enzyme than testis. However differences in specific activities of GST in ovary and testis of *C. carpio* were statistically insignificant. GST increased with the increase of mammalian ovarian follicle maturation [60]. Also a variation in GST expressed during the different stages of ruminant oestrus cycle [61].

In *Oreochromis. nilotica* (*O. niloticus*), low enzymatic activities of ovarian GST were reported. Also GPx, GR and CAT in ovary had low activities compared to that of testis [27]. In the present study, only GR and CAT activities were significantly lower in ovary compared to testis of *C. carpio*. Trenzado *et al.*, [62] showed a variation in the activities of CAT and GPx between trout and sturgeon. This variation is referred to lower activity of sturgeon compared to trout. The authors interpreted the lower antioxidant enzymes activities in sturgeon to its lower oxygen consumption. Similar results were also shown by Atli *et al*., [63] since carp exhibited lower CAT and SOD activities and higher GPx activity compared to trout fish. The study referred the variation of antioxidant enzymes to the differences in the metabolic activities and ecological needs among fish species [64]. Lipid peroxidation (LPO) regarded as a major contributor to cell damage under oxidative stress. Our results indicated a significant elevation of MDA formation in ovarian *C. carpio* compared to testis. This could account for accumulation of ROS during oocyte maturation [57].

Purified GST from ovary and testis approved by SDS-PAGE analysis to be almost homogenous with approximate molecular weight of 24 KDa. Different species of fish showed similar molecular weights of the main isoforms of cytosolic GST. Molecular weights of GST ranged from 22.4 to 26.9 KDa as reported by many investigators [65, 27, 22]. In the present study GST isolated from testis and ovary of *C.carpio* showed homodimeric configuration as seen in most fish, mammals and bivalves [65, 66]. However GST heterodimers in *Clarias lazera* (*C. Lazera*) ovary and flat fish have been reported [52].

In the current study the highest ovarian GST activity obtained at pH 8.0 when CDNB used as a substrate. GST enzyme activity of testis showed maximum activity at pH range from 8.7 to 9.2. In a previous study by Guneidy *et al.* [52], optimum activity of GST enzyme from purified *C. lazero* gonads obtained at pH 8. The teleost *Monopteru salbus* liver GST exhibited optimum enzyme activity in between pH range 7.0-7.5 [65]. Cytosolic visceral mass GST of the bivalve Asiatic clam and liver cytosolic GST of leaping mullet (*Liza saliens*) each exhibited two pH optima (pH 7.2 and pH 7.6) and (pH 7.5 and pH 11), respectively [64].

The kinetic characteristics of the purified GST from gonadal *C. carpio* were studied using different concentrations of GSH and CDNB. The affinity (K_m^{GSH}) of ovary GST was 2.3 times higher than of testis. Meanwhile catalytic efficiency (k_{cat}/K_m) of testis GST toward CDNB was two times higher than of ovary GST. Carp fish testis GST K_m^{GSH} (0.72) was similar to GST alpha in zebra fish (an important vertebrate model species). GST Alpha class showed high expression in intestine and gonads in comparison to other zebra GST classes. GST Mu class in zebra fish showed high expression in brain and gonads. Zebra fish and human GST Alpha clusters are syntenic [18]. One may recall the information regarding GR, GPx activities and total GSH concentrations in ovary and testis where higher amount of GSH in ovary was detected. The higher affinity (lower K_m for GSH) of testis GST and the active GR, GPx cycle may indicate the presence of active cycle producing GSH. The K_m^{GSH} value for ovarian GST has lower affinity for GSH which used higher content of GSH available for GST to be active efficiently.

Since GST is involved in detoxification and the biosynthesis of a number of metabolites such as prostaglandins and leuktriens, studying the effect of naturally occurring substrates and inhibitors is required. Most of the substrates so far identified are synthetic compounds, showing promising activities in vitro experiments. This is hardly achieved in biological systems due to its lack of specificity [67]. The highest specific activity for gonadal GST towards different substrates was obtained with the isothiocyanate substrate phenethyl-ITC. Ovary of *C. carpio* showed higher GST specific activity for phenethyl- ITC compared to testis. The same trend was observed for all studied substrates. High reactivity of the three used organic isothiocyanate (natural plant biodegrading products) to conjugate with the reduced form of GSH was seen compared with other substrates (figure 4). The detoxification of the majority of electrophilic xenobiotics has been attributed to the conjugation of the sulfhydryl group of GST to an organic electrophile. Organic thiocyanates (phenethyl- ITC, Allyl-ITC,….etc) conjugated with GSH enzymatically and non enzymatically to form dithiocarbamates. The detoxification of ITCs by GSH is governed by GSTs that catalyze the conjugation of thiol group from GSH with the electrophilic central C atom in ITCs (-N=C=S-). ITCs released from their biologically inert precursors glucosinolate enzymatically at the disruption of plant cell [68]. CDNB and NAD-Cl showed some specific activity in both ovary (2.34 and 0.595) and testis (0.24 and 0.1), respectively. Gonadal GSTs did not show any enzymatic activity with respect to other substrates (AD, pNPA, NPB and EPNB). In the present study CDNB which considered as the classical model substrate for analysis of GST activity did not give the highest specific activity. Similar results obtained with isolated ovary from *O. niloticus* where styrene oxide substrate showed higher relative activity percent (250%) than CDNB substrate. Also specific activity of GST isolated from *O. niloticus* testis towards EPNP substrate was found to be 466.5% higher than CDNB [48].

Ovaries of *C. lazera* showed higher activity on Phenethyl-ITC followed by benzyl-ITC with 4.93 and 3.37 fold over activity on CDNB. Also the same behavior was observed for testis with 18.3 and 5.89 fold over CDNB. On the contrary of this behavior activity on Allyl-ITC was 6.9 fold over CDNB for *C. lazera* testis. Ovary GST activity on Allyl-ITC represented 61% of that on CDNB [52]. High activity on Benzyl ITC with 71.4, 115 and 36 fold over CDNB for liver of human, rat and mouse GSTT1-1 [69]. In our study GST activity on Benzyl ITC was 7.1 fold high over CDNB for testis and 10.47 for ovary of *C. carpio*. However, the highest activity was found when Phenethyl-ITC with 11.34 and 6.35 for testis and 53.4 and 6.75 for ovary.

Inhibition studies of the different isozymes have been helped in distinguishing various GST isozymes [70]. Bromosulphophthalin is a Mu class substrate and uses as an inhibitor in the present study. It showed highest IC_{50} than showed for *Synodontis eupterus* [66] by 94-fold increase. Cibracron blue showed the same IC_{50} towards testis and ovary *C. carpio* GST as bromosulphophthalin in the present study. Hematin is a less effective inhibitor of both *C. carpio* gonads. The IC_{50} for the testis and ovary *C. carpio* GST was 11.87 and 5.74µM, respectively. Hematin also showed IC_{50} of *Synodontis eupterus* GST of 5.0 µM [66] and also less effective on Plaice GST [71]. The chosen organotin compounds in this work are the most potent inhibitors of both testis and ovary GST (table 7). TBT was reported as endocrine disruptor and inhibitor of steroidogenesis in mammals [72]. The syndrome imposex in female gastropod molluscs is one of the most well-known adverse effects caused by TBT [73]. In teleost fish masculinization also reported [74] due to inhibition the conversion of androgens to estrogen. The inhibition of GST can originate through the direct conjugation of TBT with reduced glutathione that would otherwise link with an SH group and become available for enzymatic activity [75]. TBT high lipophility induces cytotoxicity through rapid membrane permeability, affecting the intracellular region. Lipid peroxidation and DNA damage can be detected in different fish species founded in impacted areas [76, 77].

CONCLUSION

Glutathione level and its reducing oxidation cycle showed marked species specific differences. GSH content and GST K_m^{GSH} in *C. carpio* gonads are supporting the role of GSH in protecting against ROS elevated during gonads maturation. Enzymes levels of GSH cycle may indicate the role of GST in steroids biosynthesis. Isothiocyanates (natural substrates produced as a result of cellular lipid peroxidation) are efficient substrates for gonadal GST. Tin compounds (steroidogenesis inhibitors) are strong inhibitors for GST of both testis and ovary indicating GST sensitivity to their compounds besides suggesting GST role in hormone synthesis. More investigation should be done to evaluate the role of gonadal GST in *C. carpio* fish which has a great effect in polyaquaculture.

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