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Molecular cloning and characterization of a glutathione *S*-transferase from largemouth bass (*Micropterus salmoides*) liver that is involved in the detoxification of 4-hydroxynonenal

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Abstract

We are currently investigating the role of detoxification pathways in protecting against the sublethal effects of chemicals in largemouth bass (*Micropterus salmoides*). To this end, previous work in our laboratory indicated a remarkable ability of bass liver glutathione *S*-transferases (GSTs) to detoxify 4-hydroxynonenal (4HNE), a common mutagenic and cytotoxic α,β-unsaturated aldehyde produced during the peroxidation of lipids. In the current study, we observed that GST-mediated 4HNE conjugation in bass liver follows high efficiency single-enzyme Michaelis–Menten kinetics, suggesting that an individual GST isoform is involved in 4HNE detoxification. Using 5′ and 3′ rapid amplification of cDNA ends (RACE), a full-length GST cDNA of 957 base pairs (bp) in length, containing an open reading frame of 678 bp and encoding a polypeptide of 225 amino acids, has been cloned. Interestingly, a search of the BLAST protein database revealed the presence of homologous GST proteins in the plaice (*Pleuronectes platessa*), European flounder (*Platichthys flesus*) and fathead minnow (*Pimephales promelas*), but not in other fish species. Furthermore, the bass GST protein exhibited little homology with the mammalian GSTA4 subclass of proteins which rapidly metabolize 4HNE. The recombinant 6x*His*-tagged expressed GST protein showed high catalytic activity towards 4HNE, while showing moderate or low activity toward other class specific GST substrates. HPLC-GST subunit analysis, followed by sequencing, demonstrated that the isolated bass liver GST subunit constitutes the major GST protein in bass liver, with a molecular mass of 26.4 kDa. In summary, the presence of a highly expressed GST isozyme in bass and several evolutionarily divergent fish species indicates the conservation of an important and distinct detoxification protein that protects against oxidative damage in certain aquatic organisms.

Keywords: Enzyme kinetics; Glutathione *S*-transferase; cDNA cloning; Liver; 4-Hydroxynonenal

1. Introduction

The glutathione *S*-transferases (GSTs) are a multigene family of dimeric enzymes that are ubiquitously distributed and comprise approximately 2–4% of total cytosolic proteins [1]. The GSTs are currently grouped into eight classes (alpha, mu, pi, theta, sigma, omega, kappa, and zeta) based on protein sequence homology [2]. The range of compounds detoxified by GSTs is remarkably diverse and includes a number of exogenous xenobiotic carcinogens, anticancer drugs, and environmental chemicals. Most of the enzymatic reactions catalyzed by GSTs involve the conjugation of toxic electrophiles with the tripeptide glutathione [3]. Because a number of the compounds detoxified by GSTs are mutagenic, the level of GST isozyme expression can be an important determinant of sensitivity to carcinogenesis [4].

In addition to their protective activities toward electrophilic chemicals, certain GST isozymes can catalyze the reduction of cellular peroxides to their corresponding alcohols, as well as conjugate endogenous genotoxic unsaturated aldehydes formed during the peroxidation of membrane lipids [2]. Accordingly, the GST pathway can

Abbreviations: ADI, Δ5-androstene-3,17-dione; BME, β-mercaptoethanol; CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; ECA, ethacrynic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; GST, glutathione *S*-transferase; 4HNE, 4-hydroxynonenal; NBC, nitrobutyl chloride; RACE, rapid amplification of cDNA ends; TFA, trifuoroacetic acid

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thus be viewed as an integral component of the cellular antioxidant defense system. Of the reactive intermediates produced during oxidative stress, 4-hydroxynonenal (4HNE) is a reactive α,β-unsaturated aldehyde that is generated during lipid peroxidation [5] as a result of the degradation of ω-6 polyunsaturated fatty acids. Because of its high reactivity, 4HNE rapidly forms covalent adducts with biomolecules containing nucleophilic sites, such as sulphydryl groups of glutathione, cysteine, lysine, and histidine residues of proteins, and nucleophilic sites of nucleic acids. In rodents and humans, the α class GSTA4 subclass displays uniquely high catalytic activity towards 4HNE and other α,β-unsaturated aldehydes [6–8], suggesting that these enzymes may have distinctively evolved as a secondary line of defense against oxidative injury [8].

Because fish tissues have high levels of polyunsaturated fatty acids, protection against breakdown products of lipid peroxidation may greatly influence their survival and adaptation. Proteins related to mammalian alpha, pi, and theta GSTs have been described in various fish species [9]. Interestingly, studies with the marine fish plaice (*Pleuronectes platessa*), have revealed the presence of a GST enzyme (termed GST-A by George et al., but devoid of relation to the alpha class GSTs) that is a relatively efficient catalyst for the conjugation of a series of unsaturated alkenals and hydroxyalkenals, including 4HNE, but displaying little or no activity toward model substrates for mammalian GST [10]. We had previously demonstrated the presence of a bass liver GST that appeared to share extensive sequence identity to the aforementioned 4HNE-metabolizing GST-A isolated from plaice [11]. Subsequent studies from our laboratory have also shown that largemouth bass hepatic cytosolic fractions have a remarkable ability to detoxify 4HNE, and specifically at rates that exceed those of several mammalian and aquatic species [12]. In continuing with our efforts to elucidate the role of detoxification pathways in protection against chemical injury in bass, the present study was conducted to characterize the GST isoform responsible for 4HNE conjugation in bass liver. Our results suggest that a major GST form in bass liver, homologous to GST proteins in several other fish, but not in mammals, is involved in the detoxification of 4HNE, and represents a conserved protective mechanism against oxidative damage in aquatic species.

2. Experimental

2.1. Materials

All custom designed primers, platinum *Taq* polymerase, and T4 DNA ligase were obtained from Invitrogen. *Bam*H I and *Hin*d III were purchased from New England Biolabs. Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Promega. Synergel was purchased from Diversified Biotech. Agarose was obtained from Bio-Rad. Anti-rat GST Ya (rGSTA1-1; alpha class GST) and anti-rat GST Yb (rGSTM1-1; mu class GST) were purchased from Oxford Biomedical Research. Anti-rat GSTT1 (rGSTT1-1; theta class GST) was generously donated by Dr. John Hayes, University of Dundee. Anti-rat GSTP1 (rGSTP1-1; pi class GST) was a generous gift from Dr. Theo Bammler from University of Washington. Δ5-Androstene-3,17-dione (ADI) was purchased from Steraloids and 4-hydroxynonenal from Cayman Chemical. All other chemicals were obtained from Sigma–Aldrich or Fisher Scientific and were of reagent grade quality or better.

2.2. Animals, RNA isolation, and subcellular fractions

Aquacultured juvenile largemouth bass (less than 1 year of age, 200–300 g) was purchased from American Sportfish Hatchery, Montgomery, AL. Upon arrival, fish were maintained in a 1760-gallon flow-through tank receiving dechlorinated water and acclimated for 1 month at a constant temperature of 28 ± 1 °C. Livers were excised, rinsed in ice-cold PBS, and frozen in liquid nitrogen. Total RNA was isolated from liver by the method of Chomczynski and Sacchi [13], using the Trizol reagent (Invitrogen). Liver subcellular fractions of bass and a male Sprague–Dawley rat and were prepared by differential centrifugation as previously described elsewhere [14], and stored at –80 °C.

2.3. Cloning of the full-length GST cDNA

5′ and 3′ RACE systems for rapid amplification of cDNA ends (Invitrogen) were used for completing the 5′ and 3′ ends of a previously isolated 471 base pair (bp) sequence (bass-471) of a GST cDNA from bass liver [11]. Gene-specific primers for 5′ and 3′ RACE were custom designed based on the partial bass-471 sequence. First-strand cDNA was synthesized from 1 to 5 μg of bass liver total RNA using the antisense gene-specific primer 5′-GTATCCC-TCCCACAGCTTGAC-3′ with SuperScript II Rnase H^- reverse transcriptase in a 25 μl reaction. The cDNA (10 μl) was 3′ tailed with a poly$(C)^+$ tail using recombinant terminal deoxynucleotidyl transferase. A 5-μl aliquot of the 25 μl of tailed cDNA was directly used in a PCR reaction containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 400 nM of each primer, and platinum *Taq* DNA polymerase (50 μl reaction). PCR reactions consisted of 30 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, with the provided sense deoxyinosine containing anchor primer and the antisense gene-specific primer 5′-TCAGCACTCAGGAC-GTCTCT-3′.

For 3′ RACE, first-strand cDNA was synthesized from 1 to 5 μg of bass liver total RNA using an oligo(dT)-containing adapter primer provided with the kit using SuperScript II RNase H^- reverse transcriptase in a 20 μl reaction. A 2-μl aliquot of cDNA was then subjected to PCR for 30 cycles, under similar conditions used for 5′ RACE. The

only difference was that 200 nM of the sense gene-specific primer 5′-GGCTCTTTCCTGGCAGG-3′ and an antisense universal amplification primer (UAP) provided with the kit were used. The primary PCR product was re-amplified using the nested sense gene-specific primer 5′-TTCTCCCTGG-CTGATGTG-3′ and the UAP. PCR products were separated on a 2% synergel/agarose gel, purified using the Bio-Rad Prep-A-Gene kit, and sequenced at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) DNA Sequencing Core, Gainesville, FL.

In order to obtain the full-length GST, custom designed primers were deduced from the full-length 957 bp bass sequence predicted from RACE-PCR; the forward primer 5′-GGATCCATGGCTAAGGACATGACT-3′, contained an additional *Bam*H I restriction site, and the reverse 5′-AAGCTTGGTGAGAGCAGTGCAATG-3′ contained a *Hin*d III restriction site. The resulting PCR products were electrophoretically separated, purified and sequenced as previously described. The final PCR product containing the full-length bass GST cDNA was then subcloned into a pGEM-T Easy Vector (Promega).

2.4. Expression of the recombinant GST protein

The GST-containing pGEM T-easy plasmid and the expression vector pQE-30 (Qiagen) were double digested using *Bam*H I and *Hin*d III and directionally ligated using T4 DNA ligase following standard protocols [15]. Competent *Escherichia coli* strain M15[pREP4] (Qiagen) were prepared and transformed with the ligation mixture. Individual colonies were grown and expression of the 6x*His* tag recombinant protein was induced by 1 mM IPTG. Cells were harvested by centrifugation at 4000 × *g* for 15 min and stored at −80 °C until use. Harboring of the expression plasmid by bacterial colonies was confirmed by restriction digestion analysis of an aliquot of the culture. Upon thawing, cells were lyzed on ice in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 1 mM β-mercaptoethanol, 1 mg/ml lysozyme), containing 10 mM imidazole, for 30 min. Following incubation, cells were sonicated six times for 10 s with 5 s pauses in between, followed by centrifugation at 10,000 × *g* for 25 min at 4 °C. The supernatant was then loaded onto a Ni-NTA silica spin column (Qiagen), and centrifuged at 700 × *g* for 3 min. The columns were washed twice with buffer A containing 20 mM of imidazole, and 6x*His*-tagged protein eluted from the column using a higher concentration buffer (buffer A containing 250 mM imidazole), followed by a three-fold dilution with 50 mM Tris–HCl, pH 7.6, 1 mM BME buffer. The purified proteins were immediately used in enzymatic assays or stored at −20 °C prior to Western blot or HPLC analysis.

2.5. GSH-affinity purification of hepatic GST

GSH-affinity purification of bass hepatic cytosol was accomplished by using a micro-spin column and GSH Sepharose 4B matrix (Amersham Pharmacia Biotech). Approximately 300 μl of soluble protein was adjusted to 5 mM of dithiothreitol and 1 mM phenylmethylsulfonyl-fluoride (PMSF) and incubated at room temperature for 15 min. The eluate was spun at low speed (100 × *g*) for 30 s and washed with 500 μl of cold PBS. After the PBS wash, the cytosol was eluted with GSH elution buffer (50 mM Tris–HCl, 150 mM GSH, 1.4 mM BME, pH 9.6) and incubated at room temperature for 15 min. The affinity purified bass cytosol was dialyzed in PBS for 2 days to remove excess GSH and stored at −80 °C until further analysis.

2.6. Enzymatic and immunological analysis of the recombinant GST protein

Recombinant bass liver GST (*rec*GST) catalytic activities toward CDNB, CuOOH, ADI, DCNB, ECA, and NBC were assayed using a 96-well microplate reader as described in Gallagher et al. [16]. GST-4HNE activity was determined by the spectrophotometric method of Alin et al. [17]. The *rec*GST was examined for cross-reactivity toward class-specific rat GST antibodies using Western blot analysis according to Gallagher et al. [18]. Rat liver cytosol was used as a positive control for western analyses, and affinity purified bass liver cytosol was included for comparative purposes where appropriate.

2.7. Analysis of 4HNE conjugation in liver cytosol

Michaelis–Menten enzyme parameters (K_m and V_{max}) for GST-4HNE conjugation were determined in a separate experiment using GSH-affinity purified bass liver cytosol and a broad range of electrophilic substrate concentrations (4HNE, 0.006, 0.12, 0.24, 0.48, 0.96, 0.196, and 0.392 mM) at a constant nucleophile concentration (GSH, 0.5 mM). Michaelis parameters for GST-4HNE catalysis were obtained using non-linear regression analysis and proportional (1/*y*) weighting with Sigma plot enzyme kinetics software (SPP Inc.). Velocity (*V*) versus substrate concentration (*S*) and Eadie–Hofstee plots (*V* versus *V*/*S*) were calculated using Sigma Plot enzyme kinetics software (SPP Inc.).

2.8. Tissue-specific expression of GST

First-strand cDNA was synthesized from total RNA from bass liver, heart, gonad, upper GI tract, lower GI tract, brain, and muscle tissue using the Retroscript kit (Ambion) following the manufacturer's protocol. Multiplexed PCR (non-quantitative) was accomplished using primers custom designed to amplify partial cDNAs from bass liver GAPDH (475 bp) and also a 776 bp fragment of the bass GST liver. The GAPDH sequence was amplified with primers 5′-CGCATCGGTCGTCTGGT-3′ and 5′-AATGATGCCGA-AGTTGT-3′. The primers used for amplification of the 776 bp of the bass GST were 5′-GGATCCATGGCTAAG-

GACATGACT-3′ and 5′-AAGCTTGGTGAGAGCAGTG-CAATG-3′. Each multiplex PCR reaction included total first-strand cDNA from the aforementioned tissues, 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 100 ng of GAPDH and GST primers. After a 5-min incubation at room temperature, *Taq* DNA polymerase was added to each reaction. Multiplex PCR was carried for 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C; and 5 min at 72 °C. PCR products were separated on a 2% agarose gel and visualized on the Bio-Rad Fluor-S imaging system.

2.9. HPLC analysis of GST subunits, protein sequencing, and HPLC-electrospray mass spectometry

Reverse-phase HPLC was used to characterize bass cytosolic GST subunits from an affinity purified GST preparation, using a 150 mm × 4.6 mm Vydac 214TP C4 column (Grace Vydac) equilibrated with 37% (v/v) acetonitrile in water. The column flow rate was 1.5 ml/min with 37–43% (v/v) gradient of acetonitrile containing 0.075% TFA over 25 min, followed by a 43–55% (v/v) gradient of acetonitrile and 0.075% TFA over 20 min. The polypeptide peaks were detected by UV absorption at 214 nm. The peaks were collected and dried under vacuum to remove the TFA, subjected to Endo-proteinase Lys-C digestion, and sequenced using Edman degradation. The molecular masses of the collected peaks were determined by HPLC mass spectrometry (HPLC-MS) with electrospray-ionization mass spectrometry (ESI-MS). The GST protein (approximately 60 μg) was dissolved in equal volume of water containing 0.075% TFA and injected at a rate of 1.5 ml/min into the ESI ion source. Positive ion ESI-mass spectra were acquired using a ThermoFinnigan LCQ Classic ion trap mass spectrometer. The ESI source was operated at 4.2 kV with the heated capillary at 220 °C and a relative nitrogen flow of 80%. Spectra were scanned from *m/z* 200–2000 and deconvoluted using ThermoFinnigan Navigator 1.2 software.

3. Results

3.1. Cloning of the full-length GST sequence and sequence similarity to other fish and rodent GSTs

A full-length GST cDNA clone was isolated from bass liver by RACE, based on a previously isolated partial GST

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      GGACTCTTCACTTCTTCCACCATTCACAACTTCATCTGCTTTCAAGCGTAGCAGTTCACTCCATAACC

+1    ATGGCTAAGGACATGACTCTGCTGTGGGGCTCCGGCTCTCCCCCCTGCTGGAGGGTGCAGATCGCTCTGG
      M  A  K  D  M  T  L  L  W  G  S  G  S  P  P  C  W  R  V  Q  I  A  L  E  24

71    AGGAGAAGAGCCTGCAGGGCTACAACCAAAAACTGCTCCGCTTTGATAAAATGGAGCACAAGTCACAGGA
        E  K  S  L  Q  G  Y  N  Q  K  L  L  R  F  D  K  M  E  H  K  S  Q  E   47

141   AGTGATGGACATGAATCCCAGGGGTCAGCTCCCTGCCTTCAAACATGGAAACAATGTCCTGAATGAGTCC
       V  M  D  M  N  P  R  G  Q  L  P  A  F  K  H  G  N  N  V  L  N  E  S    70

211   TATGCTGCCTGCTTGTACCTGGAGAGTGAGTTCAAGTCCCAGGGAAACAAACTGATCCCCGACTGCTCGG
      Y  A  A  C  L  Y  L  E  S  E  F  K  S  Q  G  N  K  L  I  P  D  C  S  A  94

281   CTGAGAAAGCACTGATGTACCAGCGCATGTTTGAGGGTCTCACGCTCAACCAGAAAATGGCTGATGTTAT
        E  K  A  L  M  Y  Q  R  M  F  E  G  L  T  L  N  Q  K  M  A  D  V  I  117

351   ATACTACAACTGGAAGGTCCCTGAGGGAGAGAGACACGACTCTGCTGTGAAGAGAAACAGAGACGTCCTG
       Y  Y  N  W  K  V  P  E  G  E  R  H  D  S  A  V  K  R  N  R  D  V  L   140

421   AGTGCTGAGGTCAAGCTGTGGGAGGGATACCTGCAGAAGGCATCAGGCTCTTTCTTTGCAGGAAAGAACT
      S  A  E  V  K  L  W  E  G  Y  L  Q  K  A  S  G  S  F  F  A  G  K  N  F 164

491   TTTCGCTGGCTGATGTGACGGTTTATCCATCTATCGCTTATCTCTTCCATTTTGGGTTGTGTGAAGAGCG
        S  L  A  D  V  T  V  Y  P  S  I  A  Y  L  F  H  F  G  L  C  E  E  R  187

561   TTACCCTAAACTGGCAGCTTACTATAACTCCAATAAGGACAGACCCAGCATCAAAGCCACATGGCCTCCT
       Y  P  K  L  A  A  Y  Y  N  S  N  K  D  R  P  S  I  K  A  T  W  P  P   210

631   ACCTGGCTGGAGAACCCACAGGGACAAGACCAACTGAAAGACATTTGAGATTAAAACACATTTCAGCAGA
      T  W  L  E  N  P  Q  G  Q  D  Q  L  K  D  I  *                        225

701   CACACTAACTGTGAACTGTTGTGTAATATGTAACCACTCCTTGCATTGCACTGCTCTCACCGTGTGTTTC
771   ACAAAATATGCATTTGCTGTTACTGTGGTTGTTAATTTGTTTGTGACTTAAACTGCAGATTAATCTTTTT
841   GTCTTGTAAAAAGCAATAAATGTGACTTTCAGCTTTGAAAAAAAAAAAA
```

Fig. 1. Nucleotide sequence and deduced amino acid sequences of the largemouth bass glutathione *S*-transferase (GST). The bass GST cDNA is 957 base pairs (bp) in length, containing an open reading frame of 678 bp, encoding a polypeptide of 225 amino acids. The ATG start codon is underlined and the TGA translation stop codon is marked by an asterisk.

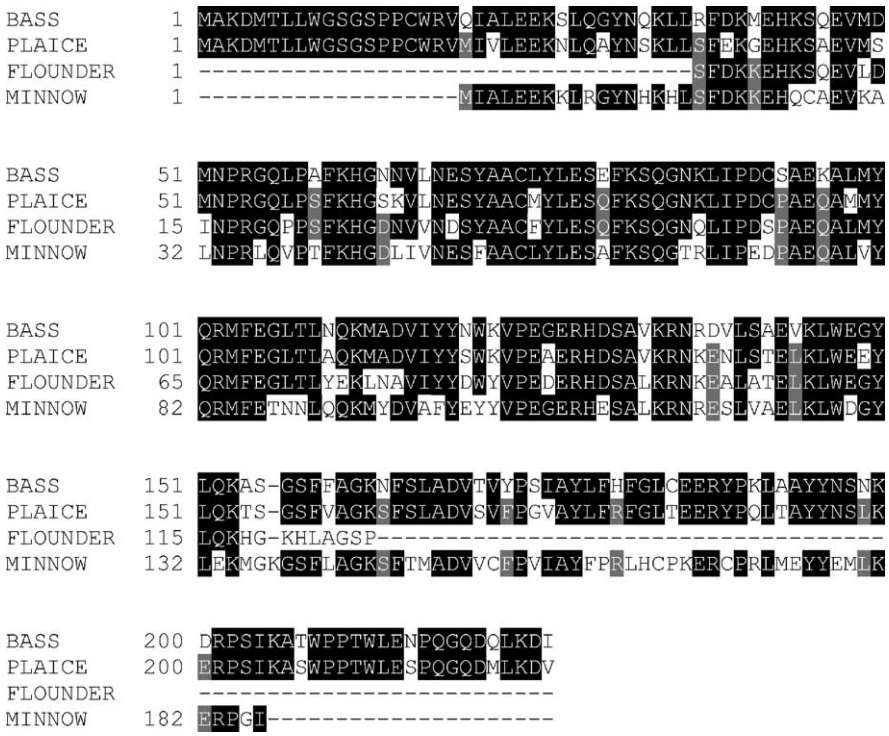

Fig. 2. Deduced amino acid sequences of largemouth bass glutathione *S*-transferase (GST; present work) were aligned against GST sequences of various fish species identified in a sequence similarity search using BLAST. The search identified similar sequences of GSTs of plaice (*Pleuronectes platessa*, GenBank X63761; 80% identity), European flounder (*Platichthys flesus*, GenBank AJ310428; 72% identity), and fathead minnow (*Pimephales promelas*, GenBank AF274054; 60% identity).

cDNA sequence [11]. Sequencing analysis showed that the bass GST clone was 957 bp in length, and contained an open reading frame of 678 bp, encoding a polypeptide of 225 amino acids (Fig. 1). The theoretical molecular mass based on the deduced amino acid sequence was calculated as 25.9 kDa. A BLAST search using the deduced 225 amino acid sequence of the bass GST revealed highest sequence similarity to GST sequences of three other fish species, the plaice (*P. platessa*; GenBank X63761), the European flounder (*P. flesus*; GenBank AJ310428), and fathead minnow (*P. promelas*; GenBank AF274054) (Fig. 2). The bass GST protein showed 80% identity (89% similarity) to the plaice GST-A, 72% identity (85% similarity) to the flounder GST, and 60% identity (74% similarity) to the fathead minnow GST (Fig. 2). Conversely, the bass GST showed little sequence identity to several mammalian GSTs, including the GSTA4-4 isoforms involved in 4HNE conjugation (Fig. 3). The bass GST shared 13% identity (28% similarity) with rat alpha class subunit rGSTA1 (GenBank NP_058709); 11% identity (21% similarity) with rat mu class subunit rGSTM1 (GenBank M11719); 21% identity (34% similarity) with rat pi class subunit rGSTP1 (GenBank X02904); 20% identity (29% similarity) with rat theta class subunit rGSTT1 (GenBank X67654); 17% identity (30% similarity) with rat alpha class subunit rGSTA4 (GenBank P14942); 16% identity (31% similarity) with mouse alpha class subunit mGSTA4 (GenBank NM_010357); and 17% identity (26% similarity) with human alpha class subunit hGSTA4 (GenBank NM_001512).

3.2. Enzymatic and immunological profile of the recombinant GST protein

The recombinant GST (*rec*GST) was found to be very labile, and enzyme activities had to be measured immediately after protein purification. Activities were normalized against CDNB activity for imidazole inhibition similar as described by Martinez-Lara et al. [19]. As observed in Table 1, the *rec*GST rapidly conjugated 4HNE (9 μmol/min/mg), and exhibited relatively high initial rate activity toward CDNB (7 μmol/min/mg). In addition, *rec*GST catalyzed the conjugation of ECA (0.5 μmol/min/mg), as well as the reduction of cumene hydroperoxide (0.5 μmol CuOOH reduced/min/mg). In contrast, no GST catalytic activity was detected using other GST substrates including ADI, DCNB, and NBC. Western blotting of affinity purified bass liver GST and the *rec*GST showed some cross-reactivity toward rat GST alpha and GST theta antibodies, and no cross-reactivity against rat GST mu or pi-class antibodies (Fig. 4).

3.3. Kinetics of GST-4HNE conjugation in bass liver

A previous study in our laboratory has investigated the ability of bass hepatic GSTs to conjugate 4HNE in

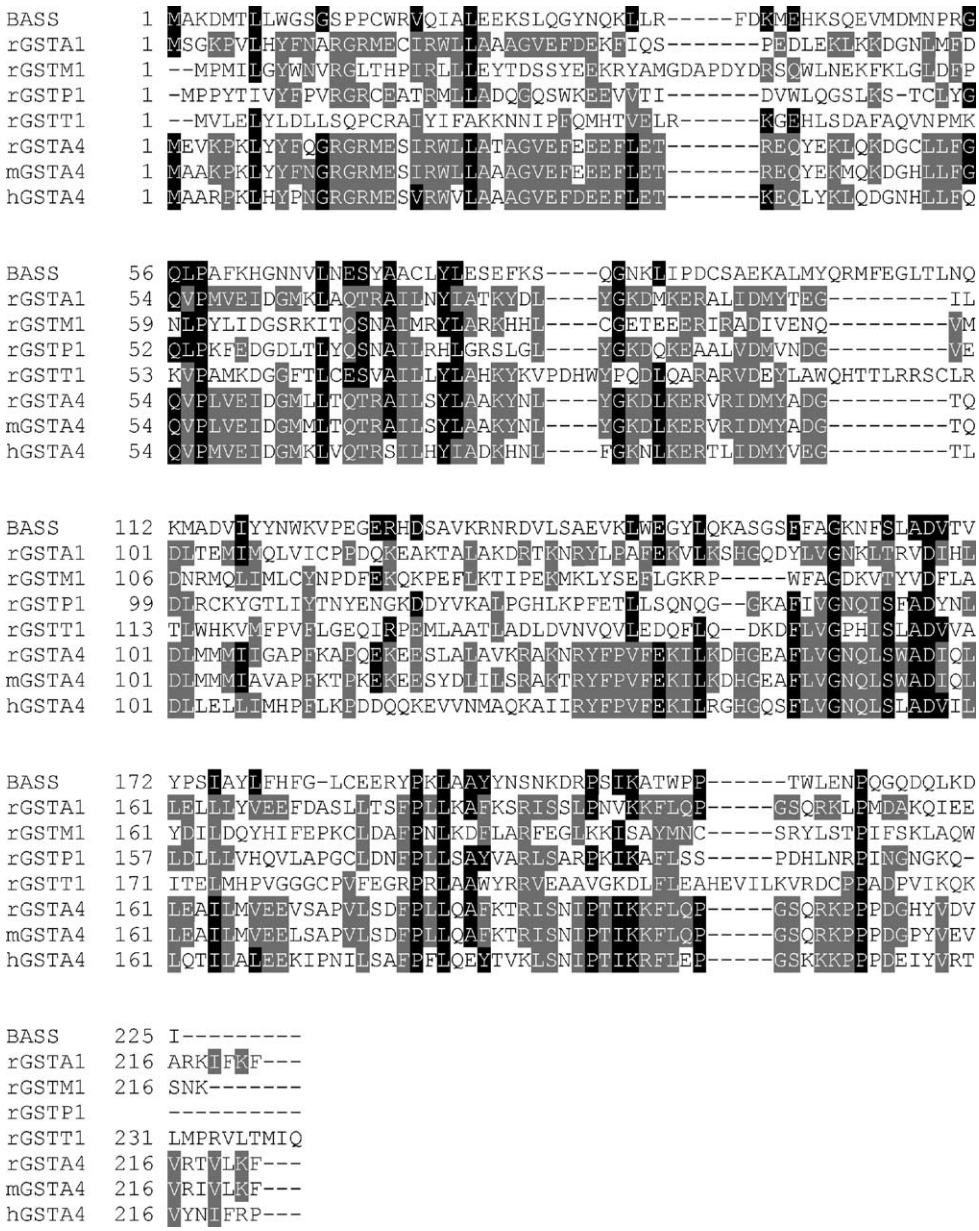

Fig. 3. Deduced amino acid sequences of largemouth bass glutathione *S*-transferase (GST; present work) were aligned against published mammalian GSTs sequences. The bass GST shared 13% identity with rat alpha class subunit rGSTA1 (GenBank NP_058709); 11% identity with rat mu class subunit rGSTM1 (GenBank M11719); 21% identity with rat pi class subunit rGSTP1 (GenBank X02904); 20% identity with rat theta class subunit rGSTT1 (GenBank X67654); 17% identity with rat alpha class subunit rGSTA4 (GenBank P14942); 16% identity with mouse alpha class subunit mGSTA4 (GenBank NM_010357); and 17% identity with human alpha class subunit hGSTA4 (GenBank NM_001512).

Table 1
Catalytic activities of recombinant largemouth bass glutathione *S*-transferase (GST) towards model GST substrates that are catalyzed by specific mammalian GST classes

Donor substrate	Class	Substrate concentration (mM)	Activity (μmol/min/mg)
1-Chloro-2,4-dinitrobenzene (CDNB)	Overall GST	1	7.0
Cumene hydroperoxide (CuOOH)	Alpha, theta	1.5	0.5
Δ5-Androstene-3,17-dione (ADI)	Alpha	0.068	<0.1
1,2-Dichloro-4-nitrobenzene (DCNB)	Mu	1	<0.1
Nitrobenzylchloride (NBC)	Mu, theta	0.5	<0.1
Ethacrynic acid (ECA)	Alpha, pi	0.2	0.5
4-Hydroxynonenal (4HNE)	Alpha	0.192	9.4

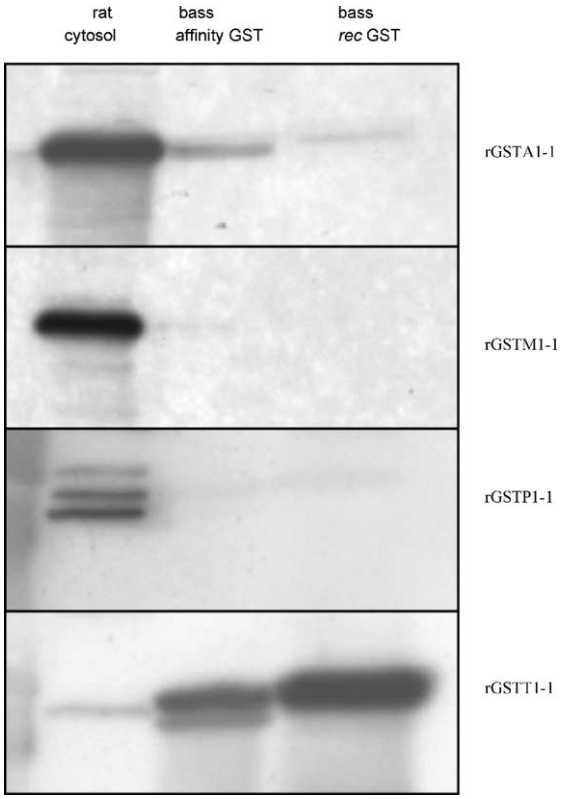

Fig. 4. Western blot analysis of affinity purified bass liver glutathione *S*-transferase (GST) and recombinant GST. The blot was probed with polyclonal antibodies raised against rodent α (rGSTA1-1), μ (rGSTM1-1), π (rGSTP1-1), and θ (rGST1-1), and rat cytosol was used as a positive control. As observed, some light cross-reactivity was observed with θ-class GST and α-class GST.

comparison to other species [12]; a review of the relevant findings is summarized in Table 2. The ratio of GST-4HNE/GST-CDNB activity in female bass liver cytosol was approximately 40% higher than observed in males, and two-fold greater than observed in rat liver. Furthermore, these GST-4HNE/GST-CDNB activity ratios were 11–15-fold greater than observed for brown bullhead catfish. GSH-affinity purification of bass liver cytosol resulted in 55-fold enrichment of GST-4HNE activity (Table 2), indicating that the GST isoform(s) contributing to 4HNE conjugation adhere to, and could be eluted from, a standard GSH-affinity column.

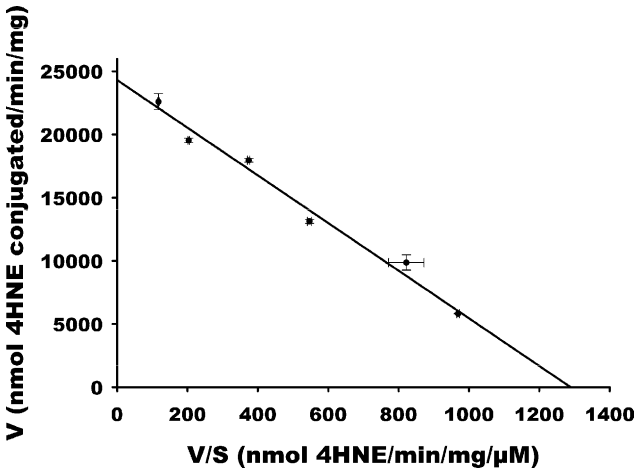

Fig. 5. Eadie–Hofstee (*V* vs. *V/S* plot) of data showing the effects of substrate concentration on the initial rate of GST-4HNE conjugation in GSH-affinity purified bass liver cytosol.

Enzyme kinetic analysis of GST-4HNE conjugation in GSH-affinity purified fractions followed linear Michaelis–Menten kinetics when GSH concentration was fixed and the 4HNE concentration was varied (Fig. 5) suggesting that a single GST isoform was involved in 4HNE detoxification in bass liver. Non-linear regression analysis of the kinetic data yielded apparent K_m and V_{max} parameters for GST-4HNE conjugation of $K_m = 18.9 \pm 1.3$ μM and $V_{max} = 24 \pm 0.5$ μmol 4HNE conjugated/min/mg, indicating a high efficiency of GST-mediated 4HNE conjugation in bass liver.

3.4. Tissue-specific expression of bass GST

The full-length size of the cloned cDNA of bass GST (957 bp) was consistent with a band (~1 kb) previously recognized in bass liver [11]. The GST fragment amplified by multiplex PCR using cDNA from various tissues of bass was approximately 776 bp in length. As observed in Fig. 6, tissue-expression analysis revealed that the bass GST is not only expressed in the liver, but also in the gonad, upper gastrointestinal tract, and brain tissue. No detectable GST expression was observed in heart, lower gastrointestinal tract or muscle tissue.

Table 2
Comparison of hepatic GST-4HNE activities in largemouth bass and other species[a]

Species (sex)	Subcellular fraction	GST-4HNE (nmol/min/mg)	GST-CDNB (nmol/min/mg)	GST-4HNE/GST-CDNB[b]
Largemouth bass (male)	Cytosol	415 ± 83[c]	402 ± 15	1.03
Largemouth bass (female)	Cytosol	415 ± 45	282 ± 73	1.47
Largemouth bass (male)	GSH-affinity purified cytosol	22,904[d]	9649	2.37
Brown bullhead (male)	Cytosol	187 ± 26	1,939 ± 214	0.096
Rat (male)	Cytosol	661 ± 26	872 ± 15	0.758

[a] A review of select findings modified from [12].
[b] GST-4HNE activities/GST-CDNB activities, no units.
[c] Mean ± S.E.M. of $n = 3$ individual animals.
[d] Mean of triplicate determinations.

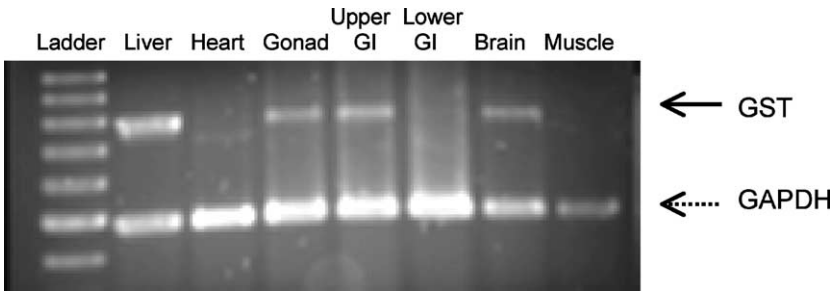

Fig. 6. Tissue-specific expression of glutathione *S*-transferase (GST) in largemouth bass. Multiplex PCR utilizing primers developed for the amplification of a 776 bp cDNA of the bass GST and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a relative control was conducted with cDNA synthesized from total RNA of liver, heart, gonad, upper and lower gastrointestinal (GI) tract, brain, and muscle. A 1 kb DNA ladder was used, and the full arrow denotes the amplified GST DNA and the dotted arrow the GAPDH (housekeeping) band.

3.5. HPLC-subunit analysis, protein sequencing, and electrospray mass spectrometry

HPLC analysis of affinity purified bass liver GST resulted in the elution of three major peaks, at approximately 11, 33, and 34 min, suggesting that bass liver may contain at least three different GST isoforms. A representative chromatogram can be seen in Fig. 7. Based upon area under the curve (AUC), the first peak constituted roughly 80% of total GST protein, assuming that the extinction coefficients for the three GST peaks were relatively similar. Sequencing of this major purified GST subunit by Edman degradation revealed that this subunit corresponding to the largest peak contained a partial sequence (ATWPPTWLENPQGQ) identical to amino acids 206–219 of the deduced sequence of the full-length bass GST. The molecular masses of the two largest collected peaks (peaks 1 and 3; Fig. 7) were determined to be 26.4 and 25.5 kDa by HPLC-MS with ESI.

4. Discussion

A previous study in our laboratory indicated that hepatic cytosolic fractions from bass rapidly catalyzed GSH-dependent 4HNE conjugation [12]. The initial rates of hepatic cytosolic GST-4HNE conjugation were similar in male and female bass, but more than two-fold greater than observed for brown bullheads, a demersal herbivore with high GST activity that is often found in similar environments with bass [16]. This finding is striking, considering that data herein and previously presented [16] have shown that bass catalyze the conjugation of other structurally diverse, class-specific GST substrates (CDNB, NBC, ECA, and ADI) at lower initial rates of GST activity than brown bullhead. The rates of hepatic cytosolic GST-4HNE activity normalized to GST-CDNB activity provide index of the proportion of specific GST catalytic activity dedicated to 4HNE conjugation relative to overall GST activity. The GST-4HNE/GST-CDNB ratios

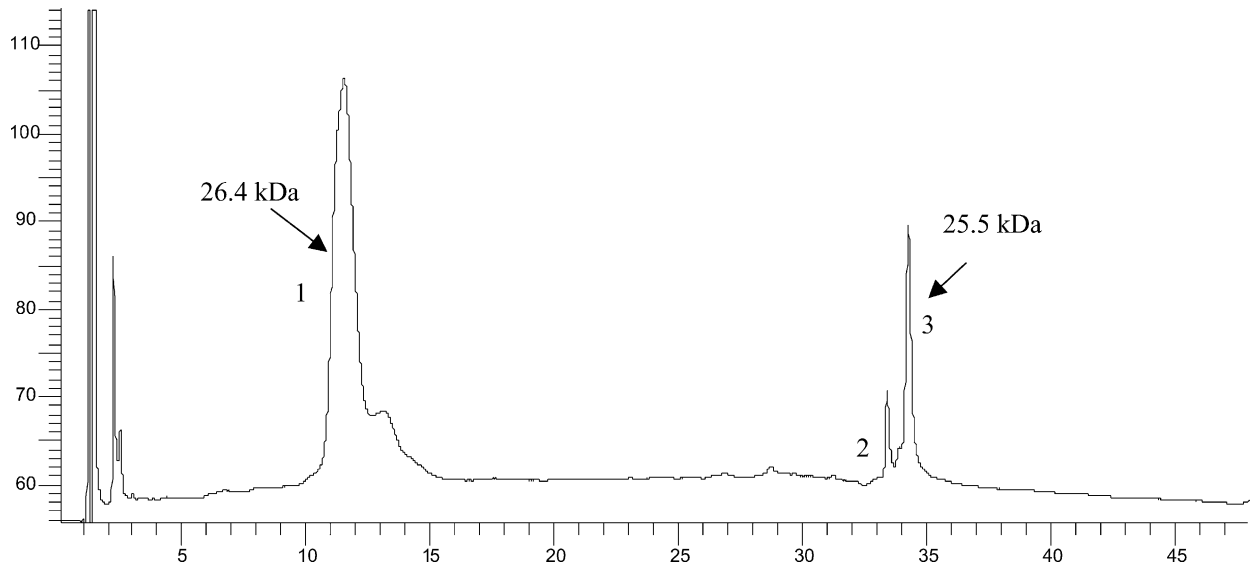

Fig. 7. Representative chromatogram of an HPLC separation of hepatic GST subunits of bass liver. Bass affinity purified cytosol used for HPLC analysis resulted in the elution of three peaks. Protein sequencing by Edman degradation of the major subunit (peak 1) revealed the partial peptide sequence ATWPPTWLENPQGQ, which was identical to bass GST-A deduced sequence at amino acids 206–219. The molecular masses of the two major subunits (peaks 1 and 3) were determined by HPLC mass spectrometry followed by electrospray-ionization analysis.

were the highest in female bass, followed by male bass, which in turn were approximately two-fold greater than observed in brown bullhead and 1.4-fold greater than observed in rat liver. These data indicate that although the overall rates of GST activity in bass may be lower compared to other species, a substantially larger proportion of activity is dedicated to 4HNE conjugation in bass liver, particularly in females. In this regard, the distinctive ability of the bass GST to efficiently detoxify 4HNE is consistent with a role in protection against peroxidative injury in this higher order predatory game fish.

The rate of GST-4HNE conjugation in bass cytosol followed monophasic kinetics, suggesting that a single GST isoform was responsible for the 4HNE activity in bass liver. The extensive sequence identity of a partial cDNA GST previously isolated from bass liver [11] to a 4HNE-metabolizing GST described in plaice [GST-A; 10, 20] indicated that this conserved isozyme could be responsible for the rapid 4HNE conjugation in bass liver. Accordingly, the bass GST in question was isolated and characterized in the current studies. The bass GST is 957 bp in length and encode a 225 amino acids polypeptide. A BLAST search using the deduced amino acid sequence of the full-length bass GST clone, as expected, revealed high sequence identity (80%) to the plaice GST isozyme. For consistency in terminology, the cloned bass GST will be referred to as GST-A, according to similarity to the plaice GST-A. A BLAST search of the nucleotide database revealed that the bass GST-A was also similar to a GST form found in two other fish species (European flounder and fathead minnow), however, similar sequences were not found in other aquatic species, including more commonly employed aquatic models such as medaka, zebrafish, or rainbow trout. Furthermore, our laboratory has been unsuccessful in demonstrating the presence of a GST-A like isoform in other fish species including brown bullhead or channel catfish. Thus, it appears that this GST isoform is not conserved among all fish species.

GST-A exhibited little sequence identity (21% or less) to several mammalian GSTs, including the rat alpha class GST form rGSTA4-4, which has a high catalytic efficiency towards conjugation of GSH with 4HNE [8]. Although the different mammalian GSTA4 isoforms have been shown to share greater sequence identity across species than with other alpha-class GSTs within the same species [6–8], the sequence identity among GSTA4 forms is still lower than 60%. Accordingly, it has been suggested that the conservation of the primary structure of the mammalian GSTA4-4 during evolution was not the most critical factor in the preservation of the protection against lipid peroxidation [8]. This assumption is supported by the findings in the current study, whereas structurally different enzymes appear to bear a similar function in evolutionarily divergent species.

With regard to substrate specificity, the bass recombinant GST-A showed relatively high activity toward CDNB, while showing very low activity towards CuOOH and ECA, lowest or undetectable activity towards ADI, DCNB, and NBC, and high activity towards 4HNE. The catalytic profile of the bass GST-A was very similar as previously described for a 6x*His*-tagged plaice GST-A [21], except for the fact that the bass recombinant GST-4HNE activity relative to CDNB was approximately 15-fold higher in comparison to the plaice. These data suggest that a substantial proportion of total bass liver GST activity is directed toward protecting against 4HNE-associated injury. Interestingly, the rate of GST-4HNE catalytic activity observed for the bass recombinant GST-A protein was relatively low compared to that observed for the GSH-affinity purified bass liver cytosol [12], which was possibly associated with the bass recombinant GST being very labile. Immunologically, some cross-reactivity was observed toward rat GST theta, and to a lesser extent, toward rat GST alpha antibodies, although the immunological profile is not consistent with either the sequence identity analysis or the substrate specificity. Thus, the sequence identity analysis, and the catalytic and immunological profile of the bass GST-A collectively indicate that the bass isozyme does not belong in any of the mammalian GST classes, but represent a distinct group of GSTs. Consistent with this hypothesis is the fact that studies with the GST-A from plaice demonstrated little homology of this fish enzyme to mammalian alpha, mu, or pi GSTs, despite the presence of some structural similarity among the amino acid sequence of plaice GST-A with plant, insect, and mammalian theta class GSTs [20]. The authors suggested that the plaice GST, along with the other theta GSTs might belong in a primitive GST class.

The bass GST-A was highly expressed in the liver, gonad, upper gastrointestinal tract, and brain tissue, but was not detectable in heart, lower gastrointestinal tract, and muscle tissue. Accordingly, the selective tissue distribution of the bass GST-A may reflect differences in organ susceptibility to oxidative damage, or alternatively, the presence of non-GST aldehyde metabolizing enzymes (e.g. aldehyde dehydrogenases, alcohol dehydrogenases) in those tissues devoid of GST-A. The pattern of tissue expression of the bass isozyme differed from that seen in the plaice, where GST-A mRNA was detected in all tissues examined, including the liver, intestine, gill, kidney, brain, gonad, heart, and spleen. The widespread tissue distribution of the plaice GST-A suggested a potential cellular ‘‘housekeeping’’ function of the gene [20]. However, because select plaice tissues (liver, intestine, gills, and kidney) had much higher steady-state mRNA levels in comparison to other tissues, a potential role in protection against toxic electrophiles was not disregarded. The distribution pattern of the bass GST-A would suggest that this enzyme does not share the ‘‘housekeeping’’ function of the plaice GST-A, although the significance of the differences in the tissue distribution of the two fish isozymes is unclear at this time.

A previous Western blotting analysis of bass liver cytosolic GST analysis conducted in our laboratory revealed the presence of at least two major GST subunits in bass liver [12]. In order to determine if the cloned GST-A reported here represented a highly expressed isoform in bass liver, we conducted HPLC-subunit analysis of affinity purified bass liver cytosol. Although at least three different subunits were identified by HPLC analysis, sequence analysis of the major peak, which constituted approximately 80% of the total GST protein, revealed identity to GST-A. Therefore, the bass GST-A appears to be the major GST isozyme in the bass liver, with a molecular mass of 26.4 kDa, and thus corresponds to the highly expressed GST form previously detected by Western blot analysis. We are currently isolating and sequencing the second major bass GST form which elutes at 34 min by HPLC (corresponding to peak 3 on our chromatograms) and exhibits a molecular mass of 25.5 kDa, as well as also characterizing the potential presence of additional bass liver GST forms.

Collectively, these results have shown that GST-A, the major GST isoform in bass liver, also expressed in select tissues, efficiently catalyzes 4HNE conjugation in this fish species. This enzyme displays unique catalytic activity and immunological cross-reactivity profiles. When evaluated in the context of low sequence identity with mammalian GST classes, this bass isozyme and its fish orthologues appear to represent a distinct group of GSTs, with a protective role against lipid peroxidation. Because fish lipids are rich in polyunsaturated fatty acids, the high GST-4HNE activity of this enzyme is likely to play a key role in protection against oxidative injury. In the case of largemouth bass, this species is a higher order predatory game fish whose diet largely consists of fish, species that are high in polyunsaturated fatty acid content. In addition, fish often need to adapt to temperature changes in the environment. In this regard, membranes are the first targets affected by changes in temperature, with a decrease in temperature resulting in a decrease in motional freedom of acyl chains of constituent phospholipids. One of the techniques used to reach this goal is by increase the unsaturation of the constituent fatty acids which results in rendering the membranes more fluid during adaptation to reduce temperature [22]. When viewed collectively, the ability of bass to rapidly detoxify aldehydes produced during the peroxidation of membrane lipids through GST-A would certainly allow for a selective advantage for survival in the wild.

Acknowledgments

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