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Purification and characterization of glutathione S-transferase of the African catfish *Clarias lazera* gonads.

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ABSTRACT

Glutathione S-transferases (GSTs) catalyze the transfer of glutathione (GSH) to a variety of xenobiotic and toxic endogenous compounds. GST was purified from ovary and testis of the freshwater fish *Clarias lazera* by affinity chromatography. Testis GST appeared to be a homodimer of two subunits each with a molecular weight of 27.5 kDa. While ovary GST appeared to be a heterodimer of two different subunits having molecular weights of 27.5 KDa and 25.1 KDa. Kinetic analysis revealed high K_m^{GSH} value (2.5 mM) for testis GST compared to 0.5 mM for ovary GST. Ovary GST showed high activity toward the substrates *para*- nitrophenyl acetate(*p*NPA), phenthylisothiocyanate (phenthyl -ITC), benzyl isothiocyanate (benzyl-ITC) and styrene-7, 8-oxide (SO) (9.39 ± 0.39, 6.96 ± 0.266, 4.76 ± 0.55 and 4.37 ± 0.075 µmol/min/mg protein, respectively). Maximum activity was obtained with SO for testis GST (215 ± 34.5µmol/min/mg protein). Hematin is the most potent inhibitor for testis GST activity however it had no effect on ovary GST. The variation in the examined characters between *C. lazera* GST ovary and testis account for structural composition difference between gonads GST and may suggest a critical role for GST in the reproduction process and sex differentiation.

Keywords: *Clarias lazera* (Karmot), gonads, glutathione transferase, enzyme kinetic, substrate selectivity, enzyme inhibitors

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INTRODUCTION

Glutathione S-transferases (EC 2.5.1.18, GSTs) form a ubiquitous super family of multi-functional dimeric enzymes with roles in Phase-II detoxification, they are found in almost every species, from plants and bacteria to animals (Sheehan *et al.* 2001). GSTs neutralize a broad range of xenobiotics and endogenous metabolic by-products via enzymic glutathione conjugation, glutathione-dependent peroxidase activity or isomerization reactions (Hayes et al. 2005; Bamidele et al. 2012). In addition to detoxifying xenobiotics, the GSTs also catalyze the conjugation of endogenous substrates, including cholesterol, prostaglandins and leukotriene A4. Broad spectrums of compounds serve as substrates for GSTs such as halogenonitrobenzenes, arene oxides, quinones, and α, β-unsaturated carbonyls (Sheehan et al. 2001).

GSTs also act as binding proteins for bile acids, steroid hormones and neurotransmitters. In addition to induction by exogenous compounds, the pattern of GST expression varies during development and aging and is, furthermore, organ-, sex- and species specific. In addition, specific hormones, including adrenocorticotrophic, growth (GH), thyroid and steroid hormones have been demonstrated to regulate the levels of cytosolic GSTs in certain target organs (Staffas et al. 1998).

Seven classes of mammalian cytosolic GSTs are known named Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta based on amino acid sequences similarities (Mannervik et al. 2005). Members of a given class may have more than 40 % sequence identity, and different classes may show less than 25 % identity (Hayes *et al.* 2005). The fish GST comprises the major GSTs such as Alpha, Mu, Pi, and Theta. They are classified based on their cDNA sequences (Frova, 2006; Blanchette et al. 2007). Some novel GST isoforms such as Omega, Kappa, Rho have also been identified from fish (Fu and Xie, 2006; Wang et al. 2006; Liang et al. 2007). Fish GST isoforms are expressed specifically in almost all the tissues, including the reproductive tissues like testes and ovaries in a discrete tissue-specific pattern (Rabahi et al.1999; Thyagaraju et al. 2005). Furthermore, like mammals, gender differences in detoxification efficacy are also reported in some fish species (Yu et al. 2008). The alterations in the GST activities directly reflect the metabolic disturbances and cell damage in specific organs of fish (Carvalho-Neta and Abreu-Silva, 2013).

Catfish *Clarias* is freshwater, belonging to the genus *Clarias*. There are 32 species of catfish belonging to genus *Clarias* are known in Africa. *Clarias lazera* and *Clarias gariepinus*are the most popular members of the inland water fishes found in Egypt. *Clarias lazera* is known locally as karmout and is commonly found in Lake Nasser and all Nile branches and streams. These species are tolerant to a wide range of water and laboratory conditions and has detritivorous behavior (Amin & Hashem, 2012).

Until now, there is no available information on GSTs of *C. lazera* gonads. Therefore, our study was concerned with the purification and characterization of the detoxifying enzyme GST from *C. lazera* ovary and testis in an attempt to identify the role of GST in gonadal differentiation and reproductive toxicology.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, 2, 4-dithiotheritol (DTT), reduced glutathione (GSH), and 1-chloro-2, 4-dinitrobenzene (CDNB) were purchased from Merck Company. Epoxy activated Sepharose 6B and molecular weight standard proteins were purchased from Pharmacia Company.1, 2-epoxy-3-(4-nitrophenoxy)–propane (EPNP), ethacrynic acid (EA), 4-nitrophenethyl bromide (4-NPB), styrene-7, 8-oxide (SO), *para*- nitrophenyl acetate (*p*NPA), bromosufophthalein (BSP), phenthylisothiocyanate (phenthyl -ITC), allylisothiocyanate (allyl-ITC) and benzyl isothiocyanate (benzyl-ITC) were purchased from Sigma Company. All other chemicals were of the highest purity commercially available.

Materials

A total of 30 mature *C. lazera* catfish of both sexes (15 males and 15 females) were collected from different local fish markets in Cairo, Egypt, throughout the period from November 2013 to February 2014 ranging in total length and total weight from 40 to 50 cm and 490 g to 1.19 kg. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Crude extract preparation

Known weights, 2 - 7 g and 2 - 5 g of *C. lazera* ovaries and testes, respectively, were homogenized using a glass homogenizer in 50% (w/v) 25 mM Tris-HCl buffer, pH 8.0 containing 1 mM EDTA and 1 mM DTT (buffer A). The homogenates were then centrifuged at 10,000 *xg* for 15 min. The supernatants (cytosol) were filtered through a plug of glass wool and the filtrates (crude extracts) were saved at -20°C for further analyses.

Purification of C. lazera GST using affinity chromatography

Reduced glutathione (GSH) was coupled to Epoxy-activated Sepharose 6B according to Simons and Vander Jagt, (1977). Crude extracts of *C. lazera* ovaries and testes were mixed individually with 15 mL of GSH-Sepharose matrix previously equilibrated with buffer A, and allowed to couple for 30 min at 4°C with 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 × 1 cm i.d.) and the bound GST was eluted with 50 mMTris-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 (1964).The SDS/PAGE was performed using 12% (w/v) polyacrylamide gel (Laemmli, 1970). Protein bands were then visualized using Coomassie brilliant blue (R-250) stain. The purified enzyme was stored at -20ºC.

Enzyme assays and protein determination

GST activities of *C. lazera* gonads (ovary and testis) toward CDNB, EPNP, EA, *p*NPA and NPB were measured as described by Habig et al. (1974). The GST activity towards phenythyl-ITC, Allyl-ITC, and Benzyl-ITC were determined as described by Kolm et al. (1995). GST activity towards SO was determined as described by Ivarsson et al. (2003). The assay conditions and substrate structures are summarized in Table 1 and Fig. 1, respectively. One unit of GST activity is defined as the formation of 1 µmol product min^{-1} at 30°C. Protein concentration was measured by the Bio-Rad (Bradford, 1976) assay, using bovine serum albumin as standard. Measurements were done on Shimadzu UV Spectrophotometer at 595 nm.

Table 1: Conditions for spectrophotometric assays of glutathione transferase

Substrate	[Substrate] (mM)	[GSH] (mM)	pH	λ (nm)	Δε ($mM^{-1}cm^{-1}$)
1,2-Epoxy-3-(4-nitrophenoxy)- propane (EPNP)	0.5	10	6.5	360	0.5
4-Nitrophenethyl bromide (NPB)	0.1	10	6.5	310	1.2
1-Chloro-2,4-dinitrobenzene (CDNB)	1.0	1.0	6.5	340	9.6
phenethylisothiocyanate (PEITC)	0.4	4.0	6.5	274	8.89
Allyl-isothiocyanate (Allyl-ITC)	0.4	1.0	6.5	274	7.45
Benzyl- isothiocyante (Benzyl-ITC)	0.4	1.0	6.5	274	9.25
Styrene-7,8-oxide (SO)	1.6	5.0	7.2	234	0.77
Ethacrynic acid (EA)	0.2	0.25	6.5	270	5.0
p-nitrophenyl acetate (*p*NPA)	0.2	0.5	7.0	400	8.3

-The final concentration of the solvent ethanol was 1.0% (v/v) in all assays.
-The 0.1M potassium phosphate buffer, pH 6.5 was used for all substrates except styrene oxide and pNPA which 0.25M Tris-HCl, pH 7.2 and pH 7.0, respectively was used at 30°C.

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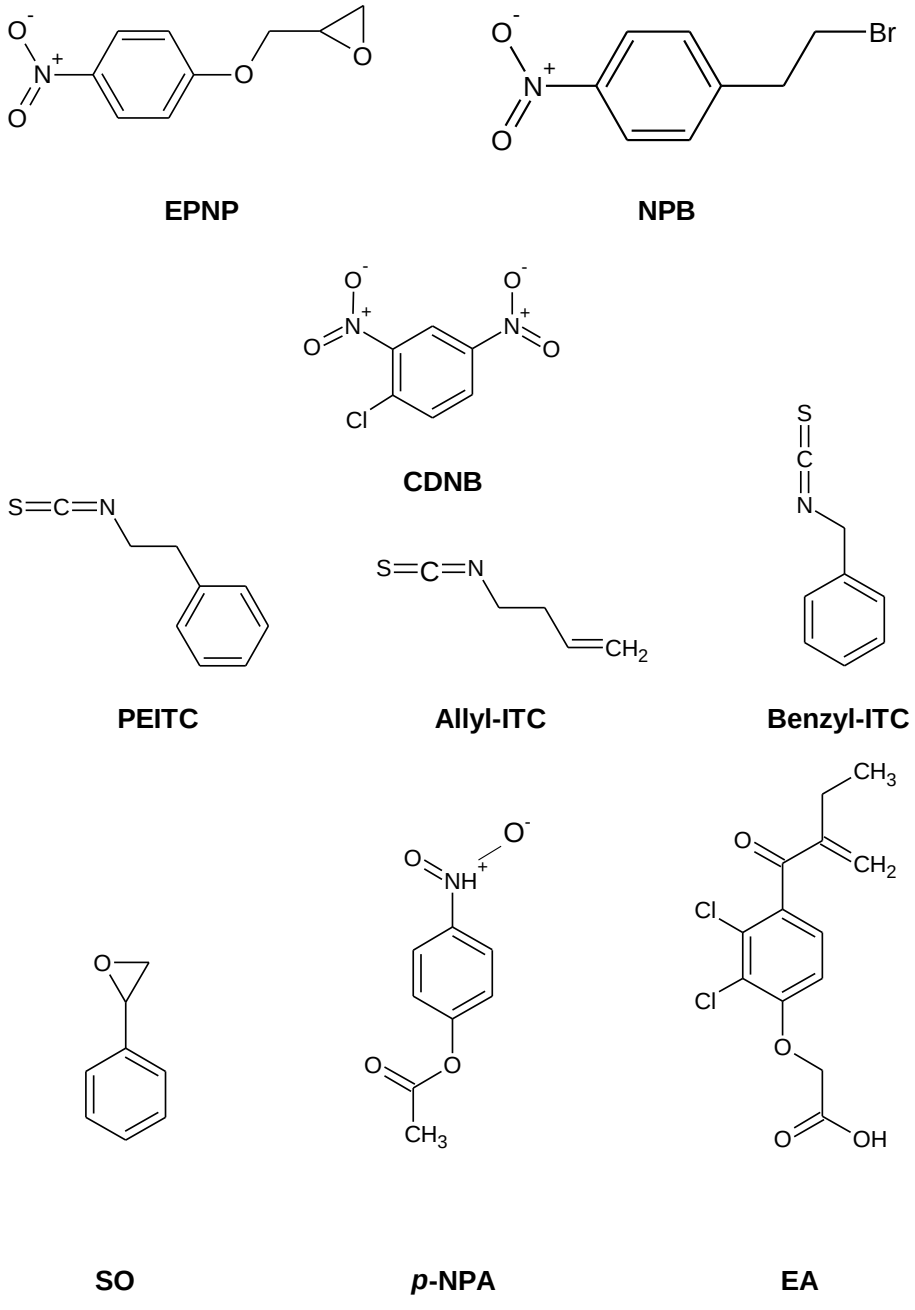

Figure 1: Chemical structures of different GST substrates

Determination of kinetic parameters

The apparent K_m and V_{max} values for GSH were determined at pH 6.5 using a GSH range from 0.4 to 2.0 mM at constant concentration of CDNB at 2.0 mM. The apparent Km and V_{max} values for CDNB were determined using a CDNB range from 0.1 to 2.0 mM at constant GSH concentration of 5.0 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, bromosulfopthalein and cibacron blue, were tested for their ability to inhibit CDNB conjugating activity of *C. lazera* purified GST. The inhibitors 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.

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Statistical analysis

All data are reported as means ± SD for n= 4-5 samples. The Student's t-test was performed to examine the difference between means.

RESULTS

Purification of gonads C. lazera GST

Total GST of the *C. lazera* gonads (ovary and testis) was purified in single step purification by affinity chromatography over a column of GSH linked to epoxy-activated Sepharose, yielding a single major peak of enzymatic activity with CDNB as substrate. The *C. lazera* ovaries and testes GST exhibited almost the same chromatographic behavior on GSH-Sepharose affinity column. The specific activity increased to 3.48 units / mg protein with 102 % recovery for *C. lazera* ovaries GST, compared to 24 units / mg protein with 42.8 % recovery for *C. lazera* testis enzyme (Table 2, 3).

Table 2: Purification of GST from *C. lazera* ovary using GSH-Sepharose affinity chromatography

Purification step	Activity (units)	Protein (mg)	Specific activity (unit/mg protein)	% Recovery	Fold
Crude homogenate (5g)	8.53	210	0.04	100	1.0
Unbound fraction	0.7	64.4	0.011	8.2	0.28
Affinity purified fraction	8.7	2.5	3.48	102	87

Table 3: Purification of GST from *C. lazera* testis using GSH-Sepharose affinity chromatography

Purification step	Activity (units)	Protein (mg)	Specific activity (unit/mg protein)	% Recovery	Fold
Crude homogenate (5g)	8.90	57	0.16	100	1.0
Unbound fraction	0.34	7.90	0.04	3.80	0.26
Affinity purified fraction	3.81	0.16	24	42.8	151

Polyacrylamide gel electrophoresis

The purified GST from *C. lazera* ovaries and testes concentrated by ultrafilteration, were examined by 7% polyacrlyamide gel electrophoresis (PAGE) and 12% SDS-PAGE gel electrophoresis followed by staining for protein using Coomassie brilliant blue. One band could be detected for both of *C. lazera* ovaries and testes GST as judged by 7% PAGE (Fig. 2, a). When the purified proteins were analyzed by SDS-PAGE, a single band corresponding to a molecular mass of 27.5 kDa was detected of the purified *C. lazera* testis. However, two bands were detected after Coomassie staining of the separated *C. lazera* ovary (Fig. 2, b). The molecular weight of the two bands was calculated from previously established slandered curve for known molecular weight protein standards (GenedirexBLUelfPrestained Protein Ladder) to be 25.1 kDa and 27.5 kDa(Fig. 2 a, b).

Characterization of the purified GST from C. lazera gonads

The effect of pH, kinetic parameters, substrate selectivity and the effect of inhibitors on the catalytic activity of the purified GST from *C. lazera* gonads were studied.

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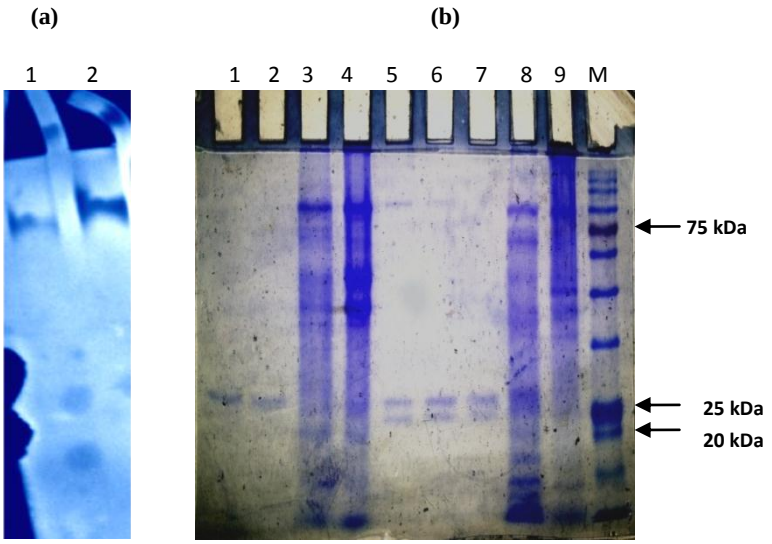

Figure 2: Polyacrylamide gel electrophoresis of *C. Lazera* gonads affinity purified GST
(a) 7% PAGE, affinity purified GST of ovary (lane 1) and testis (lane 2)
(b) 12% SDS-PAGE, crude homogenate of ovary (lane 8) and testis (lane 3), unbound affinity fraction of ovary (lane 9) and testis (lane 4), affinity purified GST of ovary (lane 5, 6, 7) and testis (lane 1, 2)

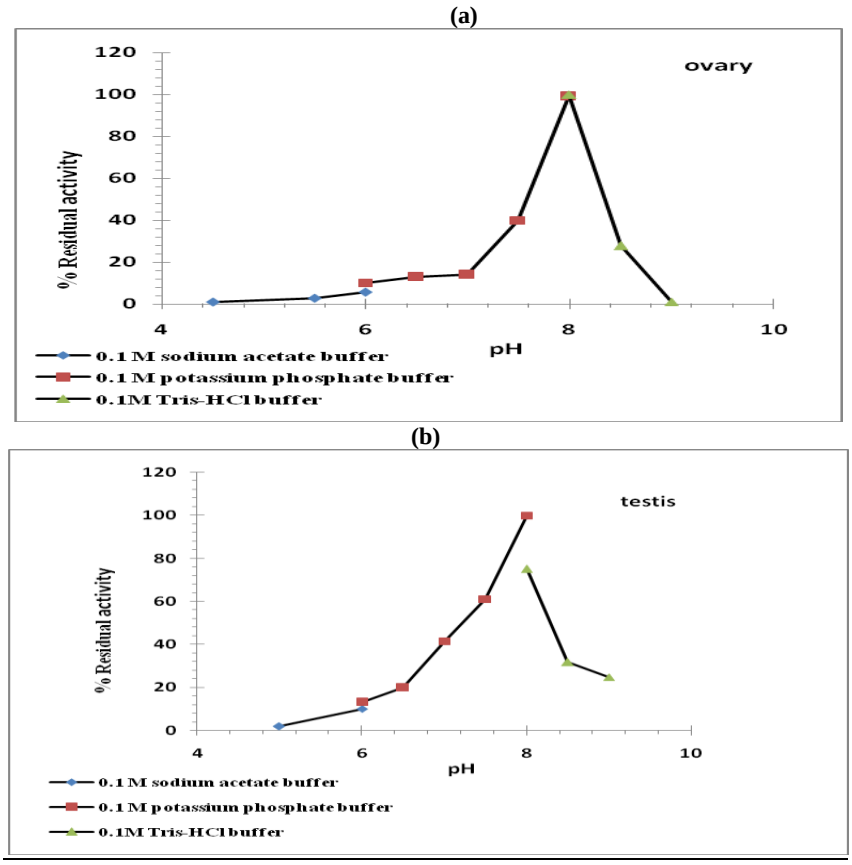

Figure 3: The effect of pH on the enzymatic activity of the purified catfish *C. lazera* GST from (a) ovary and (b) testis.

Effect of pH

The effect of pH on the purified enzyme activity from *C. lazera* ovaries and testes was examined between pH 4.5 and 9.0, using 0.1 M sodium acetate buffer for pH values 4.5 and 5.7, 0.1 M potassium phosphate buffer for pH values from 5.7 to 7.5 and 0.1M Tris-HCl buffer for pH values from 7.5 to 9.0. Both of

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the pH profiles of gonadal *C. lazera* GST exhibited a bell shape with a pH optimum at pH 8.0 and a sharp decrease at the alkaline side of the pH (Fig. 3).

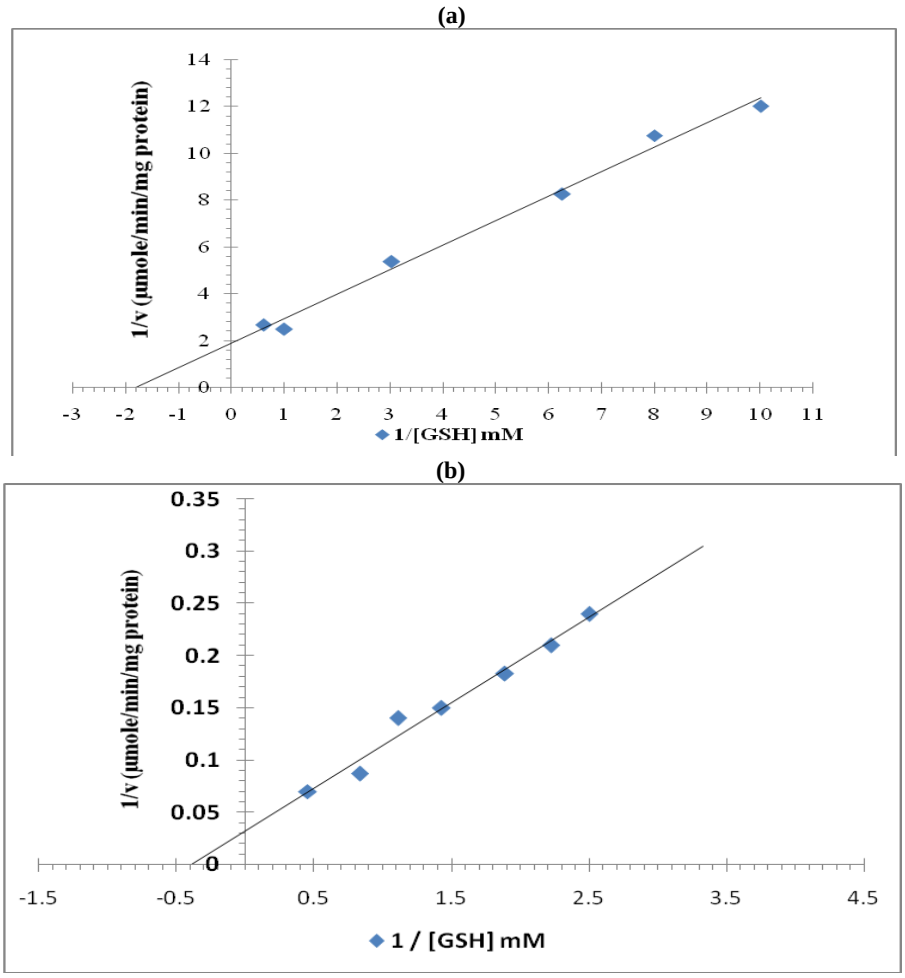

Figure 4: Lineweaver-Burk plot relating the GST activity purified from catfish *C. lazera* (a) ovary and (b) testis to GSH concentration.

Kinetic parameters

a- Effect of varying GSH concentration on GST activity of C. lazera ovary and testis

The effect of GSH concentration in the range between 0.1-2.0 mM and 0.4-2.2 mM, on GST activity of *C. lazera* ovaries and testes, respectively, was examined at a constant CDNB concentration of 2.0 mM. The enzyme exhibited a typical Michaelian behavior in both ranges of GSH concentration, i.e. a linear relationship was obtained when 1/ v was plotted against 1/[S] in both organs, Fig. 3 (a, b). The K_m value was calculated to be 0.56 mM with a V_{max} equal 0.5 µmoles / min / mg protein for ovaries, and 2.5 mM with V_{max} equal 33 µmoles / min / mg protein for testes GST (Table 4).

Table 4: The kinetic parameters of the purified GST from *C. lazera* ovary and testis

Gonad type	GSH		CDNB	
	Km	Vmax	Km	Vmax
Ovary	0.56	0.5	1.0	2.5
Testis	2.5	33.3	0.83	25

-Buffer used was 0.1 M K-phosphate, pH 6.5 at 30°C, Km values was calculated in mM and Vmax values was calculated in µmoles / min / mg protein

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b- Effect of varying CDNB concentration on GST activity of C. lazera ovary and testis

Effect of CDNB concentration on the enzyme reaction was investigated for both *C. lazera* gonads. The initial velocities were determined in the presence of constant concentration of GSH at 5mM and CDNB concentration was varied between 0.1-2 mM. In ovary and testis, a linear relationship was obtained when 1/v was plotted against 1/[S], with K_m equal 1 mM and V_{max} equal 2.5 µmol/min/mg protein compared to K_m value equal 0.83 mM and V_{max} equal 25 µmol/min/mg protein, respectively for ovary and testis GST (Table 4).

Substrate selectivity

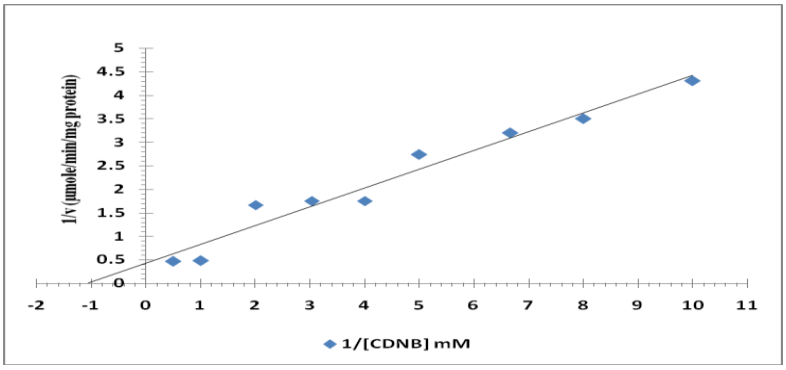
The substrate selectivity of ovary and testis GST with some known GST substrates are summarized in Table 5 and Fig. 4. The results represented in Table 5 indicated that there was a clear difference between the *C. lazera*GST activities purified from ovary and testis on the examined substrates. Ovary of *C. lazera* GST showed maximum activity for *p*NPA (32%), followed by phenethyl-ITC (24%), benzyl- ITC (16%) and SO (15%) (Fig. 4). The enzyme showed very low conjugating activity with allyl-ITC, EA and EPNB (2-3%). Both of ovary and testis GST had similar activity with the classic CDNB substrate (1.41 ± 0.084 and 1.91 ± 0.045 µmol/min/mg protein, respectively), representing 5% and 1% of the activity with the other examined substrates (Fig.4). The highest activity (75%) was obtained with SO for testis GST (215 ± 34.5µmol/min/mg protein) representing 49.2 fold increase in the catalytic activity of ovary GST (15%) with SO (4.37 ± 0.075 µmol/min/mg protein). The activity of testis GST showed 5- fold higher toward phenethyl-ITC (34.98 ± 7.24µmol/min/mg protein) compared to ovary GST. Almost the same enzyme reactivity was observed toward allyl-ITC and benzyl-ITC with specific activity equal 13.2 ± 0.973 µmol/min/mg protein and 11.2 ± 1.95µmol/min/mg protein, respectively. The enzyme isolated from testis showed very low conjugating activity with *p*NPA and EA compared to their activity with ovary GST. The ovary GST was almost 53 times more reactive towards *p*NPA than testis (9.39 ± 0.39 µmol/min/mg protein compared to 0.175 ± 0.045 µmol/min/mg protein). However, testis GST was 11.92 times more reactive toward EPNP than ovary (7.75 ± 0.22 µmol/min/mg protein compared to 0.65 ± 0.13 µmol/min/mg protein). The specific activities of ovary GST toward most of the tested substrates were lower than that of testis GST (Table 5).

Table 5: Specific activities of gonadal *C. lazera* GST using different substrates

Substrates	Specific activity (µmol/min/mg protein)	
	Ovaries	Testis
CDNB	1.41 ± 0.08	1.91 ± 0.05
phenethyl-ITC	6.96 ± 0.27	34.9 ± 7.24
Allyl-ITC	0.869 ± 0.15	13.2 ± 0.97
Benzyl-ITC	4.76 ± 0.55	11.2 ± 1.95
*p*NPA	9.39 ± 0.39	0.18 ± 0.05
SO	4.37 ± 0.08	215 ± 34.5
EA	0.74 ± 0.03	0.22 ± 0.05
EPNP	0.65 ± 0.13	7.75 ± 0.22

-Each value represents means ± S.D for three experiments

(a)



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(b)

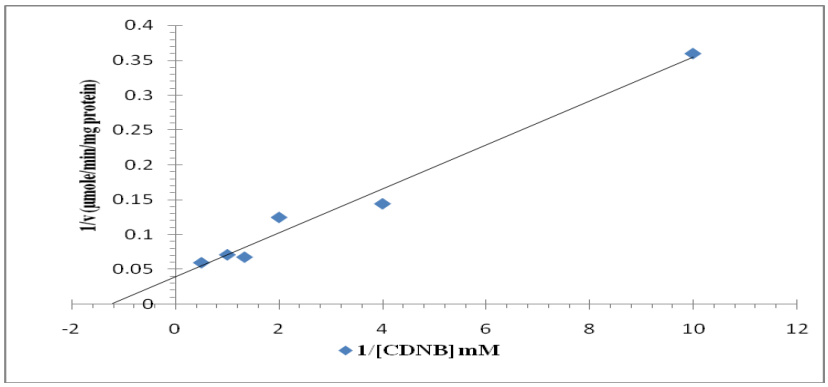


Figure 5: Lineweaver-Burk plot relating the GST activity purified from catfish *C. lazera* (a) ovary and (b) testis to CDNB concentration.

Effect of inhibitors

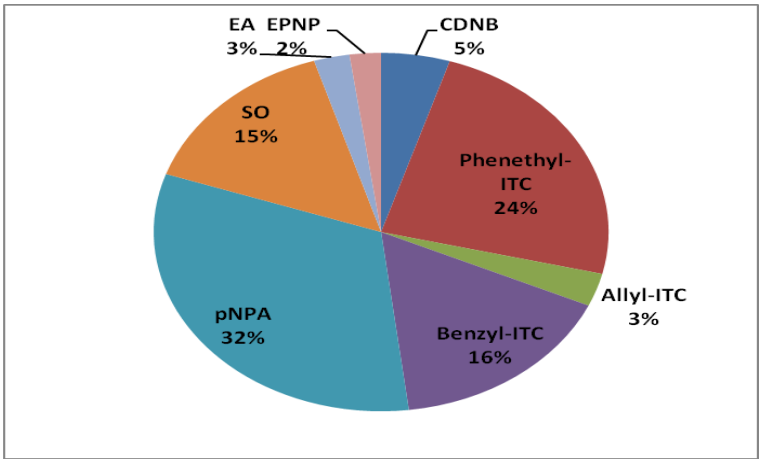
The IC_{50} values (concentration of inhibitors that will give 50% inhibition) of the activity of gonad GSTs are shown in Table 6. The *C. lazera* ovary and testis GST showed different patterns of sensitivities to the small range of inhibitors tested. Although hematin was the most potent inhibitor of testis GST activity (IC_{50} value equal 5.2 nM), no effect could be detected for hematin on ovary GST. Testis GST was almost 13.6 times more sensitive to hematin than cibacron blue (IC_{50} value equal 70.8 µM). Sensitivity of *C. lazera* ovary GST to bromosulfophtalein was almost half its sensitivity to cibacron blue (IC_{50} value equal 20 nM). However, no effect could be detected for bromosulfophtalein on testis GST (Table 6).

Table 6: Effect of inhibitors on the activity of GST from gonads *C. lazera*

Inhibitor	IC_{50} (nM)	
	Ovary	Testis
Bromosulphophethalene	20	NI
Cibacron blue	48	70.8
Hematin	NI	5.2

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 at 30°C. NI: no inhibition could be detected

(a)



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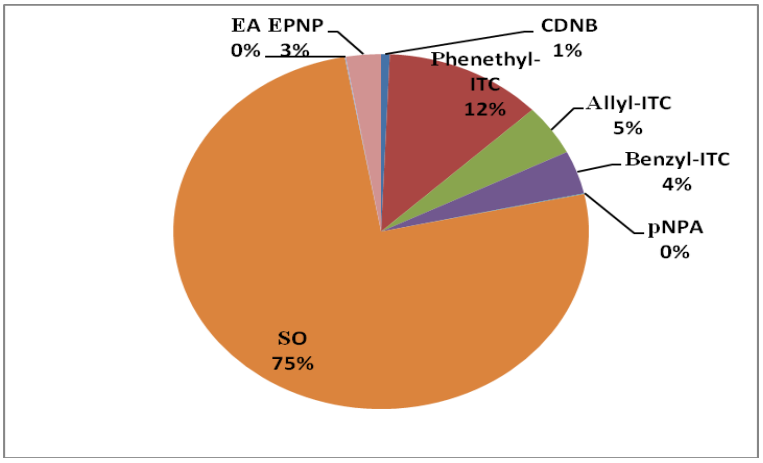

Figure 6: Substrate selectivity profile of *C. lazera* GST ovary (a) and testis (b). Pie chart show relative activities with different GST substrates

DISCUSSION

GSTs are widespread among all mammalian species, as well as in all tissues investigated to date. Endocrine organs such as the testes, adrenal and ovaries demonstrate remarkably high GST activities, which raise questions concerning possible physiological roles of these enzymes. The GSTs constitute a family of multifunctional detoxifying enzymes. Their primary known function is to conjugate compounds containing an electrophilic centre with GSH. Peroxidation and isomerization are other functions that certain GSTs are able to perform (Toft et al. 1997). GSTs have been reported to involve in steroid metabolism by catalyzing the isomerization of Δ^5-androstene-3, 17-dione to, Δ^4-androstene-3, 17-dione (Petterson and Mannervik, 2000; Johansson and Mannervik, 2001).

Hormones are regulatory biochemicals produced in all multi-cellular organisms and regulate a variety of physiological and behavioral activities. Sex steroid hormones such as testosterone and 17β-estradiol are known as key hormones that control and regulate the developmental stages of the organism including gametogenesis, fertilization, sexual development and development of primary and secondary sexual characteristics (Nelson, 2005).

Hormonal regulation of GSTs in rat adrenal, ovary and liver was investigated by Eliasson et al. (1999). They found that, GSTs (primarily GST Alpha, Pi and Theta) can act as peroxidases, for example, towards fatty acid hydroperoxides. A high binding capacity for steroids, for example, is also displayed by certain isoenzymes of GST. They suggest that, pituitary hormones adrenocoticotrophic hormone (ACTH), growth hormone (GH) and follicle-stimulating hormone (FSH) influence the levels of expression of GSTs in the organs responsive to these hormones.

Testis has high levels of GSTs. Indeed, different GST isoenzymes have been identified in the human male gonad. Specifically, GST Alpha and GST Pi have been identified in testicular somatic cells and particularly in Leydig and Sertoli cells. GST Alpha (A3-3) is primarily expressed in the mammalian placenta, adrenal glands and gonads (Johansson and Mannervik, 2001). GST Alpha may be necessary for protecting this tissue from reactive oxygen species-induced damage. The importance of GSTs in the protection against oxidative stress in testis is underscored by some studies showing that when GST activity is inhibited, products of lipid peroxidation accumulate, resulting in germ cell apoptosis (Rao and Shaha, 2000). In addition, GSTs may also exert some specific effects in the male reproductive function. GST isoenzymes exhibit a steroid binding activity and seem to be involved in male fertility, specifically in the gamete interactions (Benbrahim-tallaa et al. 2002). Ovary is subjected to continuous alterations in hormonal status and the expression of GSTs in this organ may have profound effects in terms of reproductive toxicology (Toft *et al.* 1997). The involvement of GSH and generation of reactive oxygen species (ROS) during 7, 12-dimethylbenz [a] anthracene (DMBA) induced

preovulatory follicle destruction has been evaluated. DMBA exposure increased ROS generation but did not alter concentrations of total GSH. However, GSH depletion prior to DMBA exposure resulted in increased apoptosis and cleaved caspase-3 positive follicles. Additionally, DMBA increases mRNA and protein expression of GST isoform Pi in cultured rat ovaries (Bhattacharya and Keating, 2011), potentially as a protective measure to counteract ROS generation by DMBA.

Clarias species are air breathing fishes due to the presence of accessory assistant respiratory organs beside the gills enabling it to survive for long time outside the water, otherwise debilitating hypoxic environments. *Clarias lazera* and *Clarias gariepinus* are the most popular members of the inland water fishes found in Egypt. These species are tolerant to a wide range of water and laboratory conditions and has detritivorous behavior. This means that the fish can be in contact with xenobiotics from different ways of interacting with algae from stone or sediment. These characteristics make this particular species an interesting model for ecotoxicological and biochemical studies. Moreover they are valuable bio-indicators of contamination because of their large distribution, being open swimmers, capacity to react against ecological pollution and food source for human (Kamal and Hashem, 2012).The African catfish *C. lazera* was selected for the present study because it is differ from other Nile fish (air breathing fish), it's easy to get from the natural environment and due to its sensitivity to toxicants and its ability to bioaccumulations high concentrations of residues in body tissues.

Purification of gonadal C. lazera GST

Both of ovary and testis exhibited almost the same chromatographic behavior on GSH-Sepharose affinity column, where one peak of GST bound to the column and eluted with 10 mM GSH. After affinity purification step of *Monopterus albus* GST (maGST) liver and *oreochromis niloticus* liver, a single protein band was obtained suggesting this protein was the predominant GST (Hamed et al. 2005; Huang et al. 2008).

Electrophoresis pattern in our results suggest that ovary GST is a heterodimer of two different subunits having two different molecular weights, while testis GST is a homodimer protein of two similar subunits having the same molecular weights. The results under investigation are in agreement with the molecular weights reported by the major isoforms of cytosolic GST from different species of fish possessed a molecular mass ranging from 22.4 to 26.9 KDa (Nova-Valinas et al. 2002; Hamed et al. 2004). In salmon fish livers, a similar pattern was observed where the predominant GST expressed comprised subunits of molecular mass equal 24.8 kDa (Huang et al. 2008). In another study on digestive gland of Icelandic scallop, also a single GST protein is detected by SDS-PAGE after GST chromatography. However, two and four GST isoenzymes are observed in Gilthead Sea bream livers and rainbow trout livers (Huang et al. 2008).

Mammalian, plant and microorganism GST enzymes are dimers with a molecular weight of 40,000 to 60,000 (Mozer*et al.* 1983; Kong *et al.* 1991; Nishida *et al.* 1994). In the case of heterodimers each subunit is encoded by a different gene (Buono et al. 2007). Homo- or heterodimers are known to form within a given class (Pettigrew and Colman, 2001). Pettigrew and Colman, (2001) reported for the first time that the formation of a heterodimeric species composed of one class Mu polypeptide and one class Pi subunit. The formation of these heterodimers under physiological conditions is influenced by the level of GST expression and tissue distribution (Pettigrew and Colman, 2001).

Our GST configuration is similar to those from other fish and bivalve where homodimeric GSTs are isolated. However, heterodimeric GSTs have been observed in freshwater carp and marine flatfish. To date, most GSTs isolated from fish, mammals and bivalves are homodimers and only minor cases are heterodimers. The transcriptional regulation of individual subunits is considered to influence the formation of GST homodimers and heterodimers (Huang et al. 2008).

Our study indicates that *C. lazera* ovary and testis have different GST isoenzyme expression patterns and/or the expressions of GSTs are induced by different environmental factors.

Characterization of the purified GST from C. lazera gonads

Effect of pH

The effect of pH on the enzyme activity was evaluated using CDNB as a substrate. The optimum pH for both of the purified *C. lazera* ovary and testis GST was found to be at pH 8.0. Generally optimum pH values for GST with a variety of different substrates have values ranging from 6.0 to 9.5. When CDNB is considered, a narrower range of pH 7.0-9.0 is obtained, but the mode is in the vicinity of pH 8.0 (Clark, 1989). In the study of Huang et al. (2008), the activity of freshwater fish *Monopterus albus* GST (maGST) was pH-dependent. The highest activity was observed in the pH range 7.0–7.5. At pH 6.5 and 8.5, maGST still had 62% and 72% of its maximum activity, respectively, suggesting a broad pH optimum. In the present study, the observed activity of the purified enzymes in the alkaline side may suggest a high stability in the alkaline conditions.

Kinetic parameters

The enzyme's steady state kinetics was studied in assays with various concentrations of GSH and CDNB. Kinetic analysis with GSH as substrate, revealed high K_m value (2.5 mM) for testis GST compared to 0.5 mM for ovary GST. The K_m and V_{max} for ovary and testis GST with CDNB are comparable Table 4). In the present study, ovary and testis GST kinetic studies, describing multisided enzymes having two binding sites with different affinities for GSH and CDNB substrates. The affinity of ovary and testis GST toward GSH are different with 4.5 fold increase of ovary GST over that of testis GST. However, the affinity of ovary and testis GST was almost the same with higher V_{max} value obtained with testis GST compared to V_{max} value of ovary with respect to CDNB.

The present K_m^{GSH} values are in agreement with those of other GST isoenzymes (4.2-5.9 mM for GST 5-5 rat class Theta, 0.5 mM for Rho, 2 mM for sigma, 0.11 mM for class Pi, 0.08 mM for class Alpha, A1-1 and 0.16 mM for A2-2) (Schroder *et al.* 1996).

The freshwater fish liver maGST shared similar K_m^{GSH} with *C. lazera* intestine GST (0.19 mM) (Huang et al. 2008). Important differences were also observed when considering the kinetics of two *Tilapia zilli* GSTs (tzGST) isolated from liver, tzGST2 had higher K_m than tzGST1 with values of 1.6 and 2.9 folds higher for CDNB and GSH respectively. The V_{max} value was higher for tzGST2 than tzGST1 with GSH (Bamidele et al. 2012). The differences in the kinetic parameters of *tz*GSTs in the tissue investigated probably (liver) indicated that different kinetic mechanisms are employed to handle detoxification of toxins and these may be very complex and class dependent. Thus, the structural differences within the active site may influence the type of mechanism the GST isoenzymes use to catalyze its reactions (Bamidele et al. 2012).

The present study indicates that, the high K_m value observed with testis GST is suggestive of a low affinity of the enzyme to the substrate GSH compared to ovary. However, ovary and testis GSTs have similar affinity for the substrate CDNB with different maximum velocities. Information on purification and kinetics of fish gonads GST is not reported in the available literature. The present investigation is the first report to our knowledge on kinetic properties of catfish *C. lazera* gonads.

Substrate selectivity

GST isoenzymes exhibit marked differences in their ability to conjugate GSH with various electrophiles (Gyamfi et al. 2004). The specific activities measured for both ovary and testis GST toward various substrates are listed in Table 5. The highest activity was obtained toward SO (75%) for testis GST (215 ± 34.5µmol/min/mg protein) representing 49.2 fold increase in the catalytic activity than that of ovary GST with SO (4.37 ± 0.075 µmol/min/mg protein). The isolated enzyme from *C. lazera* testis was quite efficient in catalyzing the conjugation of reduced glutathione with SO (characteristic substrate of mammalian GST Mu, M1-1). However, the isolated enzyme from *C. lazera* ovary was quite efficient in catalyzing the conjugation of reduced GSH with *p*NPA (32%). In the present study, testis GST was 11.92 times more reactive with EPNP than ovary (7.75 ± 0.22 µmol/ min / mg protein compared to 0.65 ± 0.13 µmol/ min/ mg protein). The activity of GST towards EPNP, the characteristic substrate of mammalian Theta GST, T1-1, shows great variation. Very weak reactivity of both ovary and testis GST towards EA (characteristic substrate of mammalian GST Pi) suggested that EA was a poor substrate for *C. lazera* gonadal GST. The high specific activities of liver maGST toward CDNB and NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) and no enzymatic activity towards EA and ρNPA observed in Huang et al. (2008) for GST isoenzyme purified from liver maGST were disagreed with the present work.

Unlike most GSTs, our results indicated that, both of ovary and testis affinity purified GST have relatively moderate activity towards CDNB (GST common substrate). Our results suggest that, the gonads GST preparation contains isozymes with properties similar to mammalian Alpha, Mu GST classes and almost dissimilar to Pi GST class. The gonads of *C. lazera* GST would be able to perform its xenobiotics conjugation and detoxification functions efficiently in broad substrate ranges. The variation of the substrate selectivity of *C. lazera* GST between ovary and testis may indicate a different role of the GST in the reproduction process and sex differentiation.

Inhibition

Inhibition studies of GST isoenzymes have been used in the past in distinguishing isoenzymes containing different combination of subunit and in the classification of the enzyme. In the present investigation, testis GST was strongly affected by hematin, however this inhibitor has no effect on ovary GST (IC_{50} value equal 5.2 nM). Sensitivity of *C. lazera* ovary GST to bromosulfophtalein was almost half its sensitivity to cibacron blue (IC_{50} value equal 20 nM). However, no effect could be detected for bromosulfophtalein on testis GST (Table 6).

In terms of inhibition, class Alpha is noted for a low IC_{50} value for hematin and a high value for cibacron blue (the ratio of the IC_{50} values for a given isoenzyme being ≈0.1); class Mu has a high IC_{50} for hematin and a low value for cibacron blue (the ratio of the IC_{50} values ranging from 3 to 20); class Pi have a high IC_{50} for hematin and a low value for cibacron blue (the ratio being similar to class Mu) (Mannervik, 1985).

Tilapia GSTs are sensitive to hematin and ethacrynic acid and the result agrees with the values reported earlier for the Pi-class GST (Tahir et al. 1985). The sensitivity to hematin and ethacrynic acid indicated that GSTs from liver of *T. zilli* are closely related to the Pi-class than Alpha or Mu classes. Hamed et al. (2004) indicated that GST purified from *O. niloticus* liver was strongly affected by cibacron blue and resembles the hepatic GST of the class Alpha.

The differences in sensitivity of the isoenzymes to the different inhibitors may reflect different structural features. The variation in response to inhibitors may be due to the variation in the structure of the active sites of the enzyme. In the present study, different sensitivities between ovary and testis GST to the examined inhibitors account for structural composition different between gonadal GSTs.

CONCLUSION

In conclusion, in the present study *C. lazera* ovary and testis GSTs are different structurally and kinetically in: 1) Ovary GST expressed as heterodimer protein of two different subunits, while testis GST is a homodimer of two similar subunits. 2) The k_m of GSH for ovary GST is almost 4.5 fold higher than that of testis GST, suggesting lower affinity of testis GST to the essential substrate GSH. 3) The highest activity was obtained with SO for testis GST in contrast to ovary GST having high activity with *p*NPA. 4) Testis GST was strongly affected by hematin, however this inhibitor have no effect on ovary GST.As consequence they are kinetically different, C*. lazera* ovary and testis GSTs have different role in the reproduction process and sex differentiation. It may have a role in detoxification as transferase or ligandin of the metabolites resulting during egg maturation, which is expected to be different from that during sperm maturation.

Ethical statement

All experiments were carried out in accordance with the Egyptian laws and national research center guide lines for the care of experimental animals.

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