Toxicology Reports 3 (2016) 598–607

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Catalysis of Silver catfish Major Hepatic Glutathione Transferase proceeds via rapid equilibrium sequential random Mechanism

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ARTICLE INFO

Article history:
Received 1 May 2016
Received in revised form 29 June 2016
Accepted 30 June 2016
Available online 1 July 2016

Keywords:
Silver catfish
Glutathione transferase
Steady-state
Kinetic mechanism
Inhibition

ABSTRACT

Fish hepatic glutathione transferases are connected with the elimination of intracellular pollutants and detoxification of organic micro-pollutants in their aquatic ecosystem. The two-substrate steady state kinetic mechanism of Silver catfish (*Synodontis eupterus*) major hepatic glutathione transferases purified to apparent homogeneity was explored. The enzyme was dimeric enzyme with a monomeric size of 25.6 kDa. Initial-velocity studies and Product inhibition patterns by methyl glutathione and chloride with respect to GSH-CDNB; GSH-ρ-nitrophenylacetate; and GSH-Ethacrynic acid all conforms to a rapid equilibrium sequential random Bi Bi kinetic mechanism rather than steady state sequential random Bi Bi kinetic. α was 2.96 ± 0.35 for the model. The pH profile of V_{max}/K_M (with saturating 1-chloro-2,4-dinitrobenzene and variable GSH concentrations) showed apparent pKa value of 6.88 and 9.86. Inhibition studies as a function of inhibitor concentration show that the enzyme is a homodimer and near neutral GST. The enzyme poorly conjugates 4-hydroxylnonenal and cumene hydroperoxide and may not be involved in oxidative stress protection. The *se*GST is unique and overwhelmingly shows characteristics similar to those of homodimeric class Pi GSTs, as was indicated by its kinetic mechanism, substrate specificity and inhibition studies. The rate- limiting step, probably the product release, of the reaction is viscosity-dependent and is consequential if macro-viscosogen or micro-viscosogen.

1. Introduction

Glutathione transferases (GSTs; E.C.2.5.1.18), multigene family of isoenzymes, are widely distributed in animals, plants and microorganisms [39,55]. They constitute important roles in detoxification of toxic compounds [69,5] and in non-enzymatic ligand binding functions [72]. They are linked with peroxidase and isomerase activity [27] and are also involved in the inhibition of Jun N-terminal kinase [82]. These functions make glutathione transferase superfamily important target of pharmacological and toxicological studies; and are candidates for anticancer and allergy drug therapy. GSTs catalyzes the nucleophilic addition of thiol of reduced glutathione (GSH) to a wide range of electrophilic compounds [72].

GST isoenzyme families share a common fold and are obligate dimers [84]. They are either homodimers or heterodimers with subunit sizes ranging from 23 to 30 kDa and organized into two domains: GSH binding domain (at the N-terminal) and a xenobiotic substrate-binding domain at the C terminus [8,68]. GSTs are encoded by at least nine different gene families and into 15 different classes namely- alpha, beta, delta, epsilon, kappa, lambda, mu, omega, phi, pi, sigma, tau, theta, zeta, and rho in cytosol and membrane [83,57,39,42,59]. These classes are distinguished based on sequence identity, substrate specificities, antibody cross reactivity and sensitivity to inhibitors. GSTs that differ in amino acid sequences show 50% identities within a class and less than 30% between different classes [57,72]. The diversity of GST isoenzymes provides the capability to conjugate a very broad range of compounds [52].

GSTs exhibit a remarkable degree of catalytic diversity with single isoenzyme catalyzing multiple reaction type; and, are not substrate specific but show overlapping specificities among classes [16,58]. Different forms of GST differ in their catalytic properties and indicate different kinetic mechanism of detoxification [9]. Enzymological studies on GSTs have been proven pivotal to the understanding of structure-activity relationships and physiological regulations [46,55]. The kinetic mechanism of GSTs is clearly abstruse and is isoenzyme-dependent [8,44]. The Kinetic mechanism of Class Pi has been shown to be rapid equilibrium random Bi Bi kinetic mechanism [43,44] while class Mu is a steady-state random Bi Bi mechanism [45,44]. Till date, Class Alpha, historically known as ligandin, kinetic mechanism has not being established. The extents to which these different kinetic mechanisms are applicable or extended to other GST isoenzymes class still remain unclear. Despite the conjectural position, the acceptable kinetic

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http://dx.doi.org/10.1016/j.toxrep.2016.06.006

mechanism, currently, is the random sequential model. Most GST kinetic mechanism models have been limited to conjugation of GSH with 1,2-dichloro-4-nitrobenzene but have not being extended to its other electrophilic substrates. Be that as it may, kinetic mechanism of GSTs may provide a veritable criterion to distinguish various GST isoenzymes classes, if properly explored exhaustively [43].

The potential physiological roles of piscine GSTs have been studied. Their economic value and relevance to aquatic toxicology as a potential pollution biomarker have been stressed [8,63]. Previous researches with GSTs in both aquatic and non-aquatic animals has indicated that the level of GST changes in response to certain environmental pollutants [9,8]. This is a step in understanding the metabolism of xenobiotic compounds that could pose serious ecologic and health problems in coastal areas exposed to industrial pollutions. Aquatic environment presents a unique situation, in terms of special types of living populations and exposure to pollution. The consequent examination of nature and function of hepatic GSTs from marine animals could provide clues to the survival of these organisms in adverse ecologic contexts. However, relatively little research has been done on GST isoenzymes from fish comparable to that of mammals [8,63]. Kinetic characterization of the cytosolic GSTs from several aquatic organisms has been described [76,2,21,9]. Fish GST kinetic study have complicated, inadvertently, by near silence and scanty details of fish GST kinetic mechanism and non-ideal kinetic mechanism for the isolated class.

Warri River is one example eutrophic ecosystem in oil rich Niger–Delta area of Nigeria that has been the focus of water pollution case study [23]. Exploitation of petroleum resources within the area has resulted into the discharge of petroleum hydrocarbon into the ecosystem [67]. The adjoining tributaries empty itself into this river with attendant deposition of tonnes of silts, finely divided agricultural matter and wastes. These accidentally endangered the aquatic and human lives in the area [1]. Silver catfish (*Synodontis eupterus*) is one of the catfish family widely distributed in Warri River with an important economic value. It is well known for its ability to withstand low dissolved oxygen and spawn readily. Catfish families play important economic roles in the lives of the community and serve as alternative and cheap source of daily protein. These economic roles have increased the activities of farming practices within and around Nigerian rivers to the extent that some farmers employ chemical poisons as a fishing method. Commercially important fish are sensitive to environmental pollutants [2]. Silver catfish (*Synodontis eupterus*) are known to be hardy and sturdy; and has high survival rate. The molecular basis of these attributes might be connected biochemical strategies adopted for its survival. This might be connected to the specialization of GSTs enzyme catalyzing detoxification reaction to cope with exogenous and endogenous chemical threats. The Silver catfish (*Synodontis eupterus*) GST is unknown. The present study was undertaken to determine the kinetic mechanism hepatic glutathione transferases of Silver catfish (*Synodontis eupterus*) employed in the detoxification of exogenous and endogenous organic micro-pollutants in its aquatic environment. From a comparative of view to other catfish family, this could add to body of models of toxicology research.

2. Materials and methods

2.1. Materials

Reduced glutathione (GSH), oxidized glutathione (GSSG), 1-chloro 2,4-dintrobenzene (CDNB), 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole, ethacrynic acid, 1,2-dichloro-4-nitrobenzene (DCNB), bromosulfophthalein (BSF), ρ-nitrophenyl acetate (ρNPA), phenylmethanesulfonyl fluoride, cibracron blue, hematin are products of Sigma-Aldrich Chemical, St Louis, USA. DEAE-Trisacryl was from LKB, Villeneuve-la Garenne, France. Tributyltinacetate (TBTA) and triphenyltinchloride (TPTC) were purchased from Alfa Company (Karlerushe, Germany). Glutathione Sepahrose 4B™ is from Amersham Bioscience, Uppsala, Sweden. Other specialty chemicals were obtained from Sigma in the highest purity available unless noted otherwise. All other reagents were of analytical grade commercially available.

2.2. Methods

2.2.1. Sample collection

Silver catfish (*Synodontis eupterus*) used for this study were obtained from a local "fish community" at Ikpesu market water side, Warri, Nigeria. The river has its major activities as fishing (fish farming) and sand mining/dredging by the inhabitants of the area. Silver catfish was collected from the river with the aid of fishnet. Fish were immediately transferred in a glass aquarium (120 l capacity) to the laboratory and acclimated for a week prior to the experiment. The fish were exposed to 12 h L: 12 h D and were fed with fish pellets for that period. Adult males Silver catfish (*Synodontis eupterus)* used in this project were collected in third month of the year.

2.2.2. Enzyme purification

In a typical purification, the fish were killed by cervical dislocation and the livers were immediately removed and washed in ice-cold saline (9.0 g/liter sodium chloride) to remove as much blood as possible and subsequently drained of filter paper. The diced livers (38 g) homogenized with a glass homogenizer, on ice, in 5 vols of 25 mM Tris–HCl buffer, pH 8.0, containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride acid, and 0.1 mM β-mercaptoethanol. The supernatant was clarified by centrifugation at 100 000*g* for 50 min at 4 °C using Beckman-optima-LE-80k Ultra centrifuge. The supernatant filtered through a 0.45 μm Millipore filter (Millipore, Bedford. MA) was used as the crude lysate. The lysate (about ≈150 ml) was subjected to purifications.

The crude lysate was applied to a DEAE-Trisacryl column (2.0 × 30 cm) previously equilibrated with 25 mM Tris–HCl buffer, pH 8.0 and washed with the same buffer at a flow rate of 20 ml/hour and 5 ml fractions were collected and analyzed for enzyme activity and amount of protein. The bound fractions were eluted with 0–1 M salt gradient. Bound fractions containing GST activities were pooled and concentrated with 10 MWM Millipore (10 kDa cut off membrane) then filtered through a 0.45 μm Millipore filter. This was further purified using Glutathione Sepharose™ 4 B gel column according to the protocol supplied. The bound GST was eluted with a linear gradient of GSH (0–10 mM). The pooled fractions were immediately concentrated using 10 MWM Millipore (10 kDa cut off membrane) before kinetic assays. All purification steps were performed at 0–4 °C unless stated otherwise.

The purity of the preparation was determined by SDS/PAGE [51]. Protein concentrations were determined using BIORAD protein assay kit (Bio-Rad, Hercules, CA, USA,) based on the method of [10] with BSA as a standard. A_{280} was used to monitor the protein in column effluents.

2.2.3. Glutathione transferase (GST) assay

Activities with the substrates (1-chloro-2,4-dintrobenzene, 4-chloro-7-nitrobenzo-2-oxa-1,3 diazole, ρ-nitrophenylacetate, ethacrynic acid, 1,2-dichloro-4-nitrobenzene, 4-hyroxylnonenal and *p*-nitrophenyl chloride) for characterization of the GST were measured according to published procedures [37,36,61,4]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmole of product/min/mL at 25 °C in the optimal assay condition for each substrate. Specific activity was

expressed in units/mg of protein. All initial rates were corrected for the background non-enzymatic reaction and were completed in triplicate or more. A Shimadzu UV-1600 double beam spectrophotometer was used for enzymatic and protein assay.

2.2.4. Kinetic studies

Kinetic parameters were determined for GSH and CDNB substrate (and other second substrates) by recording the activity toward a range of concentration from 0.1 to 1.2 mM for both substrates, varying the concentrations of one substrate while keeping the other constant at 25 °C. Experimental data at a fixed concentration of one substrate and varying the concentration of the second were fitted to the Michaelis-Menten equation by using the non-linear regression analysis program KaleidaGraph 4.5 (Synergy Software, Ltd). The data obtained were analyzed in accordance with data analysis devised by [15] and [26]. The apparent kinetic parameters, k_{cat}, K_M and k_{cat}/K_M, were determined. Goodness of fit of initial-velocity data to bireactant kinetic models was evaluated using the criteria described by Mannervik (1996). The pH dependence of k_{cat}/K_M of CDNB was as mentioned elsewhere [81].

2.2.5. Inhibition studies

The concentration of inhibitor giving 50% inhibition (I_{50}) of the enzyme activity assayed at pH 6.5 with 1 mM CDNB and 1 mM GSH as substrates was determined for the following compounds: Cibracron blue (0–10 μM), bromosulphophthalein (BSP) (0–10 μM), hematin (0–0.2 μM), oxidized glutathione (0–10 mM) and S-hexylglutathione (0–10 mM). Cibacron blue was made by dissolving the inhibitor in a minimal amount of 0.1 M NaOH, followed by neutralization with 0.1 M HCl and dilution with deionized water to the desired concentration. Hematin was dissolved in acetone, but dilution was made with ethanol to avoid comparatively higher inhibitory action of acetone. Stock solution as well as solution of all other inhibitors was made in 95% (v/v) ethanol [75]. The experiments were performed in duplicate and each assay was run in triplicate.

2.2.6. Viscosity dependence of kinetic parameters

The effect of micro-viscosogen (Glycerol) and macro-viscosogen (Ficoll 400) on *Se*GST second order rate constant (k_{cat}/K_M) was monitored a 25 °C. Relative solution viscosities (ηrel) were measured using all glass Ostwald viscometer (VWR, USA). The viscometer was kept isothermal by immersion in a water bath controlled at 25 ± 0.1 °C. 0.1 M potassium phosphate buffer, pH 6.5 was used as reference buffer-only solution (ηo) at 25 °C. The rate diffusion-limited processes were calculated in a manner predicted by the Stokes–Einstein relation: $[(k_{cat}/K_M)^o/(k_{cat}/K_M)] = (\eta^{rel}/\eta^o)$exp where "0" designates the absence of added viscosogen and an exponent of 1 corresponds to a diffusion-limited process. We assumed that the effect of viscosity on k_{cat}/K_M will reflect the magnitude of the rate constant from the initial binding relative to the subsequent step up to the release of products. The initial rates as a function of substrate concentration at each viscosogen were fit into:

$$v = \frac{k_{cat}S}{K_M[1 + m(\mu)]}$$

where μ is the relative viscosity $(\eta^{rel})^2$ minus 1 and m is the viscosity effect on k_{cat}/K_M

2.3. Statistical analysis

All kinetic, statistical and graphical analysis for GST catalytic activities was performed using KaleidaGraph 4.5 software (Synergy software, Reading, PA, USA) for Macintosh Computer.

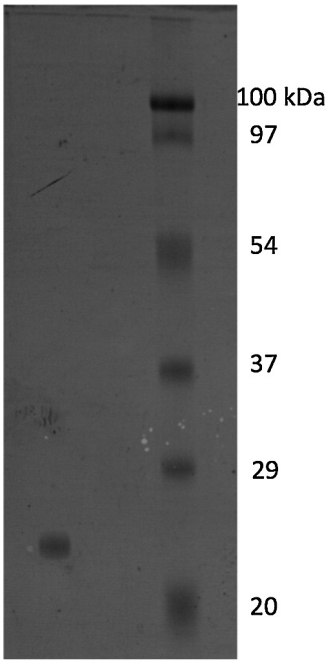

Fig. 1. SDS–PAGE of the purified GST fraction Line 2 is the molecular weight marker and Line 1 is the purified GST (total protein of 0.53 mg) from the SeGST. Analysis was performed on gels containing 12% polyacrylamide. Electrophoresis was performed for about six hours at 65 V at room temperature. Protein bands were visualized by Coomassie brilliant blue R- 250.

3. Results

3.1. se*GST purification*

The use of DEAE trisacryl anion exchange chromatography and GSH-Sepharose 4B^{TM} affinity column appear adequate for the purification of the hepatic silver catfish GST. The specific activity of the pure enzyme was 4.45 μmol/min/mg proteins. It was purified 110 times with 37.4% recovery of the enzyme activity. The enzyme showed a homogenous band under denaturing condition of 12% SDS-PAGE indicating homogeneity and purity with a molecular weight ≈25.6 kDa (Fig. 1). Gel filtration (Sephadex G100) chromatography indicated a relative molecular mass close to 53 kDa for the transferase suggesting a dimeric nature of the quaternary structure of the GST. The appearance of single migrating band during SDS-PAGE is an indication that the enzyme is homodimer of two equal subunits.

3.2. se*GST steady state kinetic*

The plots of the initial velocity versus [GSH] or initial velocity versus [CDNB] in the range of 0.1–1.2 mM GSH and 0.1–1.2 mM CDNB displayed a hyperbolic saturation curve, a Michaelis-Menten kinetics. The initial rate measurements performed over a six by five matrix with regard to the concentrations range (120 data points) gave rise to corresponding families of double reciprocal plot that were linear for both GSH and CDNB and gave a normal intersecting pattern on the third quadrant below the horizontal axis for GSH varied in the presence of several fixed CDNB concentrations (Fig. 2). This is an indication that GSH binding increases the apparent K_M of the enzyme for CDNB [66]. The secondary plots of the gradients and the intercepts of these lines against the reciprocal of the CDNB concentrations were linear (Insets). Similar results were obtained when CDNB was varied in the presence of several fixed concentration of GSH (Fig. 3). The pattern of these non-parallel lines in Lineweaver-Burk plot indicates sequential mechanism of

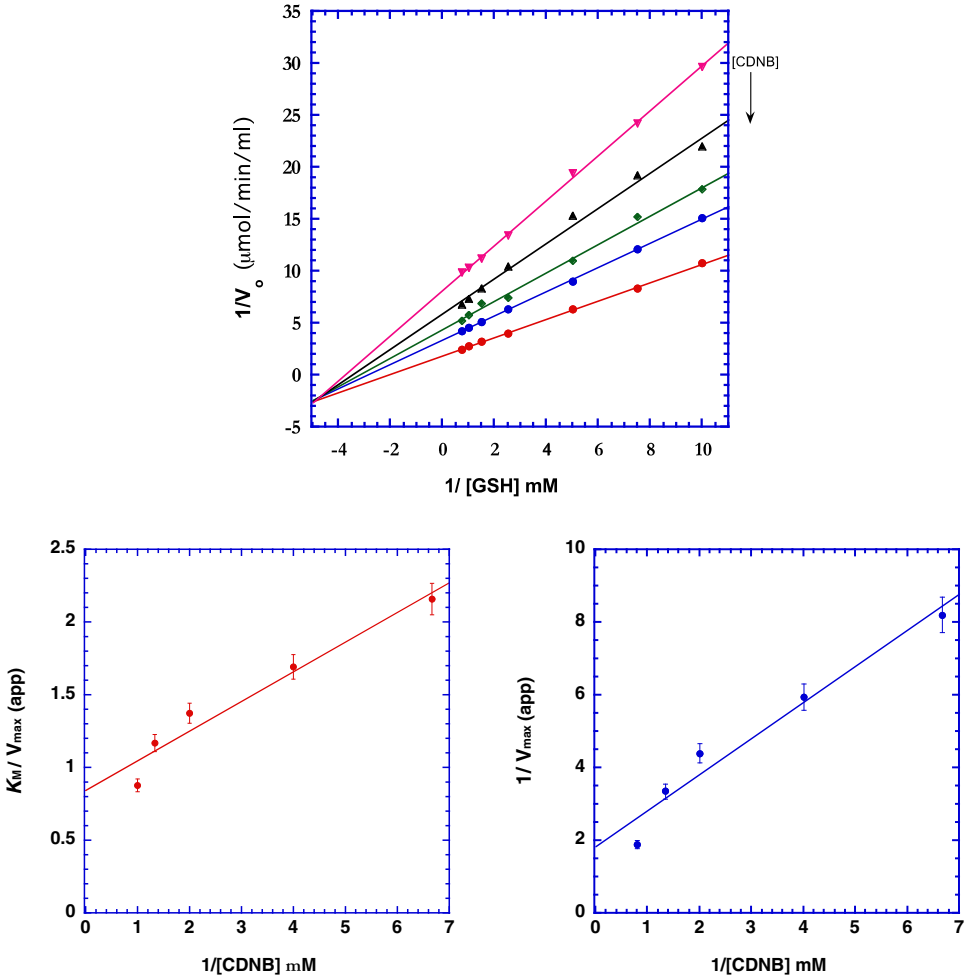

Fig. 2. Initial velocity pattern for the *Se*GST. Different GSH concentrations and fixed concentration of CDNB. The concentrations of CDNB were 0.15 mM, 0.25 mM, 0.50 mM, 0.75 mM, and 1.0 mM. A secondary plot of data from a Lineweaver–Burk plot showing the effect of varying CDNB concentration as ordinate intercept vs. 1/[CDNB].

Table 1
Kinetic constants of *se*GST reactions.

Constant	Enzyme interaction	Value
K_M [GSH] (mM)		0.53 ± 0.05
K_M [CDNB] (mM)		0.69 ± 0.17
k_{cat}[GSH] (s^{-1})		44.62 ± 1.124
K_A	E.GSH + CDNB ⇌ E.GSH.CDNB	0.64 ± 0.04
K_{IA}	E.GSH	0.14 ± 0.01
K_B	E.CDNB	1.41 ± 0.03
K_{IB}	E.CDNB + GSH ⇌ E.CDNB.GSH	0.48 ± 0.02
α^{CDNB} (K_B/K_{IB})		2.96 ± 0.35

The rate of substrate clearance (V_{max}/K_M) for GSH and CDNB are 1.85 (1/min ml) and 0.61 (1/min ml) respectively. Data are shown as means ± SD of at least three measurements.

the enzyme with both substrates adding to the enzyme before products are released. This pattern of convergence does not indicate rapid equilibrium ordered. In a rapid equilibrium ordered system with either GSH or CDBN binding first, the family of lines in the 1/v vs 1/[CDNB] plots (at fixed GSH concentrations) should intersect on the 1/v axis [18]. Kinetic constants determined from these data were summarized in Table 1. Values of K_M for GSH and CDNB were stastically indistinguishable in this study (0.53 ± 0.05 mM and 0.69 ± 0.17 mM, respectively), which may suggest a random mechanism since there is no significant difference on the substrate affinity.

3.3. Product and dead-end inhibition studies

The products of the reaction of GSH and CDNB are s-(2,4-dinitrophenyl)glutathione and chloride ion. The product inhibition studies was not encyclopedic due to our inability to successfully synthesized s-(2,4-dinitrophenyl) glutathione as earlier described elsewhere [76,43]. However, the product and dead-end inhibition studies was characterized by using KCl and methyl glutathione. The inhibition studies obtained at different fixed concentrations Cl-, when the concentrations of GSH and CDNB are varied respectively, the intersection of the double reciprocal plots indicated non-competitive inhibition with respect to GSH and also with respect to CDNB (Fig. 4a). However the possible ionic effect of high concentration of KCl was not rule out. Be that as it may, the lack of inhibition by chloride ions indicates that little specificity exists for the leaving group from the electrophile. From the secondary plot, The $K_{ii} > K_{is}$ Methyl-glutathione gave a competitive pattern for GSH and CDNB (Fig. 4b). The result of the inhibition constants is presented in Table 2. The product inhibition studied with up agree with the rapid equilibrium random sequential bi–bi mechanism. This clearly indicate the Random bi bi kinetic mechanism [26,18].

3.4. Alternate substrates kinetic studies

Kinetic analysis for GSH conjugations to structurally diverse alternate substrates were used to revalidate reaction mechanism

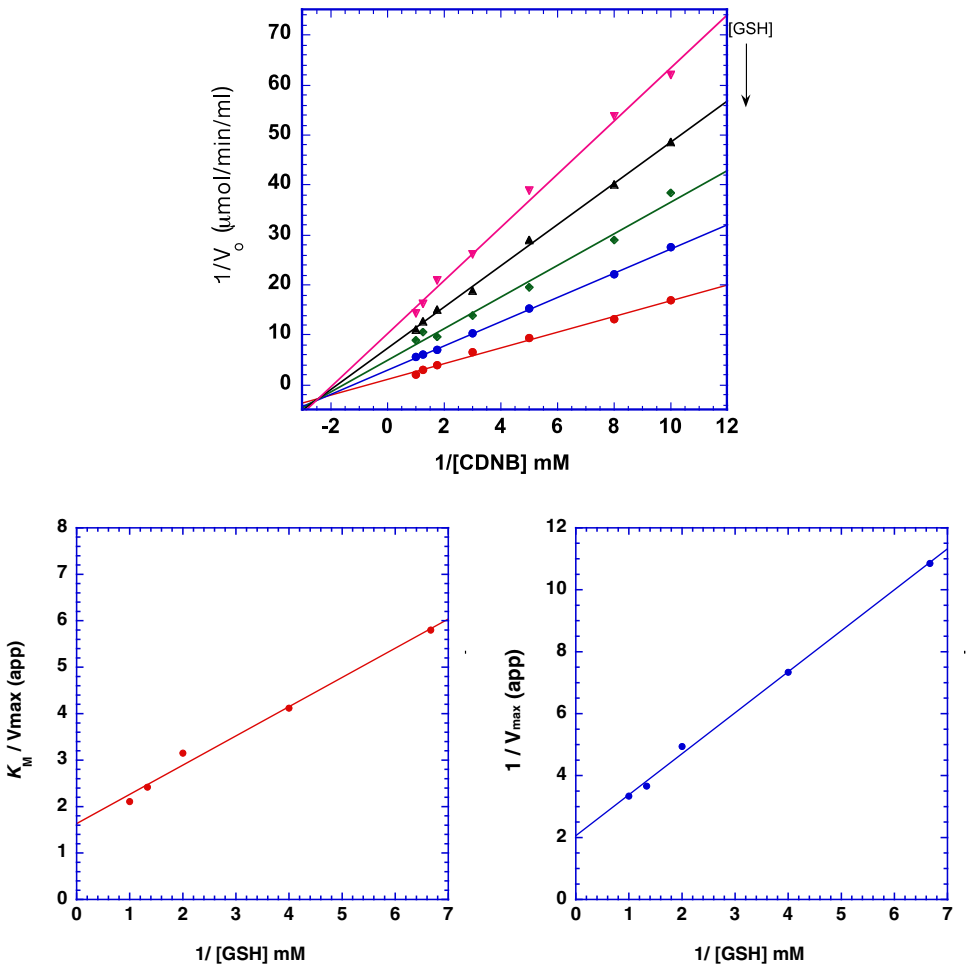

Fig. 3. Initial velocity pattern for the *Se*GST. Different CDNB concentrations and fixed concentration of GSH. The concentrations of GSH were 0.15 mM, 0.25 mM, 0.50 mM, 0.75 mM, and 1.0 mM. A secondary plot of data from a Lineweaver–Burk plot showing the effect of varying GSH concentration as ordinate intercept vs. 1/[GSH].

Table 2
Kinetic constants of the inhibition of *se*GST by KCl and 1 methyl-glutathione.

Constant	Values
K_{ii}^{KCl} (GSH varied)	5.8 µM
K_{is}^{KCl} (GSH varied)	5.6 µM
K_{ii}^{KCl} (CDNB varied)	6.7 µM
K_{is}^{KCl} (CDNB varied)	6.2 µM
$K_{is}^{1-methylglutathione}$ (GSH varied)	0.09 µM
$K_{is}^{1-methylglutathione}$ (CDNB varied)	0.16 µM

or see the changes in kinetic mechanism. The alternate substrates used were ethacrynic acid and *p*-nitrophenyl acetate. When GSH was the variable substrate with several fixed concentrations of ethacrynic acid, a families of intersecting straight line pattern of Lineweaver–Burk plot was obtained (Fig. 5), and with ethacrynic acid as the variable substrate at fixed concentrations of GSH (Fig. 5b), an intersecting pattern was again obtained. The same reaction mechanism remain undistinguishable with the use of *p*-nitrophenylacetate (Fig. 6).

3.5. pH dependence of kinetic parameter

The dependence of log V_{max}/K_M [CDNB] (rate of substrate clearance value) on pH are shown in Fig. 7. In the pH range explored, the profile are bell-shaped gave deducible pKa values of 6.88 and 9.86. Here, the asymptotes were suggested to be Histidine and Tyrosine, respectively [66,19]. These results were similar those reported by [78]. These might be linked to GST being able to lower the pKa of the bound GSH from 9.0 to about 6.5 [12]. The ionizable thiol of the glutathione and phenolic hydroxyl of tyrosine in the GST (for Alpha, Mu and Pi classes) active site were suggested to be vital for catalytic mechanism, ionization and stabilization of GST.GSH binary complex, as well as structural integrity [83,6,78,49]. This is essential for fish detoxification mechansim.

3.6. Substrate specificity

The result from the substrate specificity studies of *sc*GST for a variety of electrophilic compounds is shown in Table 3. Differences of specific activities were obtained when using three benzene substrates: 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole, 1, 2-dichloro-4-nitrobenzene and 1-chloro 2,4-dintrobenzene. The silver catfish enzyme showed maximum preference for 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole followed by 1-chloro 2,4-dintrobenzene *p*-nitrophenylchloride, ethacrynic acid and 1, 2-dichloro-4-nitrobenzene. Ethacrynic acid has specific activity of 3.17 ± 0.01 µmol/min/mg while specific activity of 4-nitrophenylacetate and *p*-nitrophenylchloride are 3.92 ± 0.05 and 1.84 ± 0.04, respectively. Bromosulfophthalein, cumene hydroperoxide and hydrogen peroxide have the lowest specific activity of 0.86 ± 0.07, 0.74 ± 0.04 and 0.71 ± 0.06 µmol/min/mg, respec-

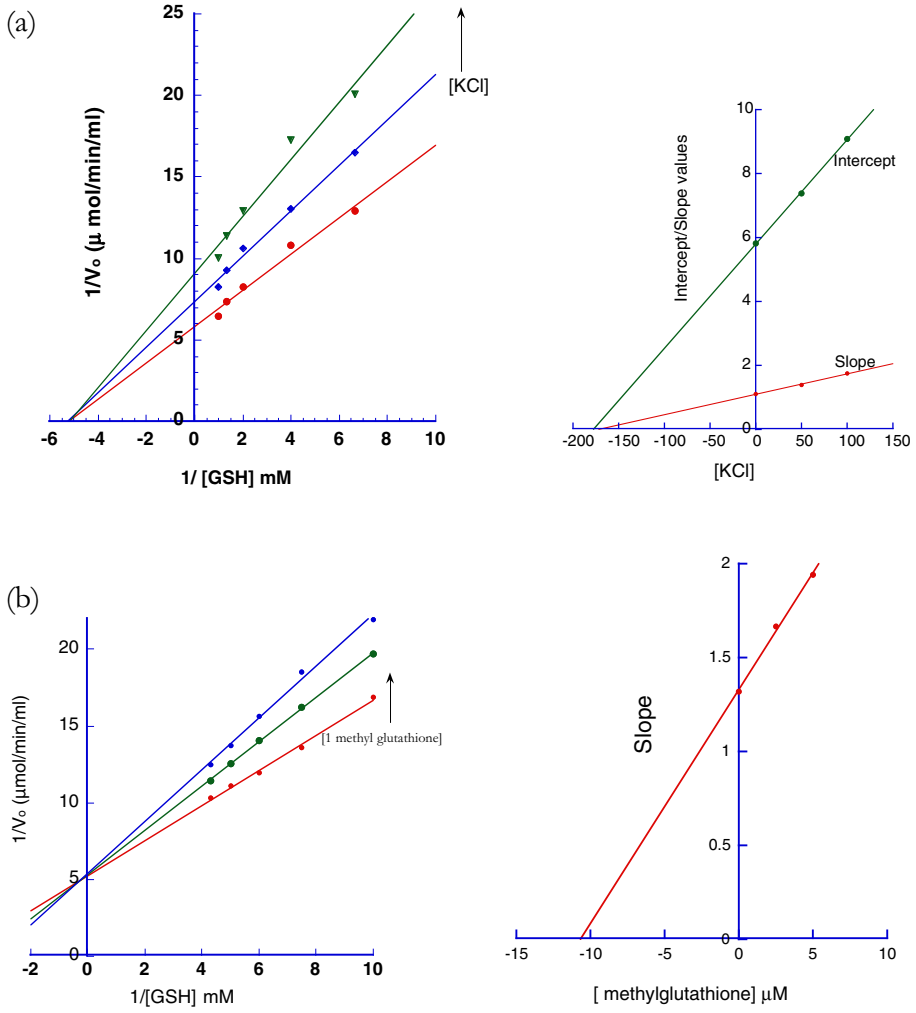

Fig. 4. (a) Double reciprocal plots for inhibition by KCl (0, 50 mM and 100 mM). CDNB concentration was fixed at 1.0 mM. GSH concentration was varied from 0.1 mM–1.0 mM. The inset showed the secondary plot of the slope and intercept of the primary plot versus [KCl] (b) Double reciprocal plots for inhibition by 1 methyl glutathione (0, 2.5 μM and 5 μM). CDNB concentration was fixed at 1.0 mM. GSH concentration was varied from 0.1 mM–1.0 mM. The inset showed the secondary plot of the slope of the primary plot versus [1 methyl glutathione].

Table 3
Substrate specificity of silver catfish.

Substrate	Specific activity (μmol/min/mg)
1-chloro-2,4-dintrobenzene	4.45 ± 0.1
1,2-dichloro-4-nitrobenzene	3.82 ± 0.04
4-Hydroxylnonenal	0.82 ± 0.07
4-nitrophenylacetate	3.92 ± 0.05
7-chloro-4-nitrobenzene-2-oxa-1,3-diazole	4.21 ± 0.09
Cumene hydroperoxide	0.74 ± 0.04
Ethacrynic acid	3.71 ± 0.06
Hydrogen peroxide	0.79 ± 0.07
ρ-nitrophenylchloride	1.84 ± 0.04

Data are shown as means ± SD of at least three measurements.

Table 4
Inhibition effect of some inhibitors on GSH-CDNB conjugation of silver catfish GST.

Inhibitors	IC_{50} (μM)
Bromosulphosptlein	47.43 ± 5.67
Cibracron Blue	0.01 ± 0.001
GSSH	35.24 ± 3.30
Hematin	5.0 ± 0.1
s-hexylglutathione	17.6 ± 1.3
Tributyltin acetate	0.02 ± 0.007
Triphenytin chloride	0.05 ± 0.005
Ethacrynic acid	21.34 ± 3.22
1-methyl glutathione	16.56 ± 1.58

Data are shown as means ± SD of at least three measurements.

tively. The specific activity of silver catfish GST towards 4-HNE (0.82 ± 0.00 μmol/min/mg).

3.7. Inhibition studies

The values for IC_{50} (concentration of inhibitor that gives 50% inhibition of the conjugation of CDNB with GSH catalyzed by the glutathione S-transferase) were determined for *se*GST and a selection of inhibitors (Table 4). Cibracron blue, tributyltin acetate and triphenyltin chloride gave similar inhibitions for the fish GST. GSSH and s-hexylglutathione were weaker inhibitors with respect to the others. Inhibition by bromosulphospthlein was the least with *se*GST.

3.8. Viscosity/Rate-limiting step in the GST catalyzed reaction

Differences between macroscopic and microscopic viscosity in the context of viscosity-dependent kinetics measurements was explored here. The relative $kcat/K_M$ value plotted as a function of the relative viscosity is shown in Fig. 8. The sensitivity of this

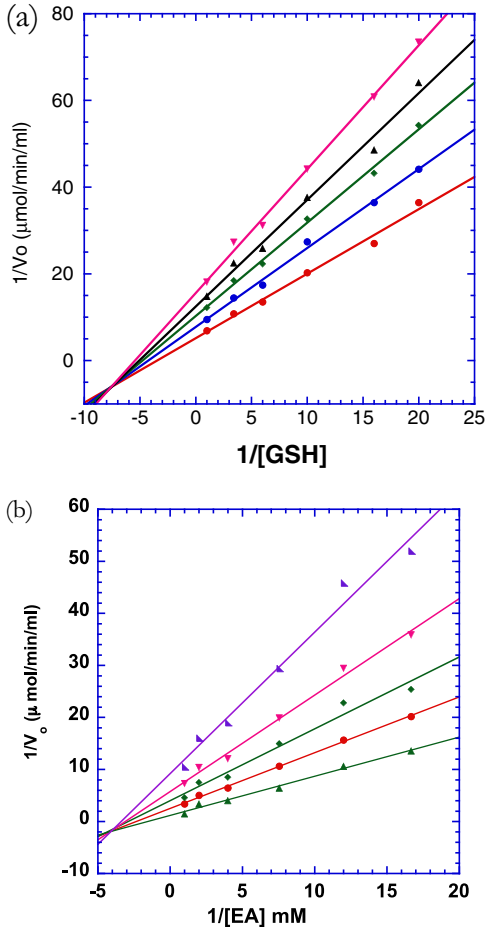

Fig. 5. (a) Double reciprocal plot for the *Se*GST. Different GSH (0–0.25 mM) concentrations and fixed concentration of ethacrynic acid (EA). Assay with ethacrynic acid was carried out at pH 6.5 (100 mM Potassium phosphate buffer) at a concentration of 0.3 mM ethacrynic acid and 0.25 mM GSH. The reaction was monitored at 270 nm. The concentrations of CDNB were 0.05 mM, 0.10 mM, 0.15 mM, 0.20 mM, and 0.30 mM. (b) Double reciprocal plot for the *Se*GST. Different EA concentrations (0–0.30 mM) and fixed concentration of GSH. The concentrations of GSH were 0.05 mM, 0.08 mM, 0.10 mM, 0.15 mM, and 0.20 mM.

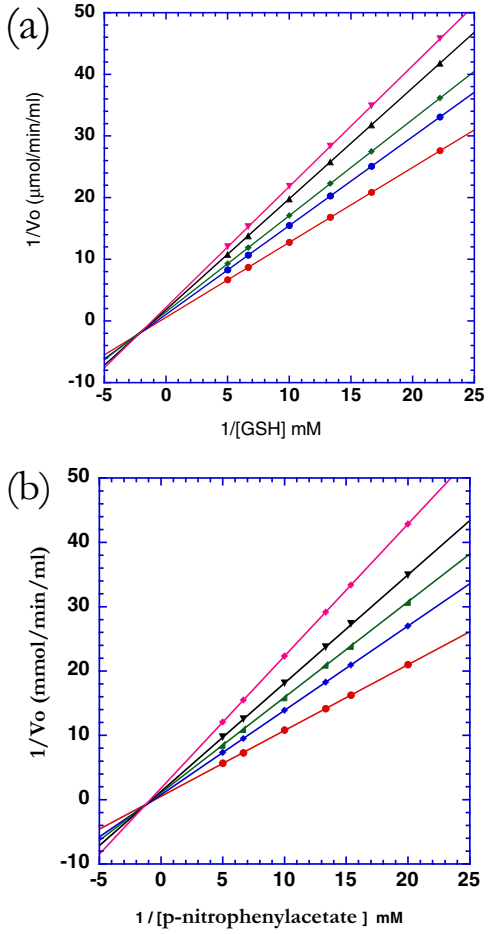

Fig. 6. (a) Double reciprocal plot for the *Se*GST. Different GSH (0–0.5 mM) concentrations and fixed concentration of *p*-nitrophenylacetate. Assay with *p*-nitrophenylacetate was carried out at pH 7.0 (100 mM potassium phosphate buffer) at a concentration of 0.2 mM *p*-nitrophenylacetate and 0.50 mM GSH. The reaction was monitored at 400 nm ($\varepsilon = 8.79\ mM^{-1}\ cm^{-1}$). The concentrations of *p*-nitrophenylacetate were 0.05 mM, 0.06 mM, 0.08 mM, 0.10 mM, 0.15 mM and 0.20 mM. (b) Double reciprocal plot for the *Se*GST. Different *p*-nitrophenylacetate concentrations (0–0.20 mM) and fixed concentration of GSH 0.05 mM, 0.07 mM, 0.08 mM, 0.10 mM 0.15 mM, 0.20 mM.

kinetic parameter to viscosity was calculated from the exponential of such plot. The possible non-Stokesian behaviour of Cl^-, a small molecule and a product of GST reaction, in the viscosogen/water mixtures were accounted for by exponential of 0.5 [38,24]. The second order rate constant ($k$cat/K_M) values decrease with increasing viscosity. The decrease of the rate constant by increasing the medium viscosity reflected the weight of diffusion events on the overall catalysis. The plot of the reciprocal of the relative catalytic efficiency [($k$cat/K_M)^0/($k$cat/ K_M)] as a function of relative viscosity (ηrel/ηo)exp with glycerol, as micro-viscosogen, gave an exponential value of 0.227. While the use of Ficoll 400, as macro-viscosogen, the exponential value was 0.291. The viscosity effect of 0 mean the rate of the reaction is completely independent of solvent viscosity whereas the effect of 1 indicate a completely diffusion–limited event while viscosity effect >1 indicate a conformational change accompanying binding of the substrate [24].

4. Discussion

The result from this study has showed the existence of Silver catfish hepatic glutathione transferase. The GSH affinity bound fractions were used in this study for kinetic characterization. It has been reported that some GST do not bind to GSH affinity matrix

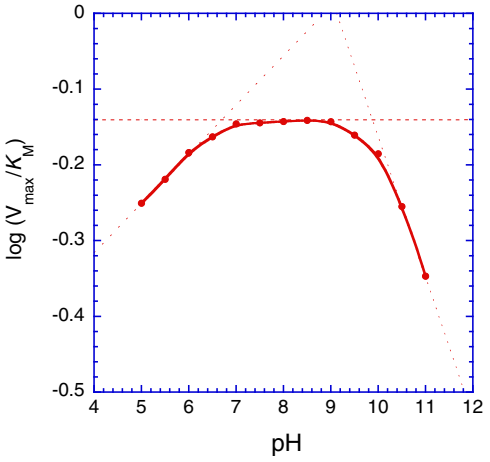

Fig. 7. Plot of log V_{max}/K_M [CDNB] versus the pH using 0.1 M MES (from pH 5.0–6.5), 0.1 M PIPES (from pH6. 5–8.0) and 0.1 M MOPS (from pH 8.5–11.0) The rates of all measured enzymatic reactions were corrected for the corresponding non-enzymatic reactions. triplicate determinations of initial velocity were made at each CDNB concentration.

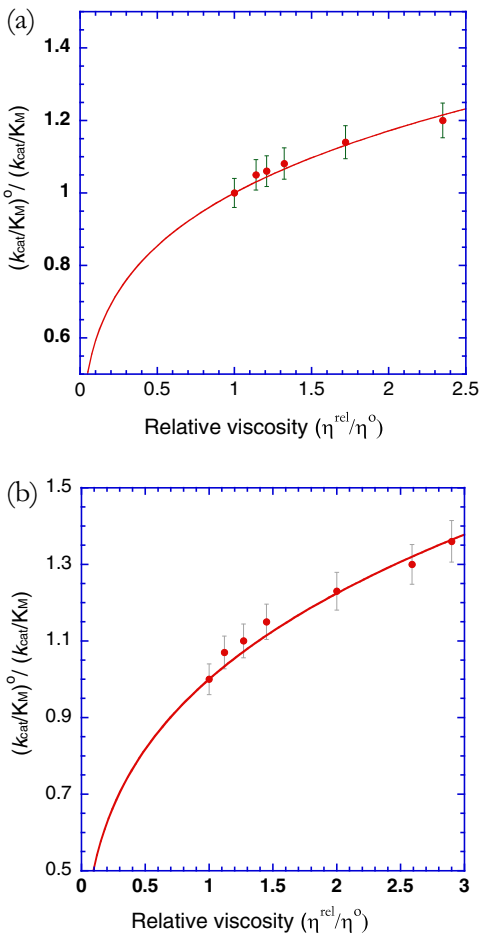

Fig. 8. Effect of viscosity on relative second order rater constant k_{cat}/K_M of seGST. (a) Glycerol was used as micro-viscosogen glycerol (b) Ficoll 400 was used as macro-viscosogen. The second order rate constant data were obtained as reported under Methodology. Experiments were performed in triplicate, and S.E. for each point does not exceed 7%.

except Alpha, Mu and Pi classes [14]. This bound fraction characterized could be either of the classes. The unbound fraction eluted from the affinity column has higher protein content and very low GST activity compared to the affinity bound fraction. The GST was purified to apparent homogeneity allowing for enzymological characterization and also underscores the efficiency of the purification procedure in contrast to the low efficiency of the purification procedure adopted by [11]. An interesting aspect of the GSTs isolated from mammals and invertebrates is the large number of isoezymes capable of conjugating GSH with a variety of electrophiles. A subunit molecular weight of approximately 25.6 kDa was observed for the affinity-bound *se*GST fraction. This value is not invariant to 25.2 kDa grey mullet (*Mugil cephalus*) liver GST, 25 kDa rainbow trout GST [62] and 25.5 kDa–27 kDa Plaice liver heterodimmer GST [33]. Gel filtration showed that the GST is homodimeric with a molecular weight of 53 kDa. This was not different for those of other mammalian, plant and microbes earlier reported; all of which were dimer with a molecular weight of 40–60 kDa [5,72]. The Silver catfish (*Synodontis eupterus*) hepatic GST is dimeric.

The kinetic mechanisms of the GSTs isoenzyme have received a good deal of attention. The bi-reactant kinetic mechanism has been, most often than not, random sequential type that could either be random, rapid-equilibrium model or random, steady-state model. The kinetic mechanism obtained from this study is in consonant with a sequential mechanism. This sequential mechanism is not unusual. It agrees with available kinetic mechanism so far reported for aquatic animals [70,54,3]. Till date, there has been no report on the possible Ping-Pong mechanism of GST; however, [17] broached over the possibility of Ping-Pong mechanism at lower concentration of <0.1 mM GSH. Since the point of intersection is below the horizontal axis, the binding of GSH increases the apparent K_M of the CDNB, and vice versa [66]. The K_M values of GSH (0.53 ± 0.05 mM) and CDNB (0.69 ± 0.17 mM) statistically showed very close substrate affinity. This gave a quantitative and qualitative evidence for the reaction being random sequential mechanism. The K_A/K_{IA} is less than unity, the binding of GSH increases the affinity of the enzyme for the CDNB and vice versa. It also demonstrate that *se*GST is sensitive to slight concentration changes with electrophilic substrate and GSH. These K_M values obtained both for GSH and CDNB are higher than those reported for non-vertebrate organisms [14]. This is in agreement with *L. vannamei* K_M values [85]. The turnover number, k_{cat}, and the catalytic efficiency, k_{cat}/K_M, for *se*GST obtained are in good agreement with the data previously reported for other aquatic animals [8,41,2]. This is consistent with the broad specificity of GSTs, resulting in a variety of leaving groups [72].

The inhibition of *se*GST by Cl-also suggests the formation of an E-GSH-Cl-dead end complex, although the possibility that the high concentration of KCl eliciting an ionic-strength effect on the kinetics was not ignored. The result from the inhibition pattern of KCl and 1 methyl glutathione showed that the reaction mechanism is random sequential Bi–Bi kinetic mechanism rather than steady state kinetic mechanism. It has been noted that steady state random Bi Bi mechanism leads to non-linearity in the reciprocal plot [76]. The ratio of K_{IA} and K_A to K_{IB} and K_B is statistically close give credence to the reaction being a rapid equilibrium. α which describes the influence of the CDNB on the binding of GSH is 2.98 ± 0.35. This value is slightly higher than human placenta of 2.1 [43] and sorghum of 1.8 [35] that were reported to be of rapid equilibrium random sequential bi bi mechanism. We assumed that change in the hydrophobic substrate would change the rearrangement of the active site residues, resulting in changes in the topology of the active site pocket and consequently affect the kinetic mechanism. The use of other electrophilic substrates (ρ-nitrophenylacetate and ethacrynic acid) did not change the pattern. HNE a specific substrate for Pi class and 4-nitrophenylacetate were used another non-specific class second substrate. Very low specific activity of the *se*GST to cumene hyroperoxide could not allow us to it as alternate hydrophobic alternate substrate. There were not different from the use of CDNB as a second substrate. The fact that the common intersection point lies the beyond the abscissa and in the third quadrant indicates that the subunits are catalytically equivalent are catalytically indistinguishable from the use of CDNB as second substrate. These results of the kinetic constant derived from the secondary plots substantiate this. This clearly suggests that the isoform could be Pi isoenzyme. This showed that non-independent binding site for the two substrates. However, steady state is generally considered more efficient than rapid equilibrium owing to smaller values of K_{cat} of the rapid equilibrium [61]. Pi class GST follow a rapid equilibrium random Bi–Bi kinetic mechanism. The physiological implication of rapid equilibrium is still a mystery over a more effective steady state. It might be plausible to assume that the functionality of the enzyme is more of "house keeping". There could be a possibility of the reaction mechanism moving to a more efficient steady state mechanism from the rapid equilibrium if the detoxification becomes overwhelming. Pi class GST has been characterized from the liver of Salmonid [86].

Substrate specificity determination showed differences the interaction of the enzyme with several hydrophobic substrates [81]. Hepatic *Se*GST substrate specificity pattern is not analogous. The apparent low substrate specificity to cumene hydroperoxide and hydrogen peroxide indicates it has inefficient peroxidase activity. There have been an increasing number of reports of fish

GSTs exhibiting low or no GSH peroxidase activity [87]. The results from this study add to this. This GST dependent low peroxidase value might not be connected to the protection of fish polyunsaturated fatty acid (PUFA) content against oxidative stress [34]. It also indicates that Silver catfish hepatic GST would be able to detoxify hydroperoxides derivatives lipids in-vivo as shown from the result from 4-hydroxylnonenal. *Se*GST exhibited high activity with ethacrynic acid, a phenylacetic acid derivative that is generated in mammals under oxidative stress [88,89]. High activity with ethacrynic acid is characteristic of mammalian pi-GSTs Mannervik and Danielson, 1988 and low peroxidase activity [55]. Mu class GST is generally associated with high peroxidase activity. These overwhelmingly showed that the hepatic *se*GST might not be Mu class GST but Pi class GST. This substrate specificity result of this *se*GST is quite distinct from Plaice GST that was earlier reported to show no detectable peroxidase activity and very low activity towards ethacrynic acid and 4-nitrobenzylchloride [33]. Inhibition studies can be very revealing [76]. Inhibition of the various isoenzymes by inhibitors has been employed in distinguishing various isoenzymes of GST from humans, mouse and rat [75]. They found that cibracron blue was most effective on the neutral isoenzyme from human liver whereas tributytin acetate was the most potent inhibitor of the basic human transferase. The IC_{50} values obtained in this study suggest that glutathione transferase from *Synodontis eupterus* might be a near neutral isoenzyme. Hematin is a less effective inhibitor of *Synodontis eupterus* GST. Hematin, a non substrate ligand for the enzyme strongly inhibited the enzymatic activity and its IC_{50} value for the enzyme was $5.0 \pm 0.100(\mu M)$. Hematin is also less effective on Plaice GST [33]. Studies have shown that diuretics ethacrynic acid (ECA) is a good substrate and good inhibitor for certain GSTs [22]. It has been used as a selective marker for Pi class GSTs, although certain other GSTs also exhibit relatively ethacrynic acid activity. The result of the present study has shown that ECA is a good substrate but a poor inhibitor for the silver catfish hepatic GST. Cibracron blue and tributyltin acetate as well as triphenyltin chloride are the most potent inhibitors. Bromosulphophthalein, a Mu class substrate, use as an inhibitor in this study, has the lowest IC_{50}. A viscosity dependence for $k\text{cat}/K_M$ was observed suggesting that substrate binding and subsequent steps up to product release could be diffusion controlled in *se*GST catalysis. The results show that the rate limiting is more of chemical step not physical as posited by [77]. The physical event is the conformational change of the ternary complex and the product release [48]. The exponential value of glycerol indicates a catalytic efficiency of 23% (0.227). Some authors [48,90,77,16] had reported that GST product release is rate-limiting step in the overall catalytic efficiency of GST catalysis. Several pieces of evidence indicate that GSTs utilize structural flexibility for attainment efficient catalysis [13,48]. We assume that the situation would be more complex when macro viscosogen is used. There was no distinction. The macro-viscosogen (Ficoll 400) gave a catalytic efficiency of 29%.

5. Conclusion

The hepatic tissue of silver catfish (*Synodontis eupterus)* obtained from not obvious polluted environment has well-expressed GST activity toward some prototypical GST substrates. This study has illuminate the role of GST in the fish and the mechanism it uses in detoxification *sc*GST might belong to the class Pi- isoenzyme, possibly a "house keeping" detoxification enzyme. The isoenzyme was very sensitive to organotin compounds. silver catfish would adapt relative to environmental xenobiotics to which it may be exposed in its ecosystem. GST as a possible tool for the bio-monitoring of polluted aquatic environment is suggested. However, the dynamics of the kinetic mechanism still remains to be unraveled.

Acknowledgments

This author thanks Akpos Iteyere Dennis for some helpful contribution, remarks and assistance during this research work. This work is partly supported by the University Research Committee (URC) of the Senate of The Federal University of Technology, Akure.

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