IDENTIFICATION AND CHARACTERIZATION OF A MAJOR HEPATIC GLUTATHIONE *S*-TRANSFASE ISOENZYME IN LARGEMOUTH BASS (*Micropterus salmoides*) THAT CONJUGATES 4-HYDROXYNON-2-ENAL.

BY

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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The glutathione *S*-transferases (GST) are a multigenic family of phase II enzymes involved in the detoxification of carcinogenic and reactive intermediates. Certain GST isozymes, including those of the mammalian alpha class, have particularly high activity toward alkenals including 4-hydroxynon-2-enal (4HNE), and other reactive by-products produced during lipid peroxidation. The ability of cells to remove 4HNE is of particular importance since 4HNE is an extremely carcinogenic and mutagenic intermediate produced at relatively high concentrations on exposure to peroxidizing chemicals.

In general, relatively little is known regarding the ability of fish species to detoxify 4HNE and related products of oxidative injury via GST. In this thesis, we

have identified and characterized the GST isoform responsible for the rapid metabolism of 4HNE in largemouth bass, a freshwater species and higher order predatory species that tends bioaccumulate lipophilic toxicants through the food chain. HPLC-GST subunit analysis revealed the presence of at least two major GST isoforms in bass liver, with the first peak (peak one) constituting 80% of the total bass liver GST protein. HPLC with electrospray-ionization of the two isolated GST subunits yielded molecular weights of 26,396 kDa and 25,515 kDa. Endo-proteinase Lys-C digestion and Edman degradation protein sequencing of this GST isoform that is similar to the plaice GST isoform that rapidly metabolizes 4HNE was termed bass GSTA. Peak one demonstrated that this major GST isoform was encoded by GSTA. Analysis of genomic DNA fragments isolated by nested PCR indicated the presence of a GST gene cluster in bass liver that is similar to plaice GST gene cluster. Using nested deletions with Exonuclease III and Mung Bean nuclease, we were able sequence the entire upstream bass GSTA promoter. Isolation of approximately 1 kb of the bass *GSTA* promoter revealed the presence of several putative response elements that may confer inducibility to endogenous and environmental chemicals. Collectively, our data indicates the presence of a major GST in bass liver involved in the protection against oxidative stress. Furthermore, this GST is part of a gene cluster that may be conserved in aquatic species.

CHAPTER 1
INTRODUCTION

Biotransformation Pathways of Detoxification

All organisms are constantly exposed to a variety of foreign chemicals, which include synthetic and natural chemicals such as chemotherapeutic drugs, industrial chemicals, pesticides, polycyclic aromatic hydrocarbons (PAH), and natural toxins from plants, molds, fungus and animals. The physical property that allows many xenobiotics to be absorbed through the skin, lungs, gastrointestinal tract, and other various parts of the body is lipophilicity. Consequently, the elimination of xenobiotics depends on the conversion of xenobiotics to water-soluble compounds by a process known as biotransformation, which is catalyzed by biotransformation enzymes in the liver and other tissues (Klaasen, 2001). The reactions catalyzed by xenobiotic biotransformation enzymes are grouped in two categories: phase I and phase II enzymes. Phase I biotransformation reactions involve hydrolysis, reduction, and oxidation reactions and results in the addition or exposure of functional groups (e.g -OH, -NH_2, -SH or -COOH). The phase I enzymes include aldehyde dehydrogenase, flavin monooxygenase, and cytochrome P450s. However, some phase I biotransformation pathways does not always lead to detoxification reactions. For example, aflatoxin B_1 (AFB_1), a known natural occurring hepatocarcinogen produced by the mold *Aspergillus flavus*, is bioactiviated by cytochrome P4501A2 (CYP1A2 isoform) to an ultimate

carcinogen AFB_1-8-9-epoxide (Eaton and Gallagher, 1994; Gallagher *et al.*, 1994).

Phase II biotransformation reactions include glucuronidation, sulfonation, acetylation, and glutathione conjugation. The phase II enzymes include UDP-glucuronosyltransferase, sulfotransferase, *N*-acetlytransferase, and glutathione *S*-transferase (GST). Phase II biotransformation reactions can result in a marked increase in xenobiotic hydrophilicity, and therefore facilitating excretion of chemicals. Of the phase II biotransformation enzymes, GSTs are a superfamily of dimeric enzymes involved in the detoxification of carcinogenic and reactive intermediates (Hayes and Pulford, 1995). Most eukaryotic species possess multiple GST isoenzymes each of which may have different affinities towards various substrates. Mammalian cytosolic GSTs have been extensively studied and are currently grouped into eight distinct classes: alpha (α), mu (μ), pi (π), theta (θ), kappa (κ), sigma (σ), omega (ω), and zeta (ζ) based on substrate specificity, immunological cross-reactivity and structural similarity (Hayes and Pulford, 1995). The structural diversity of GSTs provides the ability to conjugate a broad range of compounds. Accordingly, model GST substrates that are rapidly conjugated by a particular GST subunit are often used to identify the involvement of a particular GST isoenzymes (Hayes and Pulford, 1995). Model substrates for GST conjugation include: 1-chloro-2,4-dinitrobenzene (CDNB, overall broad specificity except for θ class), ethacrynic acid (ECA, reactive with π class rGSTP1), nitrobutyl chloride (NBC, reactive for θ class rat rGSTT1), 1,2-dichloro-4-nitrobenzene (DCNB, activity towards μ class rat rGSTM1), Δ5-

androsten-3,17-dione (ADI, selective specificity with α class rat rGSTA1 and rGSTA2), 4-hydroxynonenal (4HNE, selective for α class rat rGSTA4 and human hGSTA4), and 1,2-epoxy-3-*p*-nitrophenoxy propane (EPNP, selective for θ class rat rGSTT1) (Hayes and Pulford, 1995). Other GST substrates include carcinogens (AFB_1-8-9-epoxide), pesticides (DDT, atrazine), anti-cancer drugs (BCNU, chlorambucil) and by-products of lipid peroxidation (fatty acid hydroperoxides, α,β-unsaturated aldehydes) (Hayes and Pulford, 1995).

The mechanism of GST-mediated conjugation typically involves electrophilic conjugation with the tripeptide glutathione. The dimeric GST subunit has an active site composed of 2 distinct regions: G-site (hydrophilic binding site of substrate GSH) and an adjacent H-site (active site for variety of electrophilic substrates) (Mannervik and Danielson, 1988; Armstrong, 1997). Thus, the G-site is conserved in all GST families due to its high affinity for GSH, while the H-site shows a broad range of electrophilic substrate binding affinities can differ between GST families (Hayes and Pulford, 1995). GST catalyzes the general reaction shown:

GSH + R-X -----GST----> GSR + HX

The catalytic reaction of GST involves positioning the substrate within close proximity of GSH for binding of GSH and the electrophilic substrate to the active site of the protein, and activating the sulfhydryl group on GSH, thereby allowing a nucleophilic attack of GSH on any electrophilic center of the substrate (R-X) (Armstrong, 1997). The GSH conjugates formed in the liver can be excreted in bile, or can be converted to mercapturic acids in the kidney and excreted in urine.

The conversion of GSH conjugates to mercapturic acids involves sequential cleavage of glutamic acid and glycine (by γ-glutamlytranspeptidase and aminopeptidase M, respectively) from the GSH conjugate, followed by *N*-acetylation of the cysteine conjugate (Klaasen, 2001). Besides having catalytic activity towards reactive intermediates, certain GST isoenzymes have non-catalytic properties such as intracellular carrier proteins for steroid and thyroid hormones, bile acids, bilirubins, and fatty acids (Hayes and Pulford, 1995). Further, some GST isoenzymes show a glutathione-peroxidase-like activity (general reaction: ROOH + GSH -----GST----> ROH + GSSG + H_2O) in which organic peroxides are converted to the corresponding alcohol (Hayes and Pulford, 1995).

The expression of certain cytosolic GST isoforms can be induced by exposure to certain xenobiotics, including PAH, reactive oxygen species (ROS), Michael acceptors, phenolic antioxidants, and glucocorticoids (Hayes and Pulford, 1995). Induction of GST can involve several transcriptional mechanisms. For example, the induction of mammalian GSTs by xenobiotics can be mediated by several sequence-specific DNA motifs (xenobiotic response element (XRE), anti-oxidant response element (ARE), glucocorticoid response element (GRE), located in the regulatory regions of all genes and which respond to intracellular or extracellular stimulus by activating the transcription factors that bind to the motif and that either up- or down regulate gene expression (Dynan and Tjian, 1985). For example, the rat *rGSTA2* gene contains a xenobiotic response element (XRE: TA/TGCGTG), an anti-oxidant response element (ARE:

TGACAAAAGC), and a glucocorticoid response element (GRE: AGAACANNNTGTTCT) (Hayes and Pulford, 1995).

The XRE facilitates induction by various compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), while the GRE mediates induction by synthetic glucocorticoids such as dexamethasone. Phenolic antioxidants such as *tert*-butlyhydroquinone (TBHQ) can induce GST via ARE consensus sequences (Hayes and Pulford, 1995). In mice, mGSTA2 contains a response element related to the ARE called the electrophile response element (EPRE: TGACNNNNGC) (Hayes and Pulford, 1995). The EPRE contains two tandem arrangements of consensus sequence ARE elements that confer induction in response to certain chemicals such as β-naphthoflavone (Hayes and Pulford, 1995). Interestingly, AREs and EPREs have similar consensus sequences (TGACNNNNGC) which are often referred as AP1-like binding sites. Thus, it is proposed that a variety of chemical agents PAH, diphenols, phenobarbital and electrophilic compounds can induce mouse GST (mGSTA1) by the activation of the Fos/Jun heterodimeric complex (AP1) (Bergelson *et al.*, 1994; Hayes and Pulford, 1995).

Piscine Glutathione S-Transferases

Although mammalian GSTs have been extensively characterized, much less is known about fish GST isoenzymes. As in mammals, fish GSTs can conjugate various electrophilic environmental chemicals (George, 1994). Proteins related to mammalian alpha (α), mu (μ), pi (π) and theta (θ) class GSTs have been described in various fish species (George, 1994). In particular, the

structure and expression of three theta-like GST genes (*GSTA*, *GSTA1*, and the pseudogene, *GSTA2*) from the plaice (*Pleuronectes platessa*) have been characterized and identified (Leaver *et al.*, 1997). Analysis of the promoter of plaice *GSTA1* gene revealed multiple peroxisome proliferator response elements (PPRE) similar to murine PPRE (Zimniak *et al.*, 1994). The plaice *GSTA* and *GSTA1* gene has been found to be up-regulated after administration of perfluoro-octanoic acid (PFOA), a potent peroxisome proliferators, while only the *GSTA* gene was induced by β-naphthoflavone (BNF), a classic bifunctional inducer of phase I and II biotransformation enzymes (Leaver *et al.*, 1997). This suggests that the PPRE and ARE in plaice are regulated in a similar manner to that described for mammalian genes. The presence of a transposon-like element (PPTN) has been identified between the *GSTA1* and *GSTA* gene cluster, and the PPTN contains multiple AREs (Leaver *et al.*, 1997). Also, the presence of an estrogen response element (ERE) in the plaice *GSTA1* promoter may suggests a possible role for increasing GST-mediated detoxification of lipid peroxides during reproduction, as fish lipid membranes consist heavily of polyunsatured fatty acids (Hyllner *et al.*, 1994). In additions to the studies in plaice, Carvan et al. have developed a zebrafish transgenic model system in which DNA motifs (XRE, ARE, and EPRE) that respond to environmental pollutants by activating a reporter gene (2000). Other studies have shown that hepatic GSTs can be induced in brown bullhead and channel catfish by electrophilic agents or anti-oxidant agents (Gallagher *et al.*, 1991; Henson *et al.*, 2001). Thus, it appears that many fish

GST isoenzymes exhibit similar induction mechanisms observed for mammalian GSTs.

Role of GST in Protecting Against Oxidative Injury

Cellular respiration or oxidative process produces several reactive free radical intermediates which are the initiating factors in the decomposition of lipids, and ultimately producing α,β-unsaturated aldehydes. In this regard, the superoxide anion radical (O_2 •), hydroxide radical (OH•), and hydrogen peroxide (H_2O_2) constitute primary reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999). ROS can attack DNA, proteins, and cellular targets such as polyunsaturated fatty acids (PUFA). The process of lipid peroxidation is initiated by a hydroxy radical OH• (Figure 1). The lipid radical (L•) is converted to lipid peroxyl radical (LOO•) by an addition of oxygen, lipid hydroperoxide (LOOH) by hydrogen abstraction, and lipid alkoxyl radical (LO•) by the Fe^{+2}-catalyzed Fenten reaction (Figure 1). The end-products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and 4-hdyroxynonenal (4HNE).

Certain GST isoforms have conjugative activity towards endogenous genotoxic α, β-unsaturated aldehydes formed during lipid peroxidation (Mannervik and Danielson, 1988). In this regard, the GST detoxification pathways play an important role in protection against reactive oxygen species and their electrophilic reactive intermediates. In particular, 4HNE is the end product of arachidonic acid peroxidation, and is an extremely genotoxic, mutagenic and long-lived compound and can readily react with adjacent molecules (proteins and lipids) or diffuse to distant targets such as DNA (Esterbauer *et al.*, 1991).

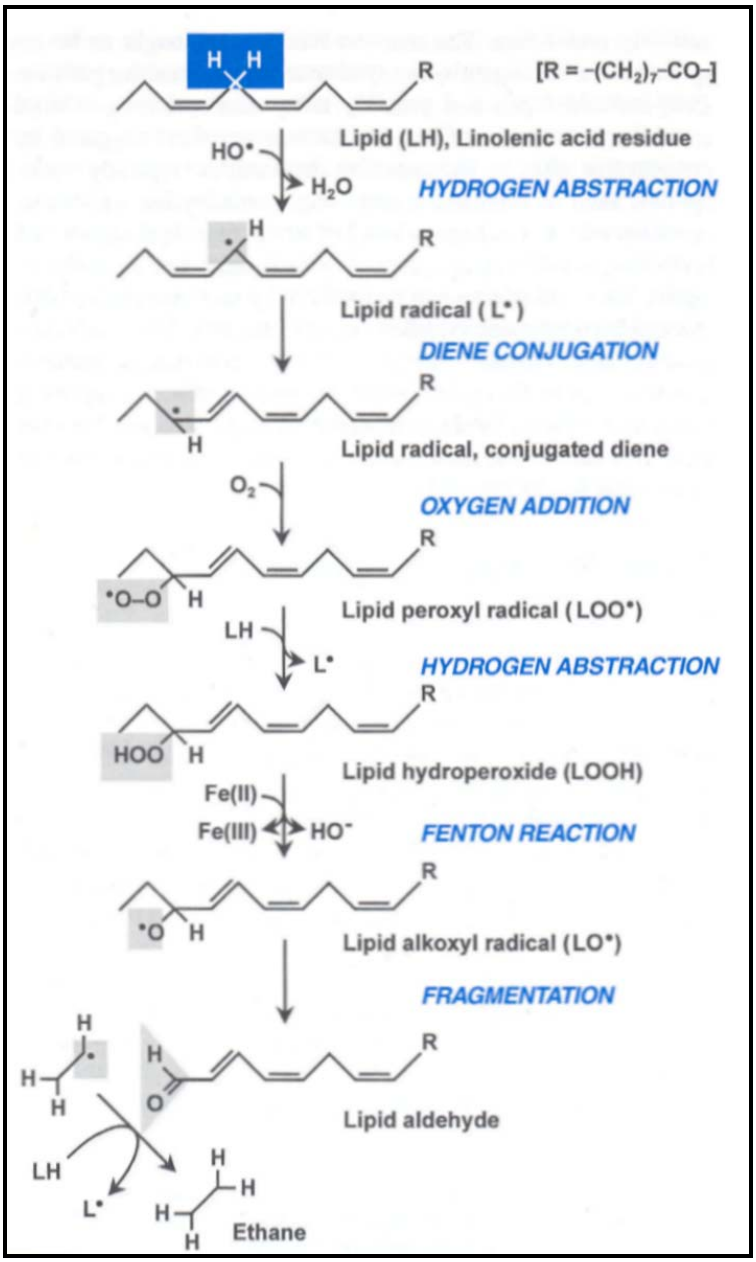


Figure 1. A brief schematic showing the initiation of lipid peroxidation by hydroxyl radical (OH•) and its end products are α,β-unsaturated aldehydes (e.g 4HNE, MDA).

The effects of 4HNE depend upon the concentration of 4HNE. At cellular levels of 100 nm or lower, 4HNE can stimulate chemotaxis and phospholipase C (Eckl *et al.*, 1993). At levels of 1-20 μM, DNA and protein synthesis are inhibited, there is an increase chromosomal aberrations and sister chromatid exchange, and cell proliferation is inhibited (Eckl *et al.*, 1993). Levels of 100 μM or above may

occur near peroxidizing membranes and can cause cell lysis and cell death (Eckl *et al.*, 1993; Halliwell and Gutteridge, 1999). Coincidently, elevated tissue 4HNE concentrations have been associated with several human diseases, including cancer (Eckl *et al.*, 1993), Parkinson's disease, Alzheimer's disease (Markesbery and Lovell, 1998), atherosclerosis (Chen *et al.*, 1995; Muller *et al.*, 1996), pulmonary inflammation (Hamilton *et al.*, 1996), rheumatoid arthritis (Selley *et al.*, 1992), ophthalmologic disorders (Esterbauer *et al.*, 1991), and liver disease (Tsukamoto and French, 1993).

Given the high reactivity and toxicological importance of 4HNE, it is not surprising that a number of enzyme systems have evolved to protect tissues from 4HNE injury (Esterbauer et al., 1991). The primary enzymatic pathways of 4HNE detoxification in adult human liver include aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), aldehyde reductase (ALRD), and GST (Figure 2) (Mitchell and Petersen, 1987; Sellin *et al.*, 1991; Hayes and Pulford, 1995).

Goals of the Present Study

We have previously described the *in vitro* kinetics of GST-CDNB conjugation in largemouth bass, a freshwater fish and a higher order predatory species that has been shown to bioaccumulate hydrophobic xenobiotics and is sensitive to the toxic effects of environmental contaminants (Gallagher *et al.*, 2000). Furthermore, we have cloned and expressed a recombinant bass GSTA protein that has high catalytic activity towards 4HNE (Doi *et al.*, 2003). This bass *GSTA* exhibits high homology to the plaice *GSTA* that also conjugates 4HNE.

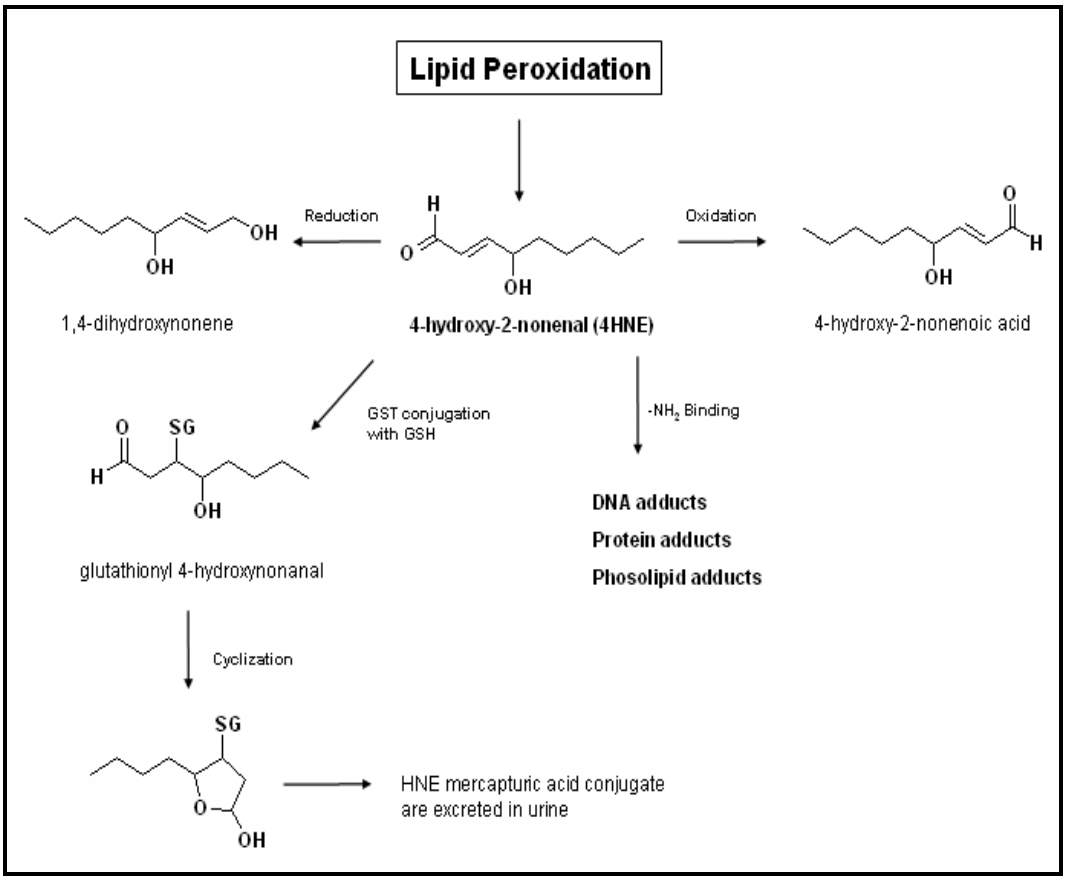


Figure 2. Enzymatic pathways of detoxification of 4HNE involve reduction via aldehyde reductase or alcohol dehydrogenase, oxidation via aldehyde dehydrogenase or conjugation with GSH via GST.

What is not known is: 1) the identity of the bass GST isoenzyme(s) involved in the high metabolism of 4HNE, 2) the enzymatic and immunological characteristics of the bass GST isoenzymes(s), and 3) genomic information on the GST gene, and specifically, the presence of regulatory elements that may confer induction by environmental compounds.

Accordingly, the specific aims and hypothesizes are the following:

Specific Aim 1: Fully characterize GST isoenzyme mediated 4HNE conjugation in bass liver.
Hypothesis: High efficiency single-enzyme Michaelis-Menten kinetics of GST-4HNE conjugation is observed in bass liver, suggesting that a single GST isoenzyme is responsible for 4HNE metabolism.

Specific Aim 2: Determine the number of major GST isoenzyme(s) in bass liver cytosol and determine if *GSTA* encodes a highly expressed cytsolic GST.
Hypothesis: Bass express multiple GST subunits, however *GSTA* is a major GST isoform in bass liver.

Specific Aim 3: Obtain at least a 1kb 5' flanking region of the *GSTA* promoter that encodes a protein that is involved in GST-4HNE conjugation. Analyze for the presence of regulatory elements that may potentially confer changes in gene expression in response to environmental chemicals.
Hypothesis: The 5' flanking region of bass *GST*A gene contains several classes of regulatory elements homologous to mammalian response elements (ARE, XRE, ERE, NF-κB, EPRE and GRE) that modulate gene transcription on exposure to environmental agents.

CHAPTER 2
EXPERIMENTAL DESIGN

Chemicals and Materials

1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), dithiothreitol (DTT), phenyl methyl sulfonamide (PMSF), bovine serum albumin and other buffers, enzymes and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO). *β*-mercaptoethanol (BME) was purchased from Fish Chemical (Farlawn, NJ). 4-hydroxynonenal (4HNE) was supplied by Cayman Chemical (Ann Arbor, MI). HPLC solvents were of analytical reagent grade and were obtained from Sigma Chemicals Co. (St. Louis, MO). Agarose (ultrapure electrophoresis grade) was obtained from Gibco/Invitrogen (Carlsbad, CA.). Immobilon polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Inc. (Bedford, MA). Primary antibodies to rat GST Ya (rGSTA1-1; alpha class GST), anti-rat GST Yb (rGSTM1-1; mu class GST) were purchased from Oxford Biomedical Research (Oxford, MI). Anti-rat GSTT1 (rGSTT1-1; theta class GST) was a gift from Dr. John Hayes from University of Dundee. Anti-rat GSTP1 (rGSTP1-1; pi class GST) was donated by Dr. Theo Bammler from University of Washington. Rabbit anti-goat IgG (conjugated to horseradish peroxidase, HRP) secondary antibodies were obtained from Sigma (St. Louis, MO). Goat anti-Rabbit IgG HRP secondary antibodies were purchased from BioRad (Hercules, CA). Enhance Chemiluminescent reagent (ECL) was purchased from Amersham-Pharmacia Corp. (Piscataway, NJ). All restriction

endonucleases were purchase from New England Biolabs (Beverly, MA). All PCR primers were synthesized by IDT DNA (Skokie, IL). The Exonuclease III / Mung Bean Deletion kit was provided by Stratagene (La Jolla, CA.).

Animals

Largemouth bass (LMB) were collected from Lake Woodruff, a non-polluted site located on a National Wildlife Refuge. Hepatic cytosolic fractions were prepared from adult, reproductively inactive (mixed sexes, aged 2-5 years) (Guillette *et al.*, 1994; Gallagher *et al.*, 2000). In addition, for some studies, aquacultured juvenile largemouth bass (200-300g) were obtained from American Sportfish Hatchery (Montgomery, Ala.). The fish were sacrificed by a blow to the head, and the brain, heart, liver, lower and upper gastrointestinal tracts, gills, and muscles were extracted, snap frozen in liquid N_2 and stored at -80^{o}C. Hepatic cytosolic fractions from brown bullheads, Sprague-Dawley rat, and adult human tissues were available from previous studies in our laboratory.

Subcellular Fractions

Affinity purification of bass liver GST

Hepatic liver cytosolic fractions were prepared as previously described (Gallagher et al. 2000) by differential centrifugation. Cytosolic fractions were prepared by using a MicroSpin^{TM} spin column and GSH Sepharose 4B matrix according to manufacturer's directions (Amersham- Pharmacia, Piscataway, NJ). For affinity purification, approximately 1.2 ml of liver cytosol was equilibrated with PBS, and 400 µl of the liver cytosolic fractions containing 5 mM DTT and 1.0 mM PMSF were applied to the purification columns. The columns were mixed gently at room temperature for 10 min, followed by centrifugation at 400 x *g* for 1 min.

The columns were then washed twice with PBS and centrifuged at 400 x *g* for 1 min, followed by elution of GST proteins with 200 µl of GSH elution buffer (GEB, 10 mM Tris-HCl, 1.4 mM BME, 150 mM reduced glutathione, pH 9.6). A second elution was performed with 100 µl of GEB and the eluates from each step were pooled. Eluates were dialyzed for 48 hr, with a change of fresh PBS every 6 hr, using a QuixSep^{TM}, micro-dialyzer system (Membrane Filtration Products, San Antonio, TX).) and Spectro-Pro membrane (MWCO 3.5 kDa, Spectrum Laboratories, IN). Protein concentrations of the affinity-purified samples were determined by the bicinchoninic-acid assay with bovine serum albumin as the standard (Smith *et al.*, 1985).

RNA isolations

Total RNA isolation from bass liver was achieved by a modified method of Chomczynski and Sacchi (1987), using the Trizol solution reagent (Invitrogen, Carlsbad, CA). Approximately 300 mg of liver was homogenized in 3 ml of Trizol and incubated at RT for 5 minutes. Approximately 200 µl of chloroform was added then transfer to a new 1.5 mL tube then equal volume of Trizol solution was added (approximately 500 µl) and incubated at room temperature for 5 minutes. An additional 500 µl of Trizol was used to further facilitate removal of proteins and other superfluous cellular materials that were not completely isolated from the first round of Trizol. The final solution was centrifuged at 12,000 g at 4°C for 15 minutes. Approximately 500 µl of isopropanol was added to the aqueous phase and subjected to another round of centrifugation. The supernatant was discarded and the pellet was washed in 75% EtOH. The purified RNA was resuspended in nuclease-free H_20 (Gibco/Invitrogen, Carlsbad,

CA.), quantified using a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA) by ∆260/280, and visualized by gel electrophoresis. RNA isolates were DNase treated (Ambion, Austin, TX) prior to first strand cDNA synthesis.

Analysis of GST catalytic activities in hepatic cytosol and GSH affinity purified cytosol

Initial rate GST enzymatic activities were performed for CDNB according to Habig et al. using a 96-well microplate reader at 340 nm and a final concentration of 1 mM GSH, and 1 mM CDNB (1981). GST activity towards 4-hydroxy-2-nonenal (4HNE) was analyzed spectrophotometrically at 224 nm and 0.5 mM GSH and 0.1 mM 4HNE. GST activity towards 4-HNE was determined according to Alin et. al, (Alin *et al.*, 1985) as modified by Gallagher et al.(1998). All GST catalytic activity assays were carried out in triplicate at 30°C and were corrected for non-enzymatic activity. GST-CDNB and GST-4HNE activities were determined using bass hepatic cytosol and GSH affinity-purified bass hepatic cytosol. Michaelis-Menten enzyme parameters (K_{max} and V_{max}) values were determined by non-linear regression analysis of GST-CDNB and GST-4HNE rate activities using Sigma Plot enzyme kinetics software (SPP Inc, Chicago, IL). A detailed kinetic analysis of the rates *in vitro* GST activity toward CDNB was performed using a broad range of electrophile concentrations (CDNB, 0.040, 0.080, 0.160, 0.320, 0.640, 1.280, 2.560, and 5.120 mM) and a fixed nucleophile concentration (GSH, 50 mM). A detailed kinetic analysis of the *in vitro* GST activities toward 4HNE was performed using a broad range of 4HNE concentrations (0.006, 0.12, 0.24, 0.48, 0.96, 0.196, and 0.392 mM) and a fixed

GSH concentration of 5 mM. All values were expressed as nmol of substrate conjugated/min/mg cytosolic protein.

Immunological cross-reactivity of bass GST with mammalian class-specific GST antibodies

Largemouth bass and male Sprague-Dawley rat cytosolic proteins (100 µg and 10 µg respectively) were separated on SDS-polyacrylamide gels (16% acrylamide, 0.09% N,N-bis acrylamide) and transferred onto Immobilon PVDF membranes. Non-specific binding was blocked by incubation of the membranes in 5% dried-milk powder in tris-buffered saline (TBS). Primary antibodies to rat (rGSTA1-1, rGSTM1-1, rGSTT1-1, and rGSTP1-1) were diluted at 1: 3000 in 5% dried-milk in TBST (TBS containing 0.1% Tween 20). Secondary antibodies (rabbit anti-goat IgG conjugated to horseradish peroxidase, HRP) was used on blots containing Ya, Yb, YB class GST, while goat anti-rabbit IgG HRP was used on the blot containing the T1 class GST antibody. All secondary antibodies were diluted at 1:10,000 with ECL as the detection reagent (Amersham-Pharmacia, Piscataway, NJ). The blots were visualized using a Flour-S Multimager and analyzed by Quantity-One Software (BioRad, Hercules, CA).

Identification of the Major Bass Liver GST Subunits

SDS-PAGE and GST Subunit Analysis

GSH affinity purified cytosolic proteins (0.14 µg, 0.26 µg, and 0.55 µg) were separated on SDS-polyacrylamide gels (12% acrylamide, 0.09% N,N-bis acrylamide) and visualized by Coomassie Blue staining. Separation of affinity purified hepatic GST isoforms was accomplished by high performance liquid chromatography (HPLC) as described by (Rowe *et al.*, 1997) with minor

modifications. Reverse-phase HPLC was used to characterize the GST subunit composition using a 150 x 4.6 mm Vydac C4 column (Grace Vydac, Hesperia, CA). Samples (approximately 60 µg) were mixed with equal volume of 0.075% trifluoroacetic acid (TFA) and injected onto the column attached to a Perkin Elmer 200 series HPLC system. The HPLC system was equilibrated with 37% (v/v) acetonitrile in 0.075% TFA. The column flow rate was 1.5 ml/min with 37-43% (v/v) gradient of acetonitrile containing 0.075% TFA over 25 minutes. This was followed by a linear increase of 43-55% (v/v) gradient of acetonitrile containing 0.075% TFA between 25-45 minutes. Polypeptide peaks were detected with a diode array detector monitoring absorbance at 214 nm. Peak area integrations were performed using Perkin Elmer Turbochrom Software. The HPLC fractions (polypeptide peaks) were collected by hand and the polypeptides were dried under reduced-pressure in a Speed-Vac centrifuge overnight to remove the TFA.

HPLC Mass Spectrometry with Electrospray-ionization Analysis

The molecular weights of the affinity-purified bass hepatic GST proteins were determined by HPLC mass spectrometry (HPLC-MS) with electrospray-ionization (ESI). The GST proteins (approximately 60 µg) were dissolved in equal volume of water containing 0.075% trifluoroacetic acid and injected at a rate of 1.5 ml/min into the ESI ion source. Positive ion ESI-mass spectra were acquired using a Thermo-Finnigan 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

acquired at 48 minutes and deconvoluted using ThermoFinnigan Navigator 1.2 software (ThermoFinnigan, Austin, Texas, USA). The HPLC fractions were subjected to Endo-proteinase Lys-C digestion and standard Edman degradation protein sequencing using Applied Biosystem Pro-Cise 494-HT sequencer by the UF Core Protein Facility to reveal amino acid sequence information.

Tissue-Specific Expression of GSTA mRNA

First strand cDNA synthesis from liver, gonad, upper and lower gastrointestinal tract, heart, brain, and muscle were prepared using Retroscript (Ambion, Austin, TX). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the house keeping gene. Primers were designed by Oligo software (Molecular Biology Insights, Cascade, CO.) to amplify partial cDNA encoding bass liver GAPDH (475 bp) and also a 776 bp fragment of bass liver GSTA. The GAPDH sequence was amplified with primers (forward primer 5'-CGCATCGGTCGTCTGGT and reverse primer 5'-AATGATGCCGAAGTTGT-3'). Bass GSTA primers (forward primer sequence 5'- CATGGCTAAGGACATGATC-3' and reverse primer sequence 5'- GATTGCACTGCTCTGACCG-3') produced a 776 bp partial fragment based on the full-length bass GSTA cDNA previously cloned and expressed by Dr. Doi (2002). These primer sets were used in a multiplexed PCR reaction in which GAPDH and GSTA primers were used to amplify both products in a single reaction. Each multiplexed 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, 100 ng of GAPDH and GST primers and 5 U of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad,CA). The PCR thermocycler parameters were: 30 cycles (94^oC for 30

sec, 56°C for 1 min, 72°C for 30 sec) and final extension of 72°C for 5 minutes. The PCR products were separated on 2% ethidium bromide agarose gel and visualized using a Flour-S Multimager system (BioRad, Hercules, CA).

Sequence Analysis of the *GSTA* Gene

Initial characterization of bass liver genomic sequences

The Universal GenomeWalker kit (Clonetech Inc., Palo Alto, CA) was used to isolate the 5' flanking region and other genomic sequences of the bass *GSTA* gene. Genomic DNA was isolated from snap frozen bass liver using the Wizard genomic DNA purification kit (Promega Corp., Madison, WI). Bass genomic DNA was subjected to an overnight digestion (to ensure complete digestion) with the following blunt-end endonuclease enzymes: Dra I, EcoR V, Pvu II, and Stu I. Following the overnight digestion, each restriction-digested bass genomic DNA fragment was subjected to a phenol / chloroform extraction to remove proteins and restriction enzymes. An additional round of chloroform extraction was used to remove residual phenol. Each batch of digested genomic DNA fragment ends was ligated into a GenomeWalker adaptor (provided in the kit) to make GenomeWalker "libraries". The GenomeWalker adaptor is a 52-mer oligonucleotide that has complementary sites for the forward adaptor primers (AP1 and AP2, provided in kit) to be used in primary and nested PCR reactions.

The GenomeWalker adaptor has three design features that are critical to the success of the PCR reaction: 1) the use of a 5' extended adaptor has no binding site for adaptor primer 1 used in primary PCR, 2) the addition of amino group at the 3' end to prevent primer dimerization with the adaptor primer, and 3) the use of a adaptor primer that is shorter than the adaptor itself would cause

"suppression PCR". The GenomeWalker DNA walking requires eight primary and secondary (nested) PCR amplications: four experimental genomic DNA libraries, two positive controls (positive control with human pre-constructed library, and the second positive control library from control human genomic DNA), and two negative controls (no genomic library templates were used in the PCR reactions) (Figure 3).

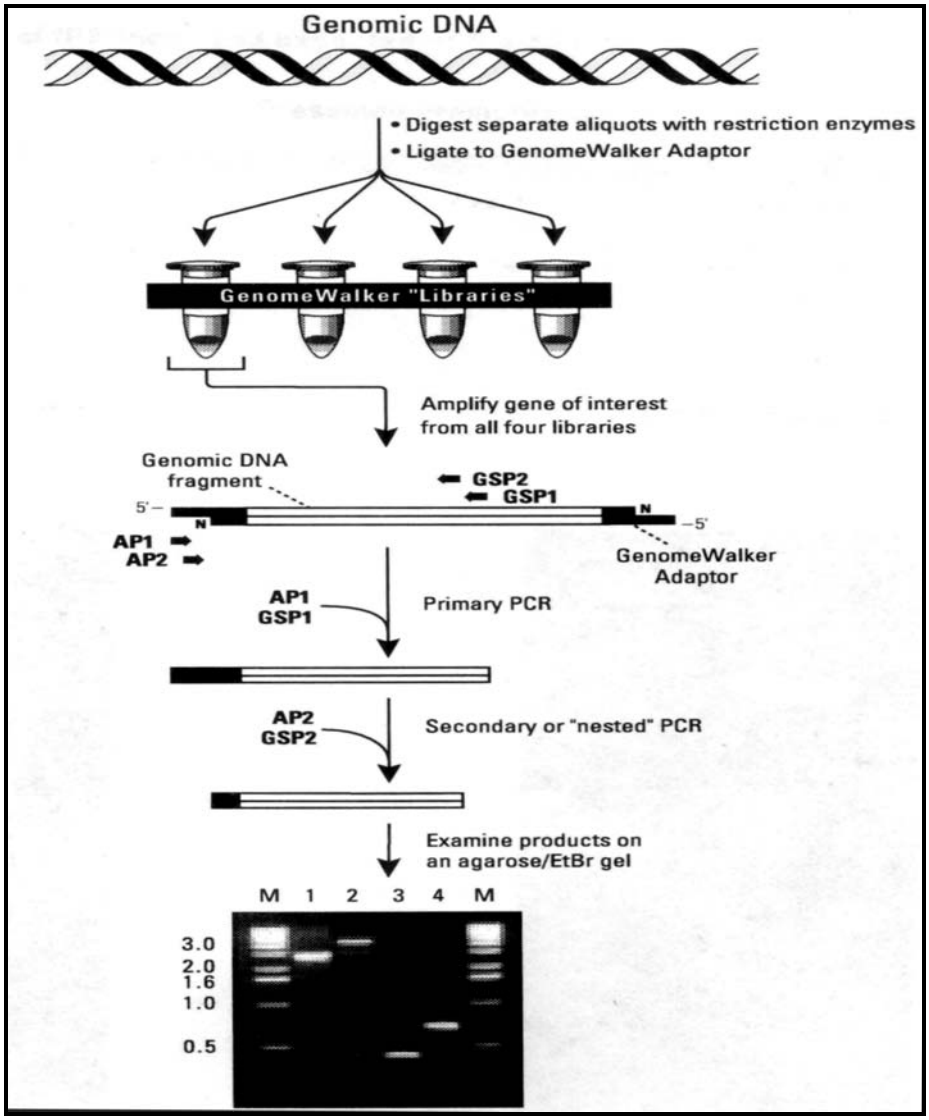


Figure 3. Flowchart of the GenomeWalker protocol for the isolation of GST genomic clones.

The first positive control reaction was performed using a pre-constructed human library to test the Advantage LD PCR polymerase and thermocycler parameters, while a second positive control with human genomic DNA was used to test the endonuclease digest reactions and GenomeWalker adaptor ligation reactions. After the primary and nested PCR reactions, all positive controls should reveal a 1.5 kb product.

Since the GenomeWalker kit is based upon PCR-based reactions, gene-specific reverse primers (GSPs) are required and that it must be designed based on a known sequence of the target gene. Previously, a full-length bass liver GSTA cDNA was cloned and expressed (Doi *et al.*, 2002) which was used to derive the primary (GSP1s) and secondary primers (GSP2s)(Table 1).

Table 1: Gene-specific primers used for primary and secondary PCR

Gene-specific primary reverse primers	Position of primer*	Gene-specific secondary reverse primers	Position of primers*
GSP1-A: TTT GTT TCC CTG GGA CTT GAA CTC ACT CTC	299-329	GSP2-E: AGG ACT CAT TCA GGA CT TGT TTC CAT GTT	249-279
GSP1-B: CTT GAA CTC ACT CTC CAG GTA CAA GCA GGC	284-314	GSP2-F: GGG ATT CAT GTC CAT CAC TTC CTG TGA CTT	197-227
GSP1-C: CAG GTA CAA GCA GGC AGC ATA GGA CTC AAT	269-290	GSP2-G**: ACT TCC TGT GAC TTG TGC TCC ATT TTA TCA**	181-210
GSP1-D**: ACT TCC TGT GAC TTG TGC TCC ATT TTA TCA**	181-210	GSP2-H: CCC CAC AGC AGA GTC ATG TCC TTA GCC	11-41

* Position of primers corresponds to nucleotide bases of the full-length bass GSTA cDNA (957bp)
** GSP1-D and GSP2-G are equivalent.

The gene-specific primers were designed by using Oligo Software (Molecular Biology Insights, Cascade, CO.). The nested PCR products were visualized on a 1.5% ethidium bromide agarose gel and purified by a gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were cloned into pGEM T-easy vector (Promega Corp., Madison, WI) and submitted for nucleotide sequencing to University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) DNA Sequencing Core (Gainesville, FL). Upon receiving sequence information, ClustalW software was used to align the sequenced fragments with the plaice *GST* gene cluster, and the BLAST nucleotide search engine was used to identify the sequence fragments.

Nested deletion analysis of GST genomic clones

Exonuclease III and the Mung Bean Deletion Kit were used to make unidirectional nested deletions on clones derived from GenomeWalker analysis (Stratagene, La Jolla, CA). One of the criteria was to select the appropriate restriction endonucleases to linearize the pGEM T-easy vector plasmid. Exonuclease III will progressively digest the 3' end of double-stranded DNA or blunt ends, but can not efficiently initiate digestion at a 3' overhang end or a 5' overhang end that is filled-in with α-thio dNTP's. To create deletions in the insert but not in the vector, the plasmids of interest were linearized by a double-digestion with a 3'-overhang restriction endonuclease and a 5'-overhang restriction endonuclease to create a substrate for unidirectional exonuclease digestion by Exonuclease III. According to the reference restriction sites of the pGEM T-easy vector, there are several unique restriction sites that can used on both sides of the inserted DNA (Figure 4). Since several 3' overhang restriction

enzymes digested the insert of the plasmid, two 5'-overhang restriction enzymes were selected: Nde I and Spe I.

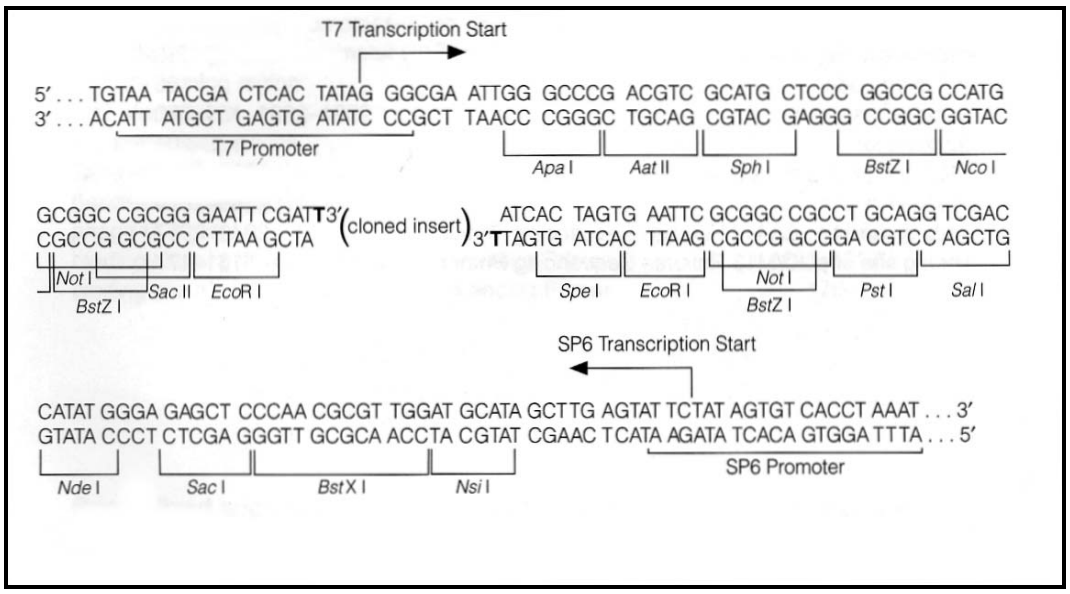


Figure 4. The promoter and multiple cloning sequence site of the pGEM T-easy vectors.

Since two 5'-overhang restriction endonucleases were used on the same side of the insert, a thioderivative fill-in with Klenow fragment was required to protect one of the sites from Exonuclease III digestion. Approximately 25 µg of clone 2F (5.5 kb inserted DNA) was digested in 500 µl reaction with Nde I for 3 hr at 37°C. After the 2F clone was completely digested, a 5' overhang fill-in reaction was performed using 1mM thio-dNTP mix and 5 U of Klenow fragment and incubated at room temperature for 10 minutes. After the fill-in reaction, the reaction was extracted using a phenol / chloroform and EtOH to remove residual restriction enzymes. Verification of the thioderivative filled-in reaction was achieved by incubating 1 µg of filled-in DNA with 20 U of Exonuclease III for 15 minutes at 37°C and visualization using a 1% ethidium bromide agarose gel. After the first digestion, the filled-in DNA was subjected to a second round of 5'-

overhang restriction digest using Spe I and another round of a phenol / chloroform and EtOH extractions as described above.

The length of restriction digested DNA converted from double-stranded to single-stranded by Exonuclease III is controlled by the reaction temperature and time of incubation. At 23°C, Exonuclease III can digest approximately 500 base pairs per 4 minutes. Accordingly, ten time points (500 bp x 10 = 5.0 kbp) were selected for analysis with each time point reaction consisting of 5.0 µg of double-digested DNA, 2X Exo III buffer, and 100 mM BME. Each time point reaction was initiated by 100 U of Exonuclease III. At every 4 minutes, aliquots were removed and heated to 68°C for an additional 15 minutes. Mung bean nucleases (45 U) were added to each time point reaction and incubated at 37°C for an additional 30 minutes. Prior to ligation, a modified version of the phenol / chloroform extraction procedure which includes 1m Tris-HCL, 8M Li-Cl, and 20% SDS was used to remove any residual Mung Bean nucleases. The Exonuclease III / Mung Bean nuclease-treated DNA was subjected to overnight ligation at 4°C and transformed into JM109 cells following the protocol from pGEM T-easy vector system (Promega, Madison, WI). Blue-white colonies were screened and plasmids from each time point reactions were purified using Wizard mini-prep kit (Promega, Madison, WI). The purified plasmids were sequenced at the University of Florida DNA Sequencing Core.

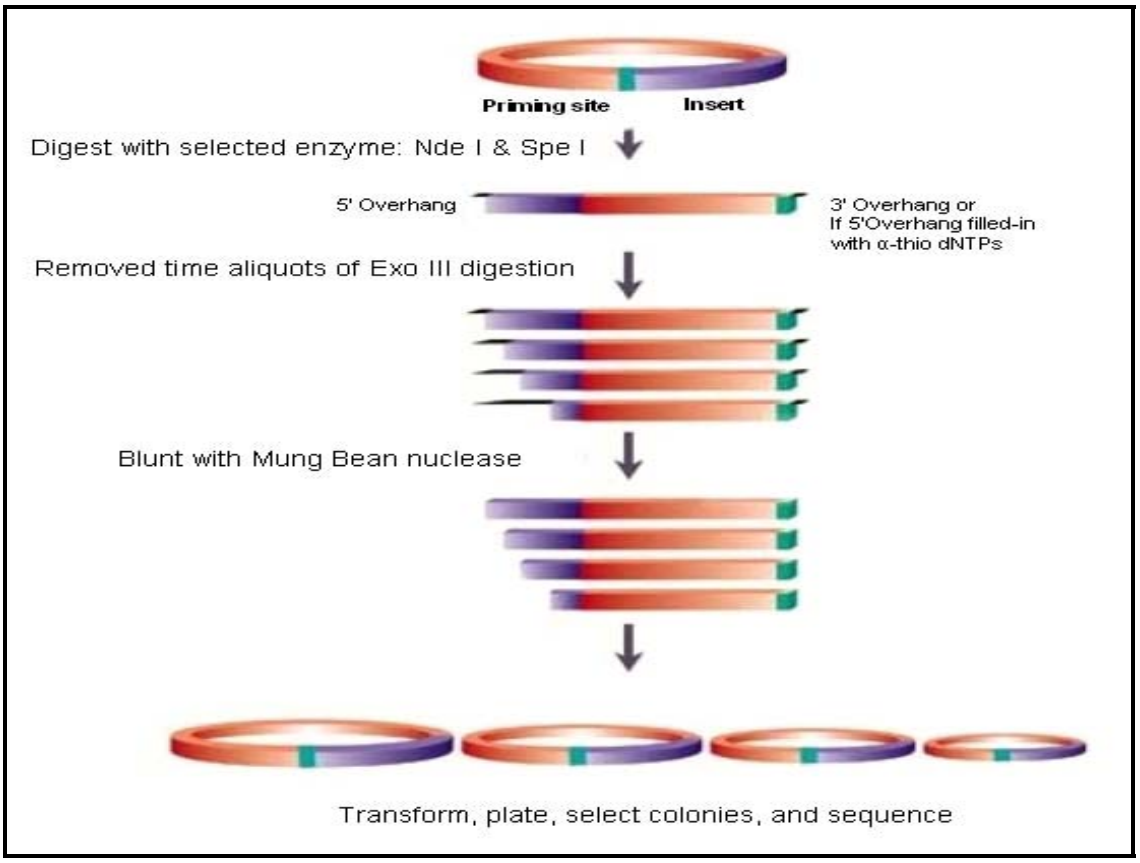


Figure 5. Exonuclease III and Mung Bean deletion kit system.

CHAPTER 3
RESULTS

GST Catalytic Activities of Bass and Other Species

A comparison of GST activities in largemouth bass and other species (brown bullhead catfish, rat, and human adult) is presented in Figure 6. No sex-related differences in GST-4HNE activities were observed among adult male and female bass (415±83 nmol 4HNE conjugated/min/mg and 415±45 nmol 4HNE conjugated/min/mg, respectively). The initial kinetic rates of hepatic cytosolic GST-4HNE activities were highest in rat liver (661±26 nmol 4HNE conjugated/min/mg cytosolic protein), followed by bass liver (male: 415±83 nmol 4HNE conjugated/min/mg cytosolic protein), brown bullhead liver (187±26 nmol 4HNE conjugated/min/mg cytsolic protein) and human liver (5 nmol 4HNE conjugated/min/mg cytsolic protein) (Figure 6). The GST-4HNE / GST-CDNB activity ratios, a numerical value which describes the relative proportion of total GST activity dedicated to 4HNE conjugation were two-fold higher in bass than rat and eleven-fold higher than in brown bullhead catfish. Furthermore, the high GST-4HNE activity of the GSH affinity purified hepatic cytosol (22,904 nmol 4HNE conjugated/min/mg cytosolic protein) was fifty-five fold higher than observed in bass hepatic cytosol (415 nmol 4HNE conjugated/min/mg cytosolic protein) which indicates that the GST isoenzyme(s) responsible for 4HNE metabolism could be readily purified by traditional GSH-affinity chromatography.

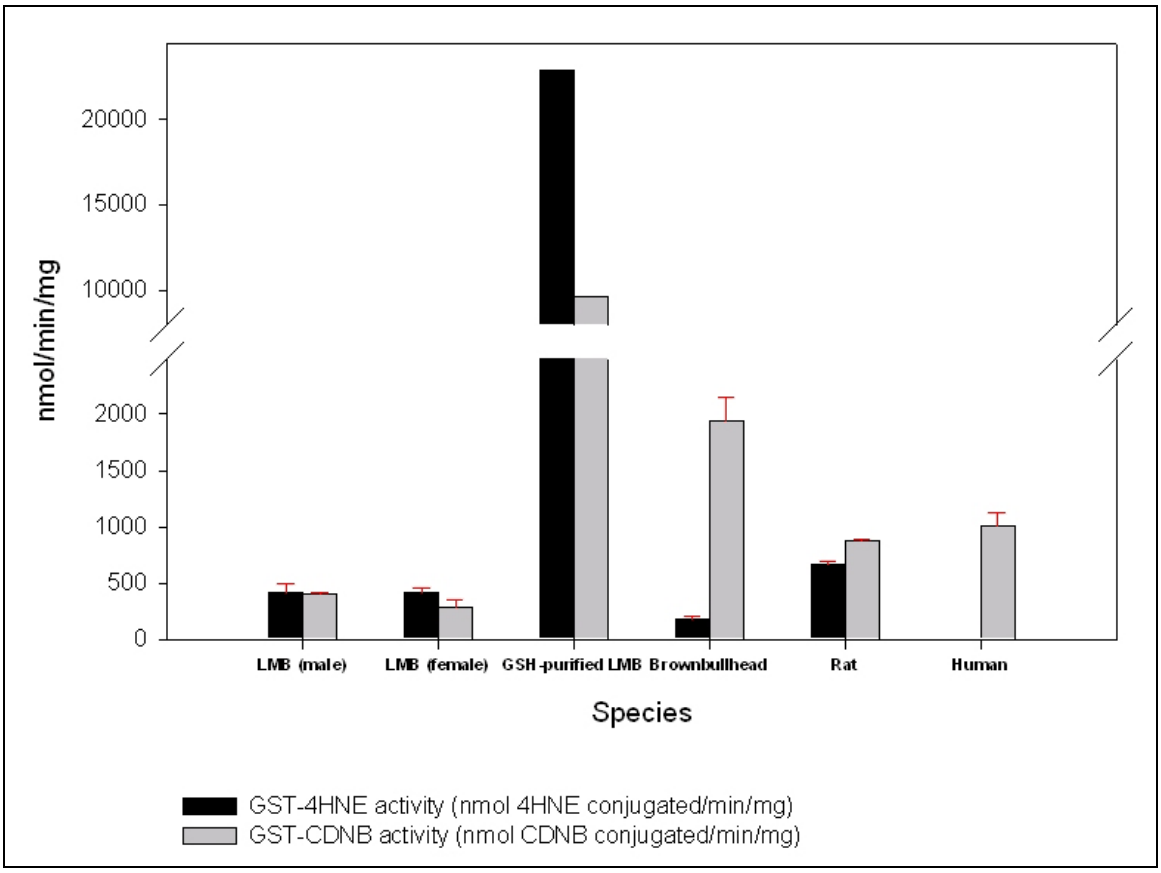


Figure 6. Comparison of hepatic GST-4HNE and GST-CDNB activities in largemouth bass and other species. Data represent the mean ± S.E.M of 3 individuals for LMB, brown bullhead, and rat while the data for GSH affinity purified fractions were analyzed from triplicate determinations. The human data was based upon upper bound of assay limit of detection of 5 nmol 4HNE conjugated/min/mg cytosolic protein.

Enzyme Kinetic Analysis of CDNB and 4HNE Conjugation in GSH Affinity-purified Hepatic Cytosolic Proteins

Because velocity versus substrate (V vs. S) plots do not clearly discriminate departures from linearity, an Eadie-Hofstee plot (V vs. V/S) was used to further analyze the GST-CDNB enzyme kinetics data (Figure 7A, 7B). The Eadie-Hofstee plot demonstrated biphasic reaction kinetics suggesting two or more GST isoenzymes may be contributing to baseline CDNB conjugating activity (Figure 7B). Non-linear regression analysis of GST-CDNB rate data points was

used to calculate the apparent kinetics parameters. The kinetics data did not fit a single-enzyme Michaelis-Menten model, however a two-enzyme Michaelis-Menten model: $V= (V_{max1} * S) / (K_{m1} +S) + (V_{max2} * S) / (K_{m2} + S)$, provided a stronger fit for the GST-CDNB activities data. The apparent K_m values for K_{m1} and K_{m2} (741 ± 63.4 µM, 658 ± 51.0 µM, respectively) and the apparent V_{max} values for V_{max1} and V_{max2} (29 ± 0.829 µmol CDNB conjugated/min/mg, 28 ± 0.716 µmol CDNB conjugated/min/mg, respectively) (Figure 7, R^2= 0.978) were calculated by non-linear regression analysis.

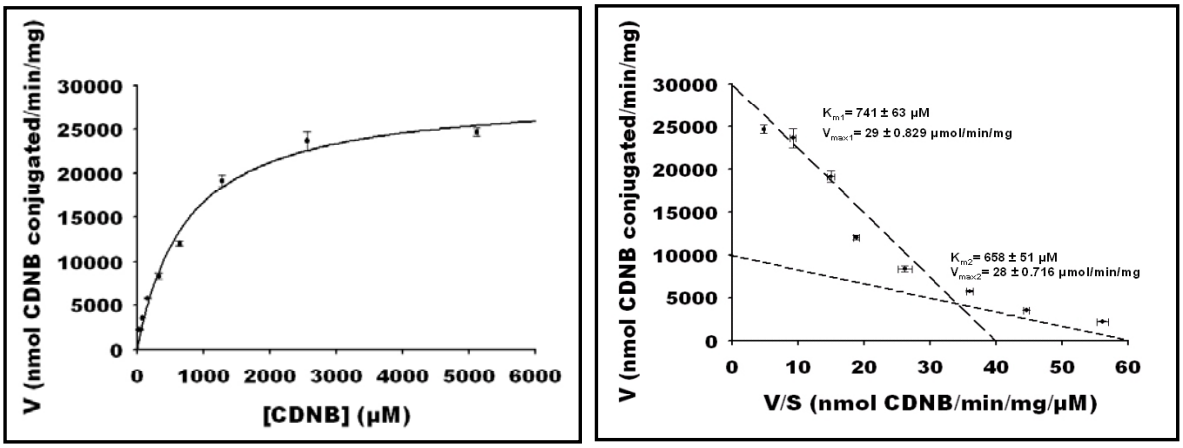


Figure 7. Initial rate enzyme kinetics of GST-CDNB activities in GSH affinity purified bass cytosol. A) V-versus-S plot. B) Eadie-Hofstee plot.

A velocity versus substrate plot (V vs. S) using 4HNE as a substrate showed a linear Michaelis-Menten enzyme kinetics plot (Figure 8A) and non-linear regression analysis yielded an apparent K_m and apparent V_{max} values of 18.9 $\pm$1.3 µM and 24 $\pm$0.5 µmol 4HNE conjugated/min/mg, respectively. An Eadie-Hofstee plot (V vs. V/S) of the largemouth bass GST-4HNE reaction kinetics data substantiated a linear relationship among substrate concentration and reaction velocity, suggesting the presence of single GST isoenzyme with

high affinity towards 4HNE metabolism present in bass liver (Figure 8B,R^2= 0.984).

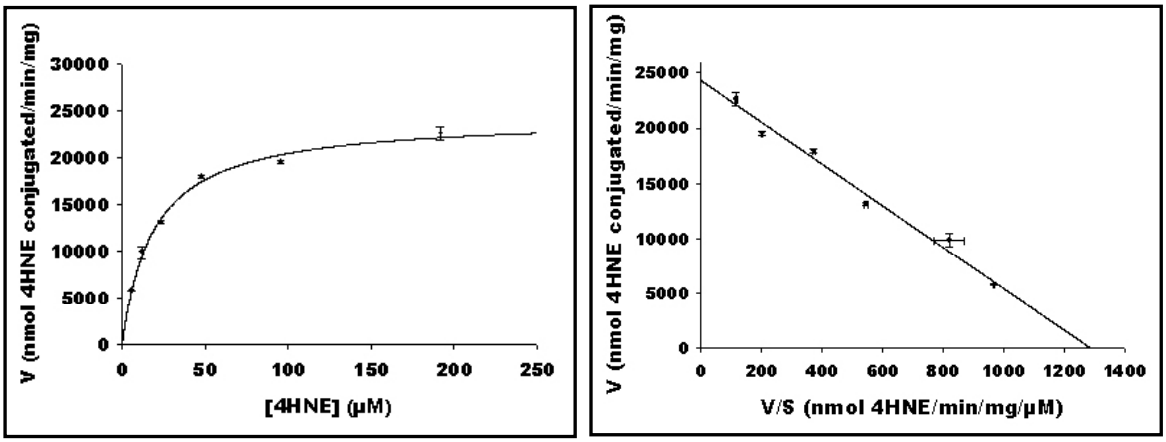


Figure 8. GST-4HNE initial rate kinetics. A) V-versus-S plot follows linear Michaelis-Menten kinetics B) Eadie-Hofstee plot shows a monophasic reaction.

Western Blot Analysis of Bass Cytosolic Proteins

Western blotting analysis was used to determine cross-reactivity and overall structural relationships between bass hepatic GST and the better-characterized rodent GSTs. As observed in Figure 9, there was no strong cross-reactivity among LMB cytosolic proteins when probed against antibodies rat μ class GST (rGSTM1-1), rat π class GST (rGSTP1-1), and rat θ class GST (rGSTT1-1). However, weak cross-reactivity was observed when LMB cytosolic proteins were probed with an antibody against rat α class GST (rGSTA1-1).

Hepatic GST Subunit Analysis

SDS-PAGE analysis of the GSH affinity-purified bass hepatic cytosol GST protein revealed the presences of two GST isoenzymes with molecular weights of 30 kDa and 27 kDa, respectively (Figure 10) which confirmed the observed biphasic reaction rates of GST-CDNB kinetics activity.

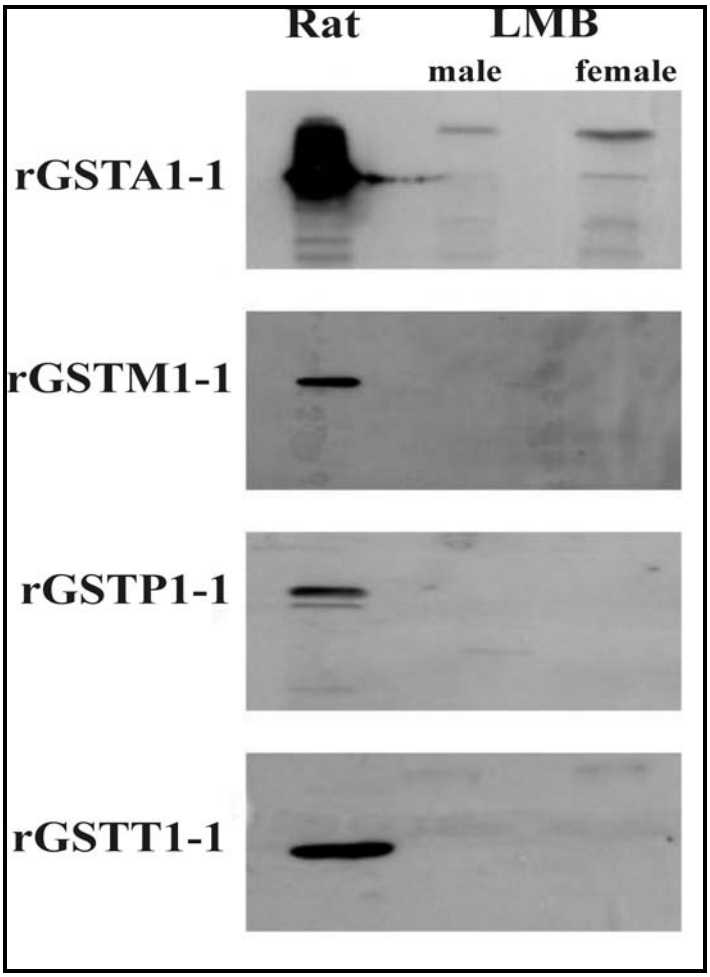


Figure 9. Western blot analysis of largemouth bass hepatic cytosolic proteins (100 µg protein per lane) using polyclonal antibodies against rodent α class GST (rGSTA1-1), rodent µ class GST (rGSTM1-1), rodent π class GST (rGSTP1-1), and rodent θ class GST (rGSTT1-1). Positive controls (5 µg rat cytosolic protein) are included in each blot.

As observed in Figure 11, HPLC-GST subunit analysis revealed the presence of at least two major hepatic fractions eluting at retention time of 11.53 minutes and 34.1 minutes (peak 1 and peak 2, respectively). Based on area under curve (AUC) and assuming similar extinction coefficients for the GST subunits, the first major peak (peak 1) constituted approximately 80% of the total affinity purified cytosolic GST mass. HPLC-MS with electrospray-ionization

yielded molecular weights of 26.3 kDa and 25.8 kDa for peaks 1 and 2, respectively.

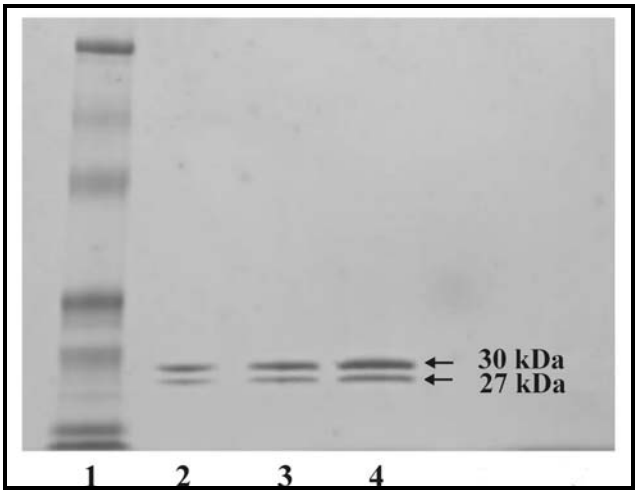


Figure 10: SDS PAGE analysis of GSH-affinity purified bass cytosol. Lane 1, Kaleidoscope marker; Lanes 2-4; 0.14 µg, 0.26 µg and 0.55 µg of GSH affinity-purified bass liver cytosolic protein, respectively.

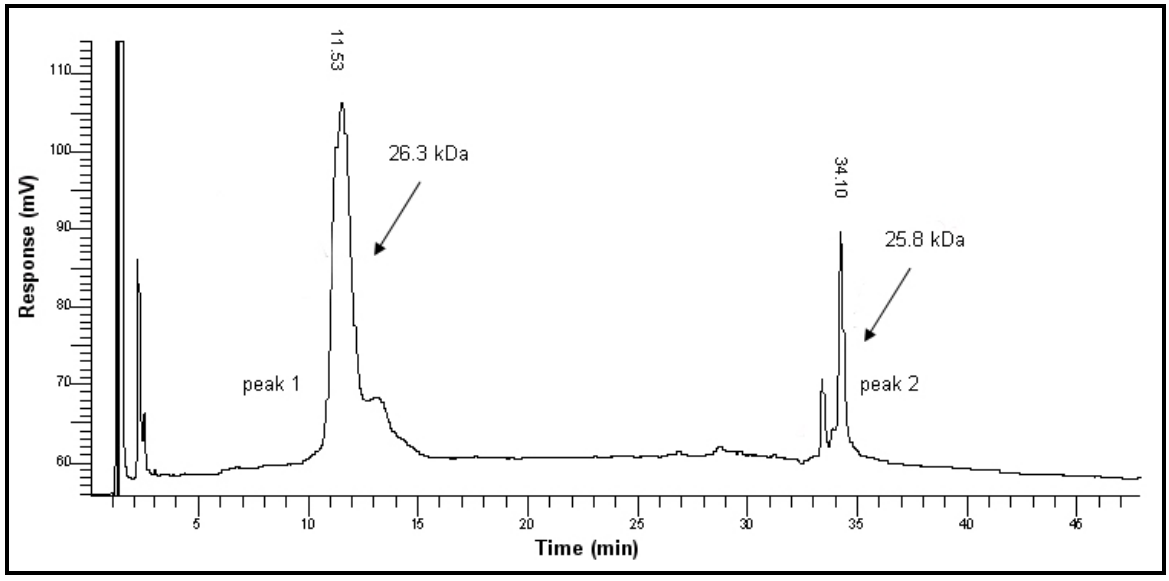


Figure 11. HPLC chromatographic analysis of hepatic GST subunit analysis of bass GSH affinity purified fractions resulted in the elution of two major peaks (peak 1 and peak 2).

Identification and Sequencing of Cytosolic GST Subunit Encoding GSTA

Concurrent with the previous study, Dr. Doi used 5' and 3' systems of rapid amplification of cDNA ends (RACE) of a previously isolated partial bass GSTA

cDNA sequence (Doi *et al.*, 2003) to clone and express the full-length GSTA cDNA clone from bass liver. Sequencing analysis showed that the bass GSTA clone was 957 base pairs in length, and containing an open reading frame of 678 bp, encoding a polypeptide of 225 amino acids with 85% identity to plaice GSTA (Figure 11).

```
LMB   1  MAKDMTLLWGSGSPPCWRVQIALEEKSLQGYNQKLLRFDKMEHKSQEVMDMNPRGQLPAFKHGNNVLNESYAACLYLESE

PL    1  MAKDMTLLWGSGSPPCWRVMIVLEEKNLQAYNSKLLSFEKGEHKSAEVMSMNPRGQLPSFKHGSKVLNESYAACMYLESQ

LMB  81 FKSQGNKLIPDCSAEKALMYQRMFEGLTLNQKMADVIYYNWKVPEGERHDSAVKRNRDVLSAEVKLWEGYLQKASGSFFA

PL   81 FKSQGNKLIPDCPAEQAMMYQRMFEGLTLAQKMADVIYYSWKVPEAERHDSAVKRNKENLSTELKLWEEYLQKTSGSFVA

LMB 161 GKNFSLADVTVYPSIAYLFHFGLCEERYPKLAAYYNSNKDRPSIKATWPPTWLENPQGQDQLKDI

PL  161 GKSFSLADVSVFPGVAYLFRFGLTEERYPQLTAYYNSLKERPSIKASWPPTWLESPQGQDMLKDV
```

Figure 12: A comparison of bass GST and plaice GSTA amino acid sequence data.

Endo-proteinase Lys-C digestion and Edman degradation protein sequencing of the major GST isoenzyme (peak 1) revealed a 14 amino acid residue of **ATWPPTWLENPQGQ**. Submission of this amino acid sequence using the BLAST protein search engine revealed that the plaice GSTA protein sequence (**ASWPPTWLESPQGQ**) at amino sequence number 206-219 had 85% identity to this sequence (peak 1) (Figure 11).

Tissue-specific Expression of Bass GSTA

The GST fragment amplified by multiplex-ed PCR using cDNA from various tissues of bass was approximately 776 bp in length. The bass GSTA mRNA was expressed in the liver, gonad, upper gastrointestinal tract, and brain tissue. No detectable GSTA mRNA expression was observed in heart, lower gastrointestinal tract, or muscle tissue (Figure 14).

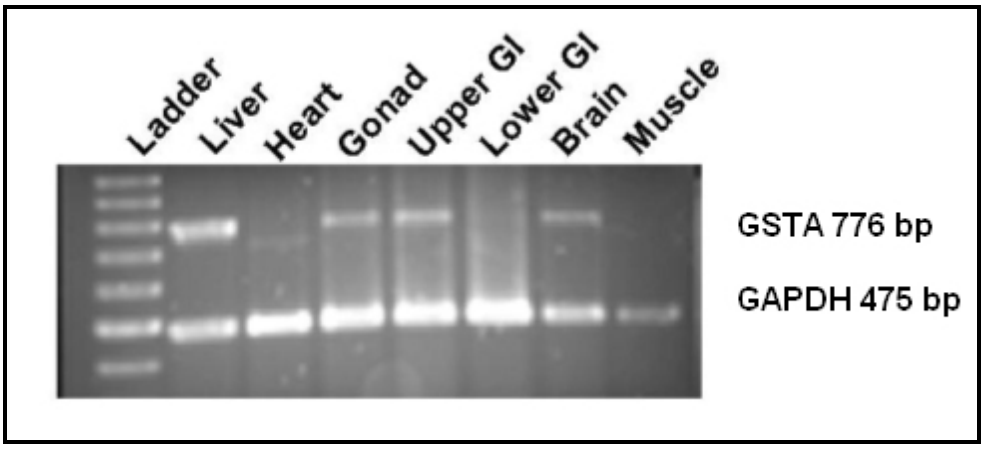


Figure 13. Tissue-specific expression of GSTA mRNA in largemouth bass. Multiplexed PCR revealed the amplification of 776 bp cDNA of bass *GSTA* and 475 bp of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Bass GSTA mRNA was expressed in liver, gonad, upper gastrointestinal tract, and brain.

Isolation of Genomic Clones and Analysis of 5' Flanking Region of the Bass *GSTA* Gene

GenomeWalker Analysis

Primary and nested PCR reactions with bass liver genomic DNA and *GSTA* gene-specific primers revealed several clones (Figure 14) termed fragment 1E (1.3 kb),1F (1.1 kb), 1H (1.0 kb) and 2F (5.5 kb). These DNA fragments were separated on 1% ethidium bromide agarose gel (Figure 14).

The fragments were gel purified and cloned into pGEM T-easy vector for sequence analysis. Sequences analysis revealed that clone 1F-SP6 (1297 bp) shared 93% identity to the 2.5 - 3.4 kb region of the plaice *GSTA2* and clone 2F-SP6 (1223 bp) was 86% identical to the 4.2 - 5.4 kb region of *GSTA1* (Figure 15). Further genomic analysis revealed that clone 2F-T7 (1192) was 80% identical to the 9.0 -10.0 kb region of *GSTA* and clone 1E-SP6 (1256) was 88% identical to the 9.7 – 11.0 kb region of plaice *GSTA* (Figure 15).

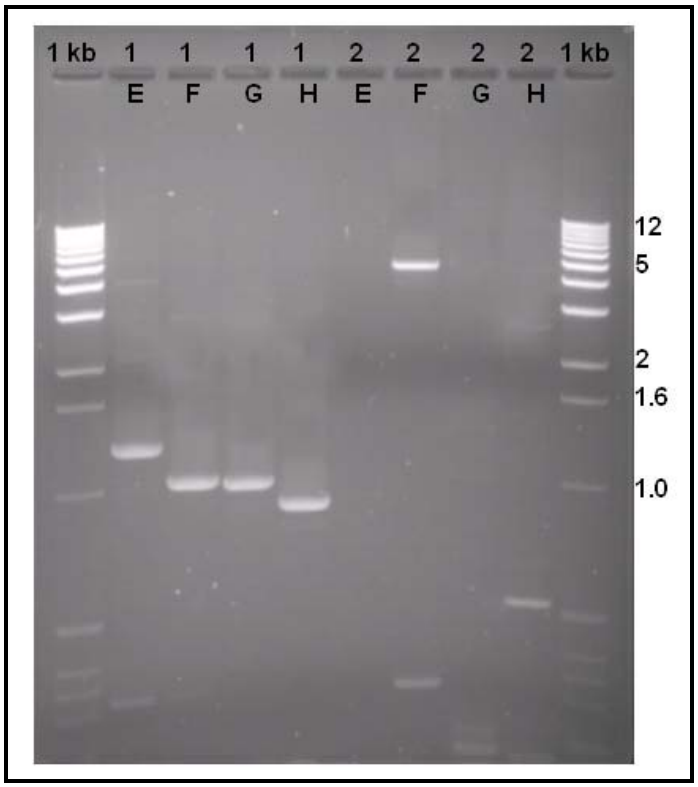
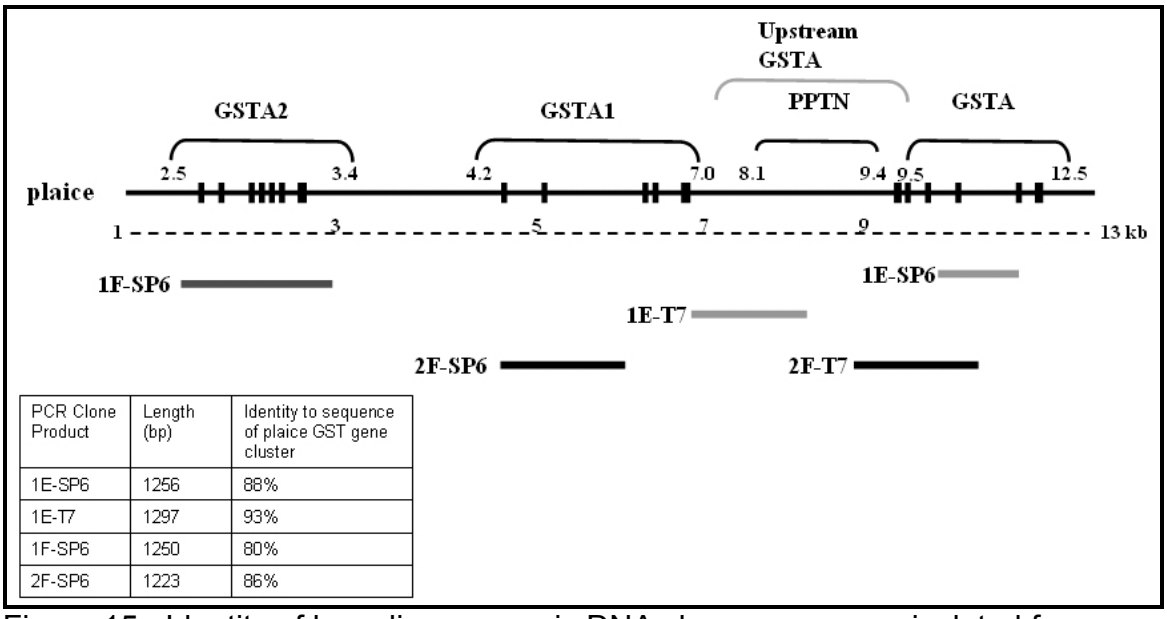


Figure 14. Secondary (nested) PCR revealed several potential bass *GSTA* clones. Clones 1E (1.3 kb), 1F (1.1 kb), 1H (1.0 kb) and 2F (5.5 kb) were gel purified and cloned.



PCR Clone Product	Length (bp)	Identity to sequence of plaice GST gene cluster
1E-SP6	1256	88%
1E-T7	1297	93%
1F-SP6	1250	80%
2F-SP6	1223	86%

Figure 15. Identity of bass liver genomic DNA clone sequences isolated from nested PCR and nucleotide sequence alignment with plaice *GST* gene cluster.

Exonuclease III / Mung Bean Nested Deletion Analysis

Exonuclease III and Mung Bean nuclease were used to create unidirectional nested deletions into the 2F clone (5.5 kb DNA plasmid) so the complete nucleotide sequence could be identified. Of the ten time point reactions, only time point reactions (1, 2, 3, 4, 5) were successfully sequenced. Using the BLAST two alignment analysis, nucleotide sequences of each time point reactions (1, 2, 3, 4, 5) showed an obvious overlap with a total nucleotide sequence of 2841 base pairs (Figure 16).

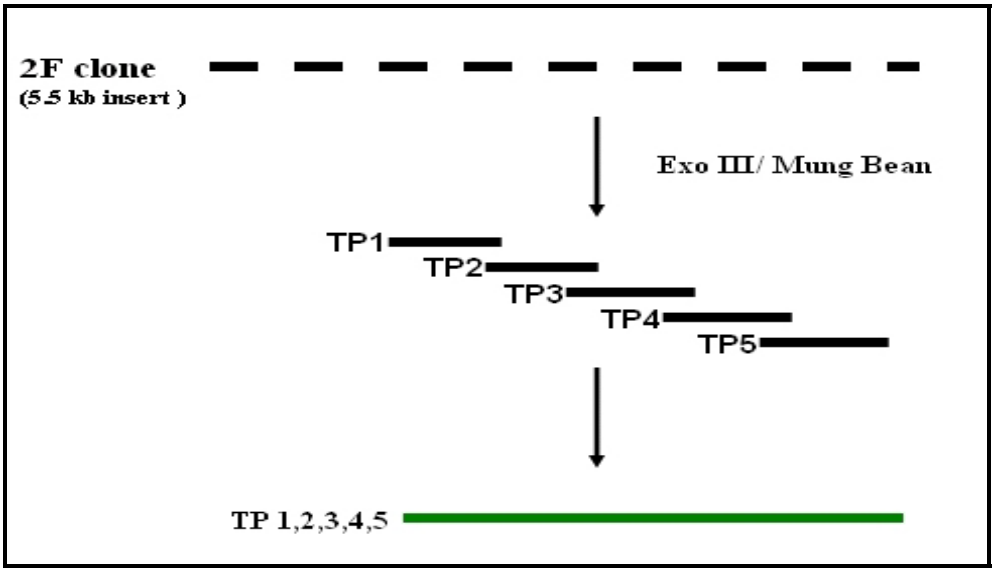


Figure 16. A schematic diagram of the Exonuclease III / Mung Bean nested deletions of the clone 2F.

Sequence alignment analysis of the nested deleted 2F clone sequences revealed a 40% identity to 6.8 kb to 9.2 kb of the 13 kb plaice GST gene cluster, which covers the middle of plaice *GSTA1* gene to plaice *GSTA* gene (including the upstream region of *GSTA*) (Figure 17 and Appendix A).

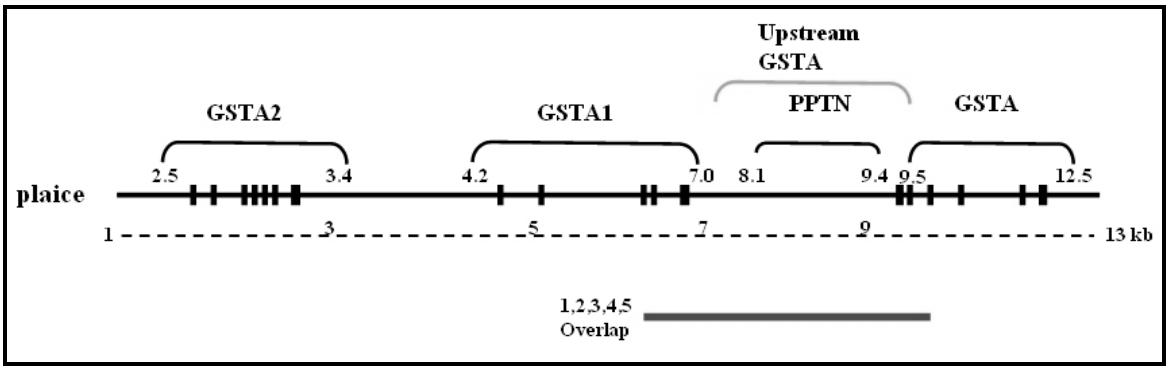


Figure 17. Nucleotide sequences of nested deleted 2F clone alignment with plaice GST gene cluster.

Nucleotide Sequence Analysis of Promoter Region of Bass *GSTA*

Table 2 shows a compilation of specific DNA motif sequences showing several classic response elements that responds to environmental or hormonal agents (Rushmore *et al.*, 1991; Hayes and Pulford, 1995).

Table 2. Sequence-specific DNA motifs important in the regulation of GST

Response Element	Consensus Sequence	Examples of genes regulated
XRE	TWGCGTG	Cytochrome P450 (1A, 1B), GSTs, quinine reductase, UDP- glucuronosyl- transferase
EPRE	TGACNNNGC	Heme oxygenase, GSTs, UDP-glucuronosyl- transferase, quinone reductase, γ-glutamylcysteine synthetase
ARE	TGACAAAAGC	Similar to those regulated by EPRE
ERE	GGTCANNNTGACC	Estrogen-responsive finger protein, vitellogenin, estrogen receptors
NF-κb	GGGRTNNCC	γ-glutamylcysteine synthetase, GSTs,
GRE	AGAACANNNTGTTCT	Superfamily of nuclear hormone receptors: progesterone, thyroid steroid hormone family, metallothionein family

W= A or T, N= A,T,G, or C, R= C, A, or T

Using these sequences as guide, sequence analysis for response elements of bass upstream *GSTA* was determined by Transfac database (www.genomatix.de/cgi-bin/gems/launch.pl). Several putative response elements were identified in the bass upstream *GSTA* region of the promoter (Figure 18). Also, a putative transcriptional start site (TSS), CAAT and TATA initiator

sequences were also identified (Figure 18). The nucleotide sequences of response elements (XRE, ARE, GRE, GRE-like and EPRE in the promoter region of the bass *GSTA*) were confirmed encompasses with the regulatory response elements found in mammals (Hayes and Pulford, 1995).

Figure 18. Nucleotide sequence of upstream region of bass *GSTA* revealed several putative response elements (XRE, GRE-like, GRE, ARE, and EPRE) putative transcriptional start site (TSS) and putative enhancer elements (TATAA and CAAT Box).

CHAPTER 4
DISCUSSION

Although much less is known about piscine GSTs relative to mammalian species, previous studies have shown that various fish species can conjugate a broad range of electrophilic substrates (CDNB, NBC, ECA and ADI) via GST, and that GST proteins related to mammalian α, μ, π and θ class have been described in several aquatic species (George, 1994). In particular, a previous study in our laboratory showed that bass hepatic GST cytosolic fractions rapidly metabolized 4HNE (Pham *et al.*, 2002), however the identity of the bass GST isoenzyme(s) responsible for the high 4HNE activity remained elusive. In the present study, we observed that the initial rates of GST-4HNE activities were relatively rapid in bass compared to other species. Furthermore, the very high GST-4HNE / GST-CDNB activity ratios indicated that a high proportion of the total GST cytosolic protein in bass is dedicated to the metabolism of 4HNE. Given that largemouth bass are higher order predators whose diet consists of lipid materials and whose lipid membranes are rich in polyunsaturated fatty acids, it likely that the high level of GST-4HNE activity functions to protect against oxidative injury.

The two-enzyme Michaelis-Menten curves of the GST-CDNB activity data suggests the presence of multiple bass liver GST isoenzymes each with different affinity for CDNB. These observations were supported by the presence of the two GST isoenzymes by our SDS-PAGE analysis. This data is also consistent

with a previous enzyme kinetics study by Gallagher et al. (2000). However, the enzyme kinetics of GST-4HNE reaction data revealed a linear relationship among substrate concentration and reaction velocity suggests the presence of single GST isoenyzme responsible for 4HNE metabolism. In addition, the apparent K_m and apparent V_{max} values (18.9 $\pm$1.3 μM and 24 $\pm$0.5 μmol 4HNE conjugated/min/mg, respectively) suggest that this GST isoenzyme has very high affinity and high catalytic efficiency for catalyzing 4HNE conjugation. Interestingly, the recombinant plaice GSTA also exhibits relatively high efficiency towards 4HNE, with Km of 150 ± 85.0 uM and a Vmax of 3.6 ± 1.8 umol 4HNE conjugated/min/mg (Leaver and George, 1998).

Based upon the immunoblotting studies, it appears that bass cytosolic GST isoenyzme(s) share little identity with the better-characterized rodent alpha, mu, pi, and theta class GSTs. This data is also supportive of the presence of one or more novel bass liver GSTs compare to rodent GSTs. Similarly, English sole and Starry flounder cytosolic GSTs exhibit high activity toward class-specific GST substrates, but do not show any strong cross-reactivity using rodent class-specific GST antibodies (Gallagher *et al.*, 1998). In most fish species, it appears that a discordance exists using class-specific substrates and antibodies directed against the rodent GSTs. Accordingly, it is evident that care should be taken for predicting or identifying the presence of GST classes in fish based upon the use of mammalian GST probes.

Our kinetics data of GST-4HNE conjugation using GSH affinity-purified cytosolic fractions suggests that a single GST isoenyzme is responsible for the

4HNE conjugating activity in bass liver. Accordingly, reverse phased HPLC analysis was used to characterize the GST subunit composition of the GSH affinity purified fractions. At least 2 peaks were eluted, the major peak (peak 1) constituted approximately 80% of the total cytosolic GST protein, assuming that the extinction coefficients were similar for the GST subunits. Furthermore, sequence analysis of peak 1 revealed a 14 amino acid sequence with high identity to the recombinant GSTA protein that was cloned by Dr. Doi et. al. and exhibiting a molecular weight of 26.3 kDa (2003). Therefore, the major peak (peak 1) appears to be GSTA and is likely the major GST isoenyzme in bass liver.

In addition to liver, bass GSTA mRNA was also present in gonad, upper gastrointestinal tract, and brain tissue. However, no detectable expression of GSTA mRNA was observed in heart, lower gastrointestinal tract or muscle tissue. This is in contrast to the studies of Leaver et. al. who demonstrated that plaice GST-A mRNA was expressed in all tissues including liver, intestine, gill, kidney, brain, gonad, heart, spleen and testis (1997). Leaver et. al. proposed that the expression of plaice GSTA mRNA in all tissues indicated a “housekeeping” function for the *GSTA* gene (1997). However, the differences in tissue expression of bass GSTA mRNA and plaice GST-A mRNA does not seem to suggest that bass *GSTA* functions as a “housekeeping” gene. This hypothesis is supported by a recent study by Hughes and Gallagher (2003) that suggested that bass GSTA mRNA expression may be altered by exposure to *β*-naphthoflavone (BNF). Furthermore, other studies indicate that “housekeeping” genes such as

glcyeraldehyde-3-dehydrogenase (GAPDH), *B*-globin, and human insulin growth factor (IGF) lack promoter elements (e.g. TATAA box and CAAT box), and most importantly, are devoid of response elements such as ARE, XRE, EPRE and GRE that may modulate gene transcription on exposure to certain chemical agents (Bird *et al.*, 1987; Kim *et al.*, 1991; McNulty and Toscano, 1995). In our study, genomic analysis revealed the presence of several putative promoter elements and putative response elements in the 5' flanking region of bass *GSTA* gene which further supports the hypothesis that bass *GSTA* is not a true "housekeeping" gene.

Interestingly, the presence of several genomic clones with high identity to the plaice GST gene cluster indicates that a bass GST gene cluster is also present. Leaver et. al, proposed a possible role for plaice GSTA involves the detoxification of potentially deleterious fatty acid metabolites (1997). With addition of the complete nucleotide sequences of 1E, 1F, 1H bass clones and partial nucleotide sequences of 2F clone, it appears that the bass GST gene cluster is similar to the plaice GST gene cluster. Accordingly, the presence of such a GST gene cluster in a freshwater species phylogenetically distant from the plaice suggests conservation of an important function such as detoxification against lipid peroxidation. Interestingly, besides the plaice GST gene cluster, no other GST gene clusters have been reported in aquatic species. However other GST gene clusters have been reported in *Anopheles gambiae* (Ortelli *et al.*, 2003), fruit fly (Sawicki *et al.*, 2003), and the human GST alpha locus (Morel *et al.*, 2002).

Analysis of the 5' flanking region of the bass *GSTA* revealed a putative transcriptional start site (TSS), a putative enhancer elements including CAAT and TATAA boxes and several putative response elements (XRE, ARE, GRE and EPRE). The presence of a TATAA and CAAT box consensus elements (approximately -120 bp and -90 bp upstream, respectively, of the transcriptional start site) does not provide conclusive identity of these enhancer sequences, due to the fact that the majority of CAAT and TATAA boxes in eukaryotic genes are generally situated about -30 to -50 nucleotides upstream from the site of transcription (Nussinov, 1987; Li *et al.*, 2000). Interestingly, Leaver et al. (1997) used primer extension to locate the transcriptional start site (72 bp upstream of the initiation codon) in plaice *GSTA*. Interestingly, we found a putative transcriptional start site 50 bp from the putative initiation codon for bass *GSTA* with identical sequences (CCGGCCCCCC) to the plaice transcriptional start site. However, the only way to determine the actual transcriptional start site of the bass *GSTA* gene will be to use such methods as SP1 protection or primer extension analysis.

The presence of a putative EPRE and putative ARE located in the upstream region of bass *GSTA* (-5 bp and -180 bp, respectively) suggest that this gene may be inducible by phenolic antioxidants. This hypothesis is supported by mammalian studies that has shown phenolic antioxidants can induce GST via ARE (Hayes and Pulford, 1995). A previous study in our laboratory that showed an induction of GST activity followed by exposure to the antioxidant, ethoxyquin, in brown bullhead (Henson *et al.*, 2001). In addition, a GRE-like element was

identified in the upstream region of bass *GSTA*. Dexamethasone, a glucocorticoid-like inducer, has been shown to induce rodent GST via the GRE (Hayes and Pulford, 1995), but little evidence has been published about GRE-mediated GST induction in fish. The presence of putative XRE (located -855 bp from the putative transcription start site), correlates with our study that showed a slight induction of GSTA mRNA expression by BNF in bass (Hughes and Gallagher, 2003) which is consistent with a presence of a single XRE in the bass *GSTA* promoter.

In summary, bass GSTA is the major GST isoenzyme in bass liver and rapidly catalyzes the GST-mediated conjugation of 4HNE. Based on sequence identity, catalytic activity and immunological cross-reactivity analysis, the bass GSTA isoenyzme differs from rodent GSTs and may be part of a novel GST family in fish. Furthermore, based on tissue expression analysis and promoter analysis, this GST isoenyzme does not appear to share any “housekeeping” gene characteristics, but is part of a bass GST gene cluster that is similar to the GST gene cluster observed in plaice. For plaice, this GST gene cluster functions in protection against lipid peroxidation and oxidative injury. However, further studies on the regulation of bass *GSTA* using a variety of model inducing agents that are targeted towards different response elements needs to be accomplished. Also of interest is the nature and identity of the second GST isoenyzme present in our HPLC analysis.

APPENDIX
SEQUENCE ALIGMENT OF BASS AND PLAICE *GSTA* GENE CLUSTER*

```
          1                                                  50
Bass----- ~~~~~ATAGT GGTTTCCTGT TTCCTGTGTG TCCTGATATG TATTGAAAAC
Plaice--- CCACTATAAT GTCTCCCTAC CTGACTGTTT CCAGGTTATC TGCAGAGAGC
Consensus -----ATA-T G--T-CCT-- -T------T- -C--G-TAT- T---GA-A-C

          51                                                 100
Bass----- TCACAGTGAC TCAGCACTCA GCAGCCCCCC CCGACACACA CACACACACA
Plaice--- TACCCTCAGC TCGGACAGTA TCACGCTCTG CTGAAGGAGA GACCCGGCAT
Consensus T--C-----C TC-G-----A -CA--C-C-- C-GA---A-A -AC-C-----

          101                                                150
Bass----- CACAGCCTGT CACAATACTT TTT....... ...TACAACA GTCAAATCTG
Plaice--- TAAAGCCAGC TGGCCCCCTC ATTGGCTGGA GAACCCCAAG GGCCAAGACG
Consensus -A-AGCC-G- -------CT- -TT------- -----C-A-- G-C-AA---G

          151                                                200
Bass----- CACTGAATGT AATAGACACC ACCTGTCTAT ATATACTTTG TTAATATATA
Plaice--- CGCTCAAAGA CATCTGAAAC TTACTCACTT TTGAAAGCTG CTTCTGTGTG
Consensus C-CT-AA-G- -AT----A-C ---------T -T--A---TG -T--T-T-T-

          201                                                250
Bass----- TACACATTTG AGACTGA..C ATTTAAAAAC TGAGCACCCA TGATTCATTC
Plaice--- TAAAGTTCAA GTTCGGATTC TCTAAGCAGC CTTTTTGCAT TTGTTCTCTC
Consensus TA-A--T--- ---C-GA--C --T-A--A-C -------C-- T--TTC--TC

          251                                                300
Bass----- AACAAGTTGT TTTGTTCTTG AAAGCTGCAG AAATCACATG AAAACCATGT
Plaice--- AAGGGTTGAG ATGATGTTCA AAATATATCA GTGTAACAAT GGCAGAAATT
Consensus AA----T--- -T--T--T-- AAA--T---- ---T-ACA-- ---A--A--T

          301                                                350
Bass----- GGCTGTAGCC GGTTTCTTCA AGCGATACAT TCAACTACTC ACCAGTCTAA
Plaice--- AGGTTTTGGA AAATAATTGT AAGAATACAT AAATAGACTG TTTTTTAGAT
Consensus -G-T-T-G-- ---T--TT-- A---ATACAT --A---ACT- -----T--A-

          351                                                400
Bass----- AATTCAAAGA ATCTGTTTCT TCATCAATAT GCAGGCTGTC TTTGTCATAG
Plaice--- GCATGTATTC ATTGGACAAA GGGTGCAAAT GAACAATAAA TAATCTGCAG
Consensus ---T--A--- AT--G----- ---T--A-AT G-A---T--- T-------AG

          401▼  Upstream region of GSTA gene                  450
Bass----- TGACATGAAG GAAAAGT... .....AATTG TGTTGAAAAC TGTTTTTCTA
Plaice--- CAACATATTA AAAGAGTTTT TACTGCATTT TGATATTTTC GATAGGTTTT
Consensus --ACAT---- -AA-AGT--- ------ATT- TG-T-----C --T---T-T-
```

```
          451                                                        500
Bass----- TTAAATGTTG TCTATATACA TGATAAATAC ACACATAACA AACATTATGT
Plaice--- AGCCATAAAC TGTATATAAA GTAGGTCCCA TCCACTGACC TGGAGGAGGT
Consensus ----AT---- T-TATATA-A --A------- -C---T-AC- ---A--A-GT

          501                                                        550
Bass----- TGGCTTGATG CCCATTCAAA TGTATTAACC TGTTTTACTC GTTTTTTAAA
Plaice--- GGGATTTATG ACCTGTACTG CCAACCACCA GGAGGGTATC GTTTGGGCAT
Consensus -GG-TT-ATG -CC--T---- ---A--A-C- -G------TC GTTT----A-

          551                                                        600
Bass----- GTCTCGTGTT TTCCTTACTG TCGGATTT.. ..GTTTGCCT ATTGCTTTTA
Plaice--- ATTCATTGTT GTATACAATC TATGGTTTTA GCCTTTGTGT TGAAAGTGGA
Consensus -T----TGTT -T----A-T- T--G-TTT-- ---TTTG--T ------T--A

          601                                                        650
Bass----- TTATCTCGTG TACACACACT AAAGAAGGGA CTGCTCTGAC TGCACCACTC
Plaice--- TCACCTCCAT CAAAGAACAC GTCTAATATA CAATATGACC TGATGTATAA
Consensus T-A-CTC--- -A-A-A---- ----AA---A C--------C TG----A---

          651                                                        700
Bass----- TGATTTAAAT AAAGGTTGAA TGATTCAATG ATTGCAGTAC ACAAAGGCTT
Plaice--- GAGGCAAACA GATGGTTGCT GAAACTCATT ATTCTATTCA GGCCTTTGTG
Consensus ------AA-- -A-GGTTG-- --A----AT- ATT--A-T-- --------T-

          701                                                        750
Bass----- TAGCTTTTTT TATATATATA GACTCTGTAG CAAGAGTGAA GTTCTCTTGA
Plaice--- CGACGCACAT AAGATAAAGG TAGTCGGAGT CTATTCATTC TATTATGTGA
Consensus ---C-----T -A-ATA-A-- -A-TC-G--- C-A------- --T----TGA

          751                                                        800
Bass----- TACATATAAA CCAGCTTATT TCAGCTTGTT GTAACACGAG GACCCATTTT
Plaice--- CCGGCAGTGC CTGAACCAGT TTTCTTACCT GAAATGACAT GTGCCATCAT
Consensus -----A---- C------A-T T----T---T G-AA----A- G--CCAT--T

          801                                                        850
Bass----- TTCAGGTTTT GATCTATTTG GCAGCATCGT GCATTTGGAC TTGCACGCTG
Plaice--- GACTGGGCAT CAGTGTATAG AGTTCATACT GTAAATATTA GCTGAGGTTC
Consensus --C-GG---T -A-----T-G ----CAT--T G-A--T---- ----A-G-T-

          851                                                        900
Bass----- ATTATCAGCA TGGGATCATC AGCATTTAGC ATCAACTCAA TTTAATTGTT
Plaice--- AGGCCCTGTC AAAG.TGGAC TTTACAGTTC TACAACAGAG GGCAGCACTG
Consensus A----C-G-- ---G-T---C ---A-----C --CAAC--A- ---A----T-

          901                                                        950
Bass----- GTGTTTTTGA AAGCTGCTGA AATCACATGA AAAACATGTG GTTTAGCTGA
Plaice--- GCATCTTGGC TTTTGAATCC GACCTGTTGA AAGTTTGGAT CCGTCTCTTT
Consensus G--T-TT-G- -------T-- -A-C---TGA AA-----G-- ---T--CT--

          951                                                       1000
Bass----- AGTCTGTTTC TTCCAGCGAT ACATTCAACT GCTGACCAGT TTTATTTTAG
Plaice--- TATTTTTTCC ACTTTGTAAC TCTGCTTCGA GAAGTGCCCA GTAAATAAGG
Consensus --T-T-TT-C -----G--A- -C-------- G--G--C--- -T-A-T---G
```

```
          1001                                                1050
Bass----- GTTTCTAAGA AGTTTGACA. AAGGCGGCCA CATCATAATT CATCCATGTT
Plaice--- ATATCATATT GTTTATCCAT ATGTAGGTAA CTTATAAATA CAACTATTTG
Consensus -T-TC--A-- --TT---CA- A-G--GG--A C-T---AAT- CA-C-AT-T-

          1051                                                1100
Bass----- TCAAGAAAAA CCCATTGAGA GTCTGAATAT ACAGTAAGTG TCCTACATTT
Plaice--- GTGTGAAATG CATTTTTTTC CAGTTGATAA AAAACAAGTG TATTTTAATG
Consensus ----GAAA-- C---TT---- ---T--ATA- A-A--AAGTG T--T--A-T-

          1101                                                1150
Bass----- GGACTTGCAG GCAGATTGTC AGCATGGGAT CATCAGTATT TTGTAAGAGG
Plaice--- TTGCCCTGAG TAACTAAGGG AAAGGTTAAC CTACTTGGAC AGGGTGTGAA
Consensus ---C----AG --A----G-- A-------A- C--C------ --G-------

          1151                                                1200
Bass----- GAGACCTGTG ACTGCAGACT ACCGGTTCAT TGATGCATGC ATGAAACTTA
Plaice--- CTATCTTGAG TCAATATCTT TGGTGATCAC TCAAAAATGG GAAGGGACTT
Consensus ----C-TG-G -C---A---T ----G-TCA- T-A---ATG- --------T-

          1201                                                1250
Bass----- AATTTCATCA CGGAGTACAA ACCCTCAAAT CAGTT.CGGT TTCCAGAAAA
Plaice--- ATTTCCACCA ATCCAGACGT GCAGGAGAGC GTGTTGCTAT ATACAGTGCC
Consensus A-TT-CA-CA ------AC-- -C-----A-- --GTT-C--T -T-CAG----

          1251                                                1300
Bass----- ATGAAATTGA TGTTTTTGAT CAAATAAGCT TTTTGATCAG CTACATTTTT
Plaice--- TTGCATAAGT ATTCACCCCC TTTGGACTTT TCTACATTTT GTCATGGTAT
Consensus -TG-A---G- --T------- -----A---T T-T--AT--- -T-----T-T

          1301                                                1350
Bass----- TCATAGTTCT AGGTAAACAC TAAATTCCTT GAATATTAGT TCGTCTGTGA
Plaice--- AACCACAGAT TAAAATTTAT TTCATCGTGA GTTTATGTAA TGGACCAACA
Consensus ----A----T ----A---A- T--AT----- G--TAT---- T-G-C----A

          1351                                                1400
Bass----- ACTGTTGAAC TGCCACTGAG AAGCATAAAG TGCATCGCTC ATGTTTCATC
Plaice--- CAAAATAGTG CATCATTTGG AAGTGGGGGG AATATTACAT GGATTTCACA
Consensus -----T---- ---CA-T--G AAG------G ---AT--C-- ---TTTCA--

          1401                                                1450
Bass----- AGAGGCTGTT GCTTGAATGG GGTAGCCCCA TGACAGTTAG CCTATTACAT
Plaice--- ATTATTTACA AATAAAAATC TGAAAAGTGT TGAGTGCATA TGTATT.CAC
Consensus A-----T--- --T--AA--- -G-A------ TGA--G---- --TATT-CA-

          1451                                                1500
Bass----- CTCTTCCAAA TAAAATACCA ACTGTCCCAG TTATCATTAG CTGATGGAGC
Plaice--- CCCCCTTTAC TGTGAAACCC CCCCTAAAAT CCATCATAAG AAAATGGA.A
Consensus C-C-----A- T---A-ACC- -C--T---A- --ATCAT-AG ---ATGGA--

          1501                                                1550
Bass----- ACAACAGGGG ACGCCCCAAC TCCCAGACAA AACAGGTAGT ..ATATAAAA
Plaice--- AGAATATGGC ACAACCGCAA ACCTACCAAG AGGAGGCCGT CCACCCAAAC
Consensus A-AA-A-GG- AC--CC--A- -CC-A---A- A--AGG--GT --A---AAA-
```

```
          1551                                                1600
Bass----- GGGCCAAGAG GAGGACTGGG ATGTCACTTT GCACATCTTC TAGGGCTGCA
Plaice--- TGAAGAGTCG GACAAGGAGA AAATTAATCA GAGAAGCAAC CAGGAGGCCC
Consensus -G---A---G GA--A---G- A--T-A-T-- G---A-C--C -AGG----C-

          1601                                                1650
Bass----- CTACTCAGTA TGTGAAAGCT GATGTACTCC TGGTAGTGTG TGCAGGATCT
Plaice--- ATGGTTACTC TGGAGGAGTT GCAGAGATCC ACAGCTGAGG TGGGAGAATC
Consensus -T--T-A-T- TG----AG-T G--G---TCC ---------G TG---GA---

          1651                                                1700
Bass----- CAACTCAAGT TTATAAGCAT GTGAAATGCC TCTACATAAG CAGGTACAGA
Plaice--- TGTCCACAGG ACAACTATTA GTCGTCTACT CCACAAATCT GGCCTTTATG
Consensus ---C---AG- --A------- GT----T-C- -C---A---- ----T--A--

          1701                                                1750
Bass----- AGTCTATTTT TTAAGTCTAA GTGATATAGG CCGACATACA GCTCAGTGCA
Plaice--- GAAGAGTGGC AAGAAGAAAG CCGTTGTTGA AAGGGATCCA TAAAAAATCC
Consensus ------T--- ---A----A- --G-T-T-G- --G--AT-CA ----A---C-

          1751                                                1800
Bass----- GCTGCTGCGA AAGTCTGAAA GACATTTTAC ATAAAATGCA AAATCATGCA
Plaice--- CGTTTGGAGT TTGCCAGAAG CCATGTGGGA GACACAGCAA ACATGTGGAA
Consensus --T---G-G- --G-C-GAA- -----T---- ---A-A---A A-AT---G-A

          1801                                                1850
Bass----- CAGCCTGCAA TACAGAGCTA TGTCACTTAT GCAAAGTCAT GACAACACAG
Plaice--- GAAGGTGCTC TGGTCAGATG AGACCAAAAT TGAACTTTTT GGCCTCAATG
Consensus -A---TGC-- T----AG-T- -G-C----AT --AA--T--T G-C--CA--G

          1851                                                1900
Bass----- AGCATTCCAC TTACCTTTG. ....TGCAGA GTGAACCAGT TGTGGCACGG
Plaice--- CAAAACGCTA TGTGTGCCGA AAACCCAACA CTGCCCATCA CCCTGAGCAC
Consensus ---A---C-- T-------G- -------A-A -TG--C---- ----G--C--

          1901                                                1950
Bass----- CCATTCAAAC AATAAAACAA ACCAGTTTGT GTCCTCAGAC ACCACATACC
Plaice--- ACCATCCCAA CAGTGAAACA TGGTGGTGGT AGCATCATGC TGTGGGGATG
Consensus -C--TC--A- -A---AA--A ----G-T-GT --C-TCA--C -------A--

          1951                                                2000
Bass----- TTTTGTAAGA GATTGTGAGA TTGAAATCTT GAGATTGACG CCAGACTTGC
Plaice--- CTTCTCTTCA GCAGGTACAG GGAAACTGGT CAGAATAGAG GGAAAGATGG
Consensus -TT------A G---GT---- ---AA-T--T -AGA-T---G --A-A--TG-

          2001                                                2050
Bass----- CATGAATATT TGGGTTAACA GTCGCGGATT TAGAGCTCAT GTGGTTAGAT
Plaice--- ATGGAGCCAA ATACAGGGAA ATCCTTTAAG AAAATCTGAT GCAGTCTGCA
Consensus ---GA----- ---------A -TC----A-- -A-A-CT-AT G--GT--G--

          2051                                                2100
Bass----- GATCGCATAA GAGCTCAACA TAAAATTGCA TGAAGTCCAA ACTTGAACAC
Plaice--- AAAGACTTGA GACTGGGGCG GAGGTTCATC TTCCAGCAGG AACATGACCC
Consensus -A---C-T-A GA------C- -A---T---- T-----C--- A-----AC-C
```

```
          2101                                                 2150
Bass----- ATTTCTTACT CCCAGACTTC AACACCTTCA AAAGGGA..C ATCTGGGTTT
Plaice--- TAAACATACA GCCAGAGCTA CAAAGGAATG GTTTGGATTA AAGAATGTTA
Consensus ----C-TAC- -CCAGA--T- -A-A------ ----GGA--- A-----GTT-


          2151                                                 2200
Bass----- TGTTTGGCCT ATGCTAGTTT AATTGTAGAG CCAATGTATG GGTTTTCAAT
Plaice--- ATGTCTTAAA ATGGCCCAGT CAAAGCCCAG ACCTCAATCC AATAGAGAAT
Consensus ---T------ ATG------T -A--G---AG -C-------- --T----AAT

          2201                                                 2250
Bass----- GTGGTGTGTA GCCTCGAAGA CATGTTTGCT TGAGTGGAGG TTTTTTTAGT
Plaice--- CTATGGCAAG ACTTGAAGAT TGCGGTTCAC AGACGGTCTC CATCCAATCT
Consensus -T---G---- -C-T--A--- ---G-TT--- -GA--G---- --T------T

          2251                                                 2300
Bass----- CAAAACACAT GGAAAACTGC TGTTATAGTG ACTTATGCAT ATATTAATAA
Plaice--- GACTGAGCTT CATCTTTTTT GCCAAGAAGA ATGGACAAAC CTTTCCATCT
Consensus -A-----C-T -------T-- ----A-A--- A---A---A- -T-T--AT--

          2301                                                 2350
Bass----- ACAGATATTG ATGTTACAAA TACACGCCAT GTATGTACGC TGGATCAACA
Plaice--- CTAGATGTGC AAAGCTGGTA GAGACATACC CCAAAAGACT TGCAGCTATA
Consensus --AGAT-T-- A--------A -A-AC----- --A------- TG-A-C-A-A

          2351                                                 2400
Bass----- ATATCACAAT ATTACATTTG GCCTGCAACC TATGCCTGTT ACCCTACAAA
Plaice--- ATTGCAGCGA AAGGGGGTTC TACCAAGTAT TGACACAGGG GGGTGAATAC
Consensus AT--CA---- A------TT- --C------- T----C-G-- -----A--A-

          2401                                                 2450
Bass----- CATCACATGC TGCACAGACC AACATTGCCT GTGTTTGTTA AATATCTGAC
Plaice--- TTAGGCACCC AACAGATGTC AACTTTTTTG TTCTCATTAT TGCTTGTGTC
Consensus -----CA--C --CA-A---C AAC-TT---- -T-T---T-- ----T-TG-C

          2451                                                 2500
Bass----- AGACACGACT TTTTGACACA CTTTTGAACT AAATATAAAA GTTAGTTTGA
Plaice--- ACAATAAAAT TTATTTTGCA CCTCCAAAGT ACTATGCATG TTTTGTTGAT
Consensus A-A----A-T TT-T----CA C-T---AA-T A------A-- -TT-GTT---

          2501                                                 2550
Bass----- CAAAACAAAA GAACTACATT TCTGCCCTTG TAACACTAAT ATATTAAGAA
Plaice--- CAAACGGGAA AAAGTTTATT TAAGTCTATT TGAATTCCAG TTAGTAACAA
Consensus CAAA----AA -AA-T--ATT T--G-C--T- T-A-----A- -TA-TAA-AA

          2551                                                 2600
Bass----- CATACTGGTG ATCTCAACAC TTTCATTCCA TGACAATATC AGAAAATTAA
Plaice--- TACA....TA ATGGGAAAAA GTCCAAGGGG GGTGAATACT TATGCAAGGC
Consensus -A-A----T- AT---AA-A- -T-CA----- -G--AATA-- -----A----

          2601                                                 2650
Bass----- AGAGCTGTAT CACATGTGAA AAGCTGGGAA CTGAAACTAC AATAAACAAA
Plaice--- ACTGTAAACA GGAGTCTGTC CCATTCCGGC CCCCCCTTCC ACCTCCTCCA
Consensus A--G------ ----T-TG-- ----T--G-- C------T-C A--------A
```

```
          2651                                                ▼   2700
Bass----- AGAGCTTTTA TAGAAACCAA ACCAAAACAT TACAGGCAGA CGTGATTGTA
Plaice--- CCACCACTCT TCTTCTCCTG AACCTCACAG GTACTGCAGC TTCTCTCTGA
Consensus --A-C--T-- T-----CC-- A-C---ACA- -----GCAG- -----T---A


          2701  CODING REGION OF GSTA gene                    2750
Bass----- CAGTGTGTGT GTGAACCTCA GATGTCTTTG AGCGTGTCTT GTCCCTTGGG
Plaice--- AACCATGGCC AAGGACAT.. GACTCTGCTG TGGGGCTCCG GCTCTCCTCC
Consensus -A---TG--- --G-AC-T-- GA------TG -G-G--TC-- G--C------

          2751                                                2800
Bass----- GTTCTCCAGC CAGTGAGGAG GCCAGCTGGC TATGATGCTG GGCCGCTCCT
Plaice--- CTGCTGGAGG GTGATGATCG TGCTGGAGGA GAAGAACCTG CAGGGCTACA
Consensus -T-CT--AG- --G------G --C-G--GG- -A-GA--CTG ----GCT-C-

          2801                                                2850
Bass----- TCAACAATGC GTAATACTCT CCCAGTTTAG GGTAATGCTC AGCAGACAAT
Plaice--- ACAGCAAATT GCTCTCCTTC GAGAAAGGGG AGCACAAGTC AGCCGA.GGT
Consensus -CA-CAA--- G---T-CT-- ---A-----G -G-A----TC AGC-GA---T

          2851                                                2900
Bass----- CTACAGAACA GAAGGTAGGA GACATTTTAA A~~~~~~~~~ ~~~~~~~~~~
Plaice--- GATGAGCATG AACCCCAGGG GTCAGGTGAG TGTGCTCCTC AAATACTATC
Consensus ----AG-A-- -A----AGG- G-CA--T-A- ---------- ----------

          2901
Bass----- ~~~~~
Plaice--- ATTTT
Consensus -----
```

*Box number 1 corresponds to nucleotide sequence 6886 coding region of exon 6 of the plaice *GSTA* gene sequence as reported by Leaver et al. (1997).

▼Bass nucleotide sequence 401 represents the first nucleotide of GST gene cluster upstream of *GSTA* gene.

▼ Bass nucleotide sequence of 2762 represents the coding region of GSTA gene.

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BIOGRAPHICAL SKETCH

I received my Bachelor of Science at the University of Florida in microbiology and a minor in chemistry in 1999. I was awarded consecutive Johns Hopkins Fellowship Research Award in 1998 and 1999. I started my graduate program at the University of Florida in 2002. After my graduation, I will be recruited as regional director for a consultant firm in Los Angeles, California.