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Altered Expression of a Glutathione S-Transferase Isoform in Creosote-Resistant Mummichog, Fundulus heteroclitus

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ALTERED EXPRESSION OF A GLUTATHIONE S-TRANSFERASE ISOFORM IN CREOSOTE-RESISTANT MUMMICHOG, *FUNDULUS HETEROCLITUS*

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Susan L. Armknecht

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ABSTRACT

Aquatic organisms often inhabit environments heavily contaminated with toxic chemicals (Weis & Weis, 1989). Mechanisms of survival in these organisms are thought to involve alteration in the biochemical processes responsible for detoxication and elimination of the xenobiotic compounds. In the Elizabeth River VA, mummichog (*Fundulus heteroclitus*) inhabit a site severely contaminated with polycyclic aromatic hydrocarbons (PAH) of creosote origin. Although chronic effects including hepatic neoplasms have been observed in adult mummichog (Vogelbein *et al.*, 1990), the fish are resistant to the acute toxicity of the contaminated sediments (Williams, 1994). Increased levels and activities of glutathione S-transferase (GST) in these fish (Van Veld *et al.*, 1991) may play a role in this resistance. GSTs are major enzymes involved in detoxication of cytotoxic and genotoxic compounds such as electrophilic metabolites of PAHs. Hepatic GSTs in resistant fish from a heavily contaminated site and in fish from a reference site were purified by S-hexylglutathione affinity chromatography. Monoclonal antibodies (MAb) were produced to a GST isoform with pI of 8.1 and subunit MW 27.2kD. This form is elevated approximately 5.8-fold in resistant fish and 2.2-fold in fish from a moderately contaminated site relative to fish from a reference site. GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) is elevated 4-fold in resistant fish and 2-fold in fish from the moderately contaminated site. There is a strong correlation between GST activity and protein levels in these fish. From SDS-PAGE gels, a 27.2 kD band corresponding to GSTs is also elevated in fish from the contaminated sites and appears to be a major cytosolic protein in resistant fish. The isoform has a blocked N-terminus but one MAb reacts with a Mu subunit from rat liver. These results indicate an elevation of GST levels and activity in fish from creosote contaminated sites and the monoclonal antibody recognizes a GST form which may contribute to resistance of fish to creosote associated contaminants.

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INTRODUCTION

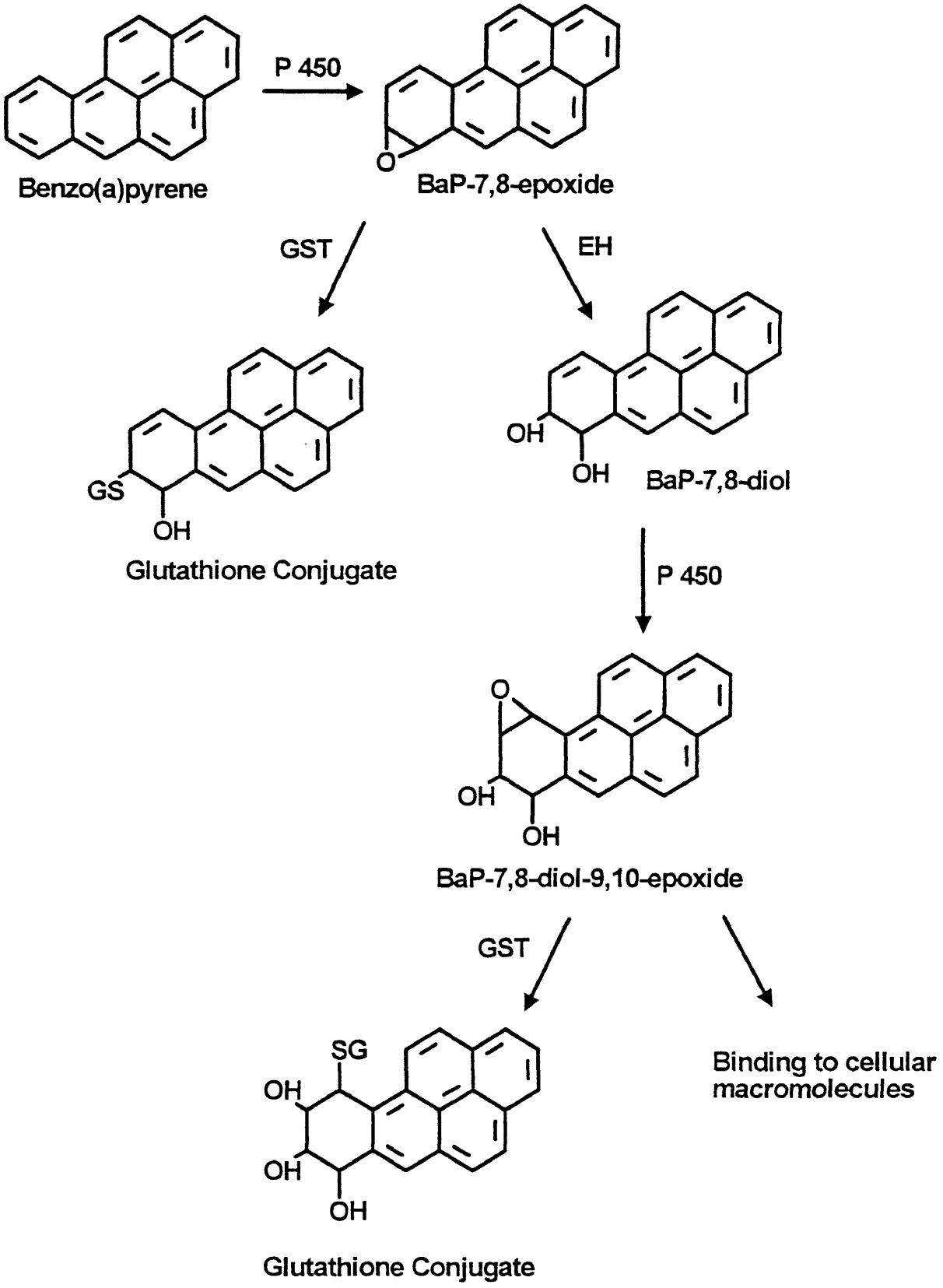
Biotransformation of lipophilic xenobiotic compounds is often a stepwise process involving phase I and phase II enzymes (Di Giulio *et al.*, 1995). Phase I enzymes are primarily cytochrome P450-mediated monooxygenases that add polar functional groups to lipophilic substrates through oxidation, reduction, or hydrolysis. The products of phase I metabolism are generally less toxic, more water soluble and therefore more readily excreted than the parent compound (Stegeman *et al.*, 1992). However, some phase I metabolites are more toxic than the parent compound. Following introduction of polar functional groups, xenobiotics are susceptible to further metabolism by phase II enzymes that link the xenobiotic to water-soluble endogenous compounds within the cell. These conjugation reactions increase the solubility, elimination, and detoxication of the xenobiotic.

Glutathione S-transferases (GSTs) are an important family of phase II enzymes involved in the biotransformation of endogenous and xenobiotic compounds (Coles & Ketterer, 1990; George, 1994; Gulick & Fahl, 1995). They perform a major role in detoxication of many lipophilic compounds, including the highly reactive and carcinogenic metabolites of polycyclic aromatic hydrocarbons (PAH) in mammals and fishes (Hawkins *et al.*, 1988; Hawkins *et al.*, 1990; Hendricks *et al.*, 1985). For example, the conversion of benzo(a)pyrene (BaP) into its ultimate carcinogenic form, BaP-7,8-diol-

9,10-epoxide (BPDE) (Varanasi *et al.*, 1986), is a three step process (Fig. 1). Some of the intermediate metabolites, as well as the ultimate form, are capable of covalently binding to critical cellular macromolecules including DNA (Kurelec *et al.*, 1991; Schnitz *et al.*, 1992; Varanasi *et al.*, 1986; Shugart *et al.*, 1987). This formation of DNA adducts creates genetic mutations and may ultimately lead to the production of mutant gene products and carcinogenesis (Barbacid, 1987; Marshall *et al.*, 1984; McMahon *et al.*, 1990). Some of the intermediate metabolites are also substrates for GSTs (Nemoto *et al.*, 1975; Hesse & Jernström, 1984; Funk *et al.*, 1995) that catalyze the nucleophilic attack of the sulfur atom of glutathione (γ-glutamylcysteinylglycine) on the electrophilic groups of reactive epoxides. These conjugation reactions prevent the epoxides from binding to cellular macromolecules (Hesse & Jernström, 1984; Quinn *et al.*, 1990). The glutathione conjugates are transported out of the cell by an ATP-dependent export pump (Ishikawa, 1992; Jedlitschky *et al.*, 1994) and excreted (Boyland & Chasseaud, 1969).

GSTs also play a role in protection against oxidative stress (Ketterer *et al.*, 1990; Hayes & Strange, 1995; Di Giulio *et al.*, 1995). Oxidative stress is caused by reactive oxygen species, or oxyradicals ($HO^{\cdot}$, $O_2^{\cdot-}$, H_2O_2) which are products of incomplete reduction of oxygen to water during aerobic metabolism. These oxyradicals oxidize membrane lipids, proteins and DNA. GSTs are believed to have arisen due to the emergence of oxygen and aerobic respiration in order to inactivate the toxic products of oxygen metabolism as well as inactivate toxic products produced by other organisms (Lee, 1991). While oxidative metabolism of endogenous substrates results in the natural occurrence of reactive electrophiles, exposure to xenobiotic compounds (Ahmad, 1995)

Figure 1. Metabolism of benzo(a)pyrene (BaP). BaP is converted to a toxic metabolite by cytochrome P450 (P 450). The toxic metabolite may bind to critical cellular macromolecules, be detoxified by glutathione S-transferase (GST) mediated conjugation with glutathione or be further metabolized by epoxide hydrolase (EH) and P 450 to the ultimate carcinogenic form. Cellular macromolecules are protected from the ultimate carcinogenic form of BaP by GST-mediated conjugation with glutathione.


P 450
Benzo(a)pyrene
BaP-7,8-epoxide
GST
EH
GS
OH
Glutathione Conjugate
OH
OH
BaP-7,8-diol
P 450
O
OH
OH
BaP-7,8-diol-9,10-epoxide
GST
SG
OH
OH
OH
Glutathione Conjugate
Binding to cellular macromolecules

and elevated oxygen conditions may increase the rate of production of these reactive compounds. For example, redox cycling of compounds such as quinones and diols (products of BaP metabolism) results in the formation of oxyradicals. An increase in oxyradical formation may then result in mitogenic effects, DNA damage, alteration of membrane fluidity, and cell damage and death. GSTs protect cells through conjugation of electrophiles produced from oxyradicals and peroxidation of DNA and lipid hydroperoxides.

In addition to their role in conjugation, GSTs detoxify xenobiotics by serving as carrier proteins, transporting toxicants between sites of storage or toxicity and sites of biotransformation (Hanson-Painton *et al.*, 1983). GSTs also form covalent bonds with reactive electrophilic toxicants themselves (Schelin *et al.*, 1983), further reducing the possibility of the compounds binding to cellular macromolecules.

GSTs have been identified in mammals, fishes and invertebrates (Fitzpatrick & Sheehan, 1993; Lee *et al.*, 1988) but have been most extensively characterized in rats, mice, and humans (Habig *et al.*, 1974; Jakoby, 1978; Mannervik, 1985; Mannervik & Danielson, 1988; Pickett & Lu, 1989; Rushmore & Pickett, 1993). The GSTs constitute a supergene family, being products of at least four gene families (Lai & Tu, 1986). Each enzyme or isoform is a dimeric protein composed of two subunits of 25-28 kDa. Each subunit contains a binding site for glutathione and a binding site for the substrate. The majority of GSTs are cytosolic although microsomal forms exist. They are found in all tissues and compose approximately ten percent of the soluble hepatic protein in the rat and three percent in humans (Jakoby, 1978). Isoform expression can vary within an

organism depending on tissue (Tahir *et al.*, 1988), age (Peters *et al.*, 1989) and sex (Hayes *et al.*, 1994), as well as between species (Mannervik *et al.*, 1985) and strains (Egaas *et al.*, 1995). In mammals, the soluble GST isoforms are grouped into Alpha, Mu, Pi, (Mannervik *et al.*, 1985) and Theta (Meyer *et al.*, 1991) classes based upon substrate specificity, immunological cross-reactivity, and protein sequence. There is a 70-80% identity in amino acid sequence between isoforms within the same class and less than 30% identity between classes (Mannervik & Danielson, 1988). Most GST isoforms react with 1-chloro-2,4-dinitrobenzene (CDNB), with the exception of Theta (Meyer *et al.*, 1991). Thus CDNB serves as a general substrate for measuring GST activity (Fig. 2). The Mu class GSTs have the highest activity with epoxides, including benzo(a)pyrene-4,5-oxide (Mannervik, 1985) an intermediate metabolite of BaP. BPDE, the ultimate carcinogen of BaP, is a Mu class substrate but is a more efficient substrate for the Pi class (Robertson *et al.*, 1986a). The conjugation reaction for Mu and Pi with diol-epoxides of PAHs is selective towards the (+)-enantiomer (Robertson & Jernström, 1986; Funk *et al.*, 1995).

GSTs can be induced in mammals using PAHs and PCBs. These compounds generally result in an induction of phase I enzymes followed by phase II enzymes. The induction of specific isoforms may also be strain- (McLellan *et al.*, 1991), organism- (Foliot & Beaune, 1994), and sex-specific (Di Simplicio *et al.*, 1989) and often involves induction of forms which are not constitutively expressed (McLellan *et al.*, 1991; Hayes *et al.*, 1991). Induction (Fig. 3) results in an increase in GST activity, GST protein levels, and GST mRNA (Clapper *et al.*, 1994; Ding & Pickett, 1985). The regulation of GSTs is

Figure 2. Conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). Glutathione S-transferase (GST) catalyzes the nucleophilic attack of the sulfur atom of GSH on the electrophilic groups of reactive compounds.

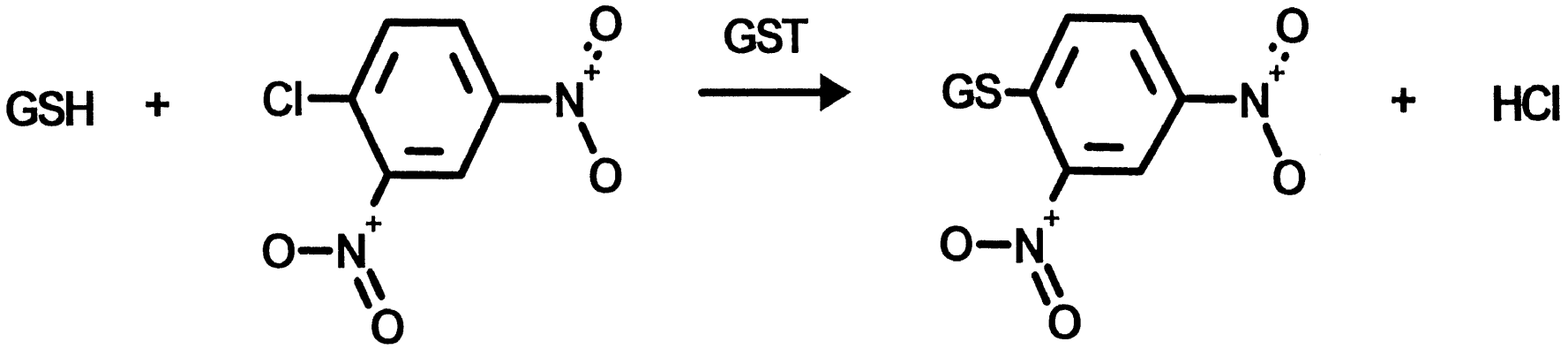
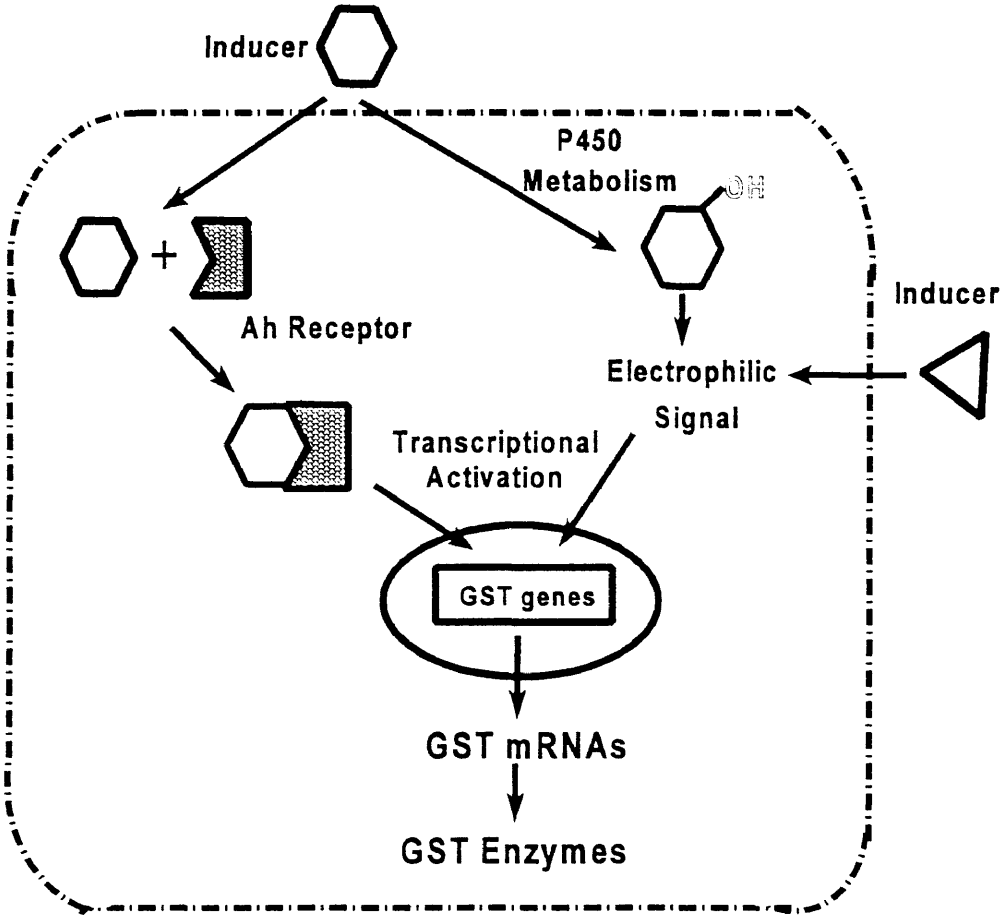

GSH
+
Cl
O
N+
O
O
O−N+
O
GST
GS
O
N+
O
O−N+
O
+
HCl

Figure 3. Mechanism of GST induction by different types of inducers. Modified from Prochaska & Talalay (1988). An inducer may enter a cell, bind to the Ah receptor, and the resultant complex activates transcription of the GST genes. The same type of inducer may enter a cell, undergo phase I metabolism and the resultant metabolite generates an electrophilic signal which stimulates transcription of GST genes. Another type of inducer may enter a cell and generate an electrophilic signal which activates transcription.


Inducer
P450
Metabolism
OH
Ah Receptor
Inducer
Electrophilic
Signal
Transcriptional
Activation
GST genes
GST mRNAs
GST Enzymes

not completely understood but there is evidence for several regulatory elements on the subunit genes (Hayes & Pulford, 1995). Planar aromatic compounds can activate gene transcription either through binding with the aryl hydrocarbon (Ah) receptor which interacts with the xenobiotic responsive element (XRE), or through an unknown interaction with the antioxidant-responsive element (ARE) which is independent of the Ah receptor (Rushmore *et al.*, 1990; Rushmore & Pickett, 1993).

GSTs have been identified in fishes and characterized in a few species (Foureman, 1989; Nimmo, 1987; George, 1994) including carp (Dierickx, 1985a), little skate (Foureman & Bend, 1984), thorny-back shark (Sugiyama *et al.*, 1981), plaice (George & Young, 1988; George & Buchanan, 1990), and rainbow trout (Ramage & Nimmo, 1984; Dierickx, 1985b). Fishes exhibit multiple isoforms in the liver, gut, gills and kidney. Isoforms have pI ranges of 5.2 - 9.5 and subunits range in size from 21.7-29.0 kDa. Reported activities of GSTs in hepatic tissues of fish using CDNB as a substrate range between 0.1 μmoles/min/mg in Atlantic salmon (George *et al.*, 1989) and 3 μmoles/min/mg in sheepshead minnow (James *et al.*, 1988). Activity may also vary with sex (George *et al.*, 1990), season (Ramage *et al.*, 1986; George *et al.*, 1990; Mathieu *et al.*, 1991), and organ (Laurén *et al.*, 1989; Leaver *et al.*, 1992; Perdu-Durand & Cravedi, 1989).

A classification scheme for fish GSTs has not been developed although some evidence suggests that fish and other non-mammalian organisms possess isoforms similar to major mammalian isoforms. Immunochemical comparison of fish GSTs with mammalian isoforms has indicated that trout, salmon and cod express a subunit that is

recognized by antiserum to the rat Pi class subunit (Dominey *et al.*, 1991; George *et al.*, 1989). Dominey *et al.* (1991) also found that the subunits from a major GST isoform in salmon displayed 65% protein sequence identity with the subunits from the rat Pi class isoform. In contrast, flounder, turbot, sea trout and salmon express a subunit immunologically cross-reactive to an Alpha class rat subunit and cod, turbot and sea trout express a subunit cross-reactive to a Mu class subunit (George *et al.*, 1989). Plaice liver has two major isoforms, one has an amino acid sequence with greatest similarity to mammalian Theta (Leaver et al., 1993) and the other is immunologically reactive with Alpha (George & Buchanan, 1990). Toad (*Bufo bufo)* embryos have a GST which is 80% identical with mammalian Pi class (Di Ilio *et al.*, 1992) and blue mussels (*Mytilus edulis*) have a GST which is 60% identical with mammalian Pi class GST (Fitzpatrick *et al.*, 1995). Two isoforms isolated from the eyes of shrimp (*Penaeus japonicus*) exhibit 57% and 40% identical with human Mu and Theta class GSTs respectively (Lin & Chuang, 1993). In two species of squid, GST sequences from the digestive gland showed greatest similarity to the rat Alpha class in *Loligo vulgaris* (Harris *et al.*, 1991) and Pi in *Ommastrephes sloani pacificus* (Tomarev *et al.*, 1993). Octopus (*Octopus vulgaris*) digestive gland also has a form most similar to Pi (Tang *et al.*, 1994).

Induction of GST levels and activity by various environmental toxicants (eg. PAHs, PCBs) has been demonstrated in mammals (Ding & Pickett, 1985). However, studies using mammalian inducers with fishes have yielded inconsistent results. Some studies report increases (1.2-3 fold) of hepatic GST activity following treatment of fishes with PAHs and PCBs (Andersson *et al.*, 1985; George & Buchanan, 1990; Zhang *et al.*,

1990; George & Young, 1986; Fair, 1986; Bernhoft *et al.*, 1994; Scott *et al.*, 1992; Chatterjee & Bhattacharya, 1984). In other studies, treatment of fishes with similar agents did not result in a significantly elevated response (James & Little, 1981; Ankley *et al.*, 1986; Lemaire *et al.*, 1992; Collier & Varanasi, 1991; Bernhoft *et al.*, 1994; Goksøyr *et al.*, 1987; Van Veld *et al.*, 1991; James *et al.*, 1988). The differences in responses seen in fishes to these classical mammalian inducers may be attributed to differences in species responsiveness, exposure period or other factors (Table 1).

Field studies have similarly led to inconsistent results with respect to GST induction. Some studies suggest that fishes collected from PAH or PCB contaminated sites exhibit elevated (2-fold) GST (Andersson *et al.*, 1988; Collier & Varanasi, 1984; Monod *et al.*, 1988) while other studies report no differences between clean and contaminated sites (Collier *et al.*, 1992; Lindström-Seppä & Oikari, 1988). Bagnasco *et al.* (1991) found that annular seabream from a polluted site exhibited a depression of GST activity compared with control site fish. Perch collected from an oil spill site had elevated GST activity four months after the spill, but rainbow trout did not exhibit an elevation in activity after four days of exposure to the oil (Lindström-Seppä & Pesonen, 1986).

Although there are no clear trends in the literature regarding induction of GSTs by environmental agents, species-specific differences in GST expression have been correlated with relative sensitivity to carcinogens. For example, a comparison of constitutive GST activity between channel catfish, a species with a low incidence of contaminant induced neoplasia, and brown bullhead, a species with a greater incidence of

Table 1. Responses of fish hepatic GSTs to inducers of mammalian GSTs.

Organism	Inducer	Dose	Route	Exposure Time	Induction**	Reference
Rainbow trout	BNF	70 mg/kg	i.p.	7 days	N	Goksoyr et al., 1987
Rainbow trout	BNF	50 mg/kg	i.p.	14 days	Y	Zhang et al., 1990
Rainbow trout	BNF	100 mg/kg	i.p.	2 weeks	Y	Andersson et al., 1985
Rainbow trout	PCB	1.5 mg	oral	9 days	Y	Bernhoft et al., 1994
Rainbow trout	PCB	1.5 mg	oral	14 days	N	Bernhoft et al., 1994
Rainbow trout	PB	50 mg/kg	i.p.	7 days	N	Goksoyr et al., 1987
Atlantic cod	BNF	100 mg/kg	i.p.	7 days	N	Goksoyr et al., 1987
Atlantic cod	PB	50mg/kg	i.p.	7 days	N	Goksoyr et al., 1987
Cod	PCB	1.5 mg	oral	9 & 14 days	N	Bernhoft et al., 1994
Plaice	PCB	-	i.p.	12 days	Y	George & Buchanan, 1990
Plaice	3-MC	-	i.p.	12 days	Y	George & Buchanan, 1990
Plaice	tSOx	-	i.p.	12 days	Y	George & Buchanan, 1990
Plaice	3-MC	10 mg/kg	i.p.	2 days	Y	George & Young, 1986
Sea bass	BaP	20 mg/kg	i.p.	14 hours	N	Lemaire et al., 1992
Sea bass	BaP	0.075-7.5 mg/kg	i.p.	3 days	Y	Fair, 1986
Catfish	PCB	100 mg/kg	i.p.	8 days	N	Ankley et al., 1986
English sole	PCB	100 mg/kg	i.p.	72 hours	N	Collier & Varanasi, 1991
English sole	PB	100 mg/kg	i.p.	72 hours	N	Collier & Varanasi, 1991
English sole	tSOx	500 mg/kg	i.p.	72 hours	N	Collier & Varanasi, 1991
English sole	BaP	5 mg/kg	i.p.	72 hours & 14 days	N	Collier & Varanasi, 1991
English sole	PAH*	0.52 & 1.04 mg/kg	i.p.	72 hours & 14 days	N	Collier & Varanasi, 1991
Flounder	3-MC	20 mg/kg	i.p.	6 days	N	Scott et al., 1992
Flounder	PCB	100 mg/kg	i.p.	6 days	Y	Scott et al., 1992
Flounder	tSOx	100 mg/kg	i.p.	6 days	Y	Scott et al., 1992
Mummichog	BNF	12.5 ug/kg	feed	2 weeks	N	Van Veld et al., 1991

*Sediment extract containing PAHs

**Induction is measured as an increase in activity using CDNB as a substrate

BNF= beta-naphthoflavone
PCB= polychlorinated biphenyl
PB= phenobarbital
3-MC= 3-methylcholanthrene
BaP= benzo(a)pyrene
tSOx= trans-stilbene oxide
i.p.= intraperitoneal

neoplasia (Hasspieler *et al.*, 1994b), revealed that the channel catfish expressed both higher levels of hepatic glutathione (GSH) and GST activity than the brown bullhead. The differences in GST activity between the two fish species suggests a role of the enzyme in protection against carcinogens. Similarly, a comparison of English sole and starry flounder from a PAH contaminated site (Collier *et al.*, 1992) indicated that English sole had a greater prevalence of hepatic lesions along with lower GST activity than starry flounder. There were also differences in GST isoform expression between the two fish species. The flounder expressed two isoforms not found in the sole.

In addition to GST induction following environmental toxicant treatment, numerous studies have revealed GST elevation in chemically induced mammalian tumors (Buchmann *et al.*, 1985). A comparison of the GST isoforms expressed in normal and neoplastic tissues indicates that Pi predominates in most tumors (Rushmore *et al.*, 1988; Shea & Henner, 1987; Tsuchida & Sato, 1992) and drug-resistant cells (Batist *et al.*, 1986; Tew, 1994) while there is altered expression of other isoforms as well (Howie *et al.*, 1990; Stalker *et al.*, 1994; Castro *et al.*, 1990; Schisselbauer *et al.*, 1990). Farber (1991) has suggested that development of cancer is an adaptive response to xenobiotic exposure. According to Farber, rare hepatocytes containing a resistant phenotype are located throughout the liver and proliferate to form hepatocyte nodules in response to toxicant exposure. These nodules may confer resistance to the acute effects of toxicants; however, they may eventually develop into carcinomas. In support of this hypothesis, Harris *et al.* (1989) found that rats bearing carcinogen-initiated tumors were resistant to doses of carbon tetrachloride which induced mortality in all control animals. Common

biochemical features believed to contribute to resistance in tumor cells are 1) over-expression of ATP-driven membrane pumps, such as P-glycoprotein (Pgp), that actively pump foreign compounds out of resistant cells (Gottesman & Pastan, 1993; Kartner & Ling, 1989); 2) decreased expression of the enzymes involved in toxicant activation (eg. cytochrome P-4501A); and 3) increased expression of GSTs (Farber, 1990; Hayes & Wolf, 1990; Wolf *et al.*, 1987).

GST expression patterns in fish tumors appear different than those observed in mammals. For example, Hayes *et al.* (1990) found reduced GST expression in pollutant-associated hepatic neoplasms in white suckers (*Catostomus commersoni*). Aflatoxin and PAH induced rainbow trout neoplasms were also deficient in GST expression (Kirby *et al.*, 1990b). Similarly, Stalker *et al.* (1991) found a decrease in GST subunit expression in hepatocellular adenomas, bile duct adenomas and carcinomas in PAH exposed white suckers. Stalker *et al.* (1991) concluded that neoplastic progression was due to loss of constitutive GSTs responsible for detoxication and elimination of PAHs in exposed fish.

Recently, a resistant population of mummichog (*Fundulus heteroclitus*) was discovered at a creosote-contaminated site (Atlantic Wood) in the Elizabeth River, VA (Williams, 1994). Although 93% of the mummichog exhibit hepatic lesions and 33% exhibit neoplasms (Vogelbein *et al.*, 1990), this population appears to thrive in the harsh chemical environment. Atlantic Wood (AW) sediments are acutely toxic to mummichog from clean (reference) sites (Vogelbein & Van Veld, unpublished). Hyperplastic liver lesions (altered foci) and neoplasms have been observed mainly in adult AW fish while the majority of juveniles do not exhibit these lesions. However, these fish are resistant to

the acute toxicity of the chemicals in their environment.

The biochemical mechanisms involved in the acute toxicity resistance of AW mummichog are an area of recent investigation. Van Veld *et al.* (1992) reported a depression of cytochrome P-4501A (CYP1A) in neoplastic and preneoplastic liver lesions of these fish. More recently, a general low constitutive level of CYP1A in non-neoplastic AW liver tissue was also reported (Van Veld & Westbrook, 1995). Cooper (unpublished) observed that levels of liver P-glycoprotein in AW fish were roughly similar to those found at reference sites. Although P-glycoprotein over-expression is believed to play a major role in chemical resistance in mammalian cells (Gottesman & Pastan, 1993; Kartner & Ling, 1989), it does not appear to explain resistance in Atlantic Wood fish.

Perhaps the most remarkable of all biochemical differences observed between AW fish and reference fish is the consistent elevation of GST levels and activity in AW fish (Van Veld *et al.*, 1991). While there were no apparent differences between GST activity in neoplastic and preneoplastic hepatic lesions and adjacent normal tissues, hepatic GST activity in these fish exceeded that of reference site fish by approximately three-fold. An inability to induce a similar level of induction in reference fish in the laboratory (Van Veld *et al.*, 1991) suggests that the elevated GSTs in the resistant AW fish may represent a genetic adaptation. This would be consistent with the demonstrated genetic nature of acute toxicity resistance in mummichog inhabiting polluted environments (Weis & Weis, 1989; Williams, 1994). Although it is apparent that there are one or more isoforms of over-expressed GST in AW fish, no information is available on the nature of the isoform(s), their mechanisms of regulation, or the role they play in

resistance.

The objectives of this project were to: 1) purify the glutathione S-transferases from Atlantic Wood (contaminated) and King Creek (reference) mummichog livers and compare the isoform composition in the two groups, 2) produce monoclonal antibodies to the elevated GST isoform(s) found in the AW mummichog, 3) quantify the relative amounts of elevated isoform in fish from three sample sites: control, moderately contaminated and heavily contaminated with PAHs of creosote origin, using monoclonal antibodies, and 4) identify the elevated isoform based upon immunological reactivity and/or protein sequence and physicochemical characteristics.

MATERIALS and METHODS

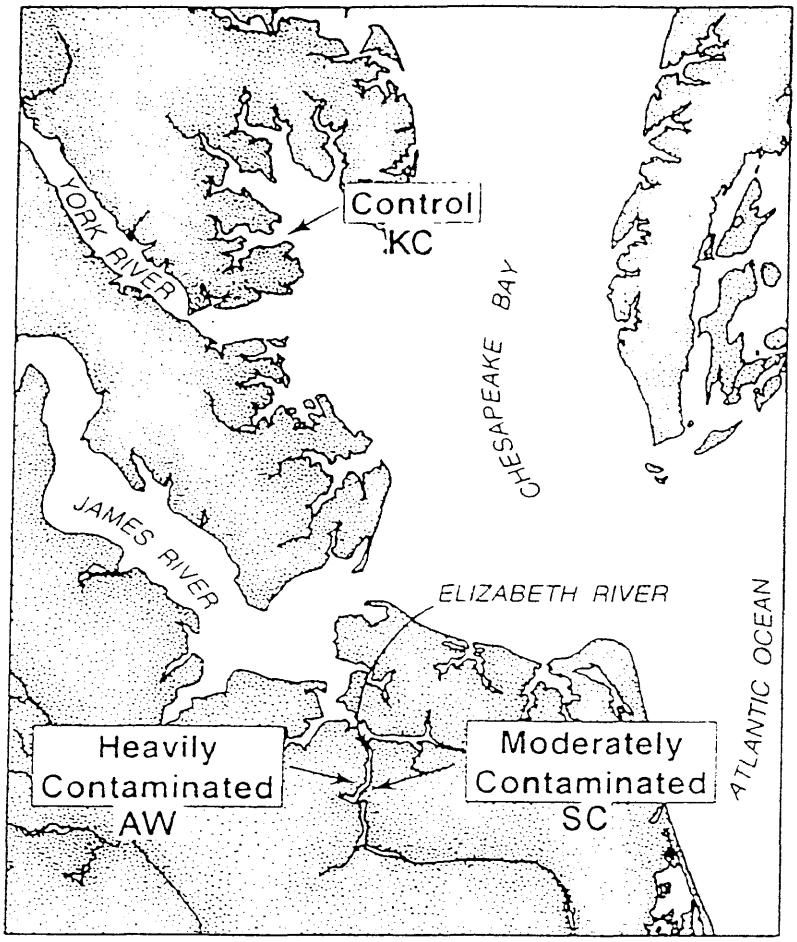
Sample Sites and Collection

Male mummichog, *Fundulus heteroclitus*, weighing 5-20g, were collected from three sites (Fig. 4): 1) Atlantic Wood (AW), a heavily creosote-contaminated site in the Elizabeth River, VA adjacent to Atlantic Wood Industries, Inc., 2) Scuffletown Creek (SC), a moderately creoste-contaminated site across the river from AW, and 3) King Creek (KC), an uncontaminated site in Gloucester County, VA. Sediment PAH concentrations at these sites have been reported as 2200, 61 and 3mg PAH/kg dry sediment at AW, SC, and KC respectively (Vogelbein *et al.*, 1990). Mummichog were caught in minnow traps and transported to the laboratory in coolers with iced estuarine water. Fish were sacrificed within 2 days of capture with an overdose of tricaine methanesulfonate (MS-222, Crescent, Phoenix, AZ). Livers were removed and frozen in liquid nitrogen before transfer to -80°C.

Cytosol Preparation & Enzyme Purification

Livers were thawed and homogenized in homogenization buffer (0.25 M sucrose, 25 mM HEPES, 1 mM EDTA, pH 7.5) using a Polytron (Brinkmann, Westbury, NY) tissue homogenizer. The homogenate was centrifuged twice at 12,000g for 11 minutes and once at 100,000g for 60 minutes in a Sorvall RC 28S (DuPont, Wilmington, DE) centrifuge at 3°C. The supernatant (cytosolic fraction) was removed from the microsomal pellet and stored at -80°C.

Figure 4. Map of mummichog, *Fundulus heteroclitus*, collection sites. The collection sites were: King Creek (KC), a clean, reference site, Scuffletown Creek (SC), a moderately creosote-contaminated site, and Atlantic Wood (AW), a heavily creosote-contaminated site adjacent to Atlantic Wood Industries, Inc. SC is approximately 600 m from AW.


Control
KC
YORK RIVER
CHESAPEAKE BAY
JAMES RIVER
ELIZABETH RIVER
ATLANTIC OCEAN
Heavily
Contaminated
AW
Moderately
Contaminated
SC

Purification of GSTs from the cytosol was performed at 4°C using S-hexylglutathione affinity chromatography (Mannervik & Guthenberg, 1981). Pooled cytosol was dialyzed overnight at 4°C in 3/4" Prepared Dialysis Tubing with 12-14kD molecular weight exclusion limit (Gibco BRL, Gaithersburg, MD) using three changes of three liters of loading buffer (10mM Tris HCl, 50mM NaCl pH 7.8). Dialyzed cytosol (4-6ml) was passed over a column (1.5cm x 8.5cm) of S-hexylglutathione agarose (Sigma, St. Louis, MO) with 4 column volumes of loading buffer. The column was washed with 3.5 column volumes wash buffer (10mM Tris HCl, 200mM NaCl, pH 7.8) and the enzyme eluted with 4 column volumes elution buffer (wash buffer with 5mM S-hexylglutathione [Sigma, St. Louis, MO]). Fractions (2.0 ml) were collected using an ISCO Foxy fraction collector (Lincoln, NE) and analyzed for protein content at 280nm and for enzyme activity (see below). Eluted fractions with GST activity >1.0 units/ml and protein absorbance greater than 0.1 A_{280} were pooled and washed in 20mM sodium phosphate buffer. The pooled fractions were then concentrated to approximately 500-600μl using a Centriprep-30 concentrator (Amicon, Inc., Beverly, MA) and stored at -80°C.

Enzyme Activity

Glutathione S-transferase activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma, St. Louis, MO) as a substrate (Habig *et al.*, 1974). The assay was conducted in a thermostated cell compartment in a Gilford RESPONSE spectrophotometer (Ciba-Corning, Oberlin, OH) at 25°C using 0.1M potassium phosphate buffer, pH 6.5, with 1mM glutathione, 1mM CDNB, and 10-20μl

cytosolic enzyme. Blanks consisted of assay buffer without cytosolic enzyme. Change in absorbance of substrate upon conjugation with GSH was monitored at 340nm over a period of 5 minutes. Enzyme activity is expressed in units which are defined as the formation of 1μmole product per minute. Specific activity is defined as the units of enzyme activity per mg protein. Total protein was estimated using the method of Bradford (1976) with bovine serum albumin as a standard.

Electrophoresis

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels (Laemmli, 1970) in a Bio-Rad Mini-PROTEAN II dual slab cell (Bio-Rad, Richmond, CA). SDS-PAGE was used for the determination of purity of the enzyme after purification, molecular weight estimation, and for western blot quantification of GST in samples from all sites.

Native (non-reducing) isoelectric focusing (IEF) was performed according to Robertson *et al.*(1987) using a Hoefer Mighty Small II SE250 Vertical Slab Unit (Hoefer, San Francisco, CA). Native IEF was used to separate the GST isoforms, estimate their isoelectric points (pI), and to screen monoclonal antibodies (see below). Denaturing (non-native) IEF in urea was performed according to Killick (1991) in order to determine the subunit composition of the GST isoforms. Ampholytes (Bio-Rad, Richmond, CA) were used to create a pH gradient in both native and non-native IEF gels.

Estimation of molecular weights and isoelectric points were determined using electrophoresis protein standards (Bio-Rad, Richmond, CA) and purified rat liver glutathione S-transferase subunits of molecular weights 28 (Alpha), 26.5 (Mu), and 25

(Alpha) kD (Sigma, St. Louis, MO). Protein standards used in SDS-PAGE gels were molecular weight markers and rat liver GST subunits (Sigma, St. Louis, MO). Standards used in IEF gels were IEF markers (Sigma, St. Louis, MO). Standards used in IEF-urea gels were 2-D markers (Bio-Rad, Richmond, CA). Gels were stained for protein using either silver stain (Polysciences, Inc., Warrington, PA) or Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA).

Western Blotting

Western blotting was performed according to ECL (Amersham Life Science, Buckinghamshire, England) Western blotting protocols . Proteins were transferred from SDS-PAGE and IEF gels to Immobilon PVDF Transfer Membrane (Millipore, Bedford, MA) for western blotting (Towbin *et al.*, 1979) in a Bio-Rad Mini Trans-blot cell. Pre-chilled buffers were used for transfer (100V for 1h, 4°C). The transfer buffer used with SDS-PAGE gels consisted of 25mM Tris, 192mM glycine, 20% methanol (pH 8.3). The transfer buffer used with IEF gels consisted of 0.7% acetic acid. The transfer buffer used with IEF-urea gels consisted of 0.7% acetic acid, 10% methanol. Following transfer of proteins to membranes, the membranes were stored overnight in TTBS (20mM Tris base, 137mM NaCl, 3.8 mL 1M HCl w/ 0.1% Tween-20, pH 8, per 1000 mL) at 4°C. The membranes were then incubated on a rocker (Reliable Scientific, Hernando, MS) with mouse anti-GST primary antibody (either polyclonal or monoclonal) diluted in TTBS for 1 hour, rinsed in TTBS and incubated with the secondary antibody, horseradish peroxidase linked goat anti-mouse antibody (G∝M-HRPO, IgG, IgM, H+L) (Jackson ImmunoResearch, West Grove, PA) for 1 hour. Detection of antibody bound to protein

bands was performed using enhanced chemiluminescence with ECL (Amersham Life Science, Buckinghamshire, England) Western blotting detection reagents. The membranes were then stripped of antibody in stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5mM Tris-HCl, pH 6.7) at 50°C for 30 minutes and stained for total protein with colloidal gold (Bio-Rad, Richmond, CA).

Monoclonal Antibody

Production of monoclonal antibodies was performed according to Goding (1983) with a few modifications (Fig. 5). Purified GST (antigen) from Atlantic Wood fish was emulsified in Freund's Complete Adjuvant (FCA) (Sigma, St. Louis, MO) and administered to four 5-week old female BALB C/J mice (Jackson Labs, Bar Harbor, ME). Each mouse received one subcutaneous and one intraperitoneal (ip) injection (approx. 40μg protein/100μl FCA/mouse). Serum was derived from each immunized mouse prior to immunization (pre-bleed) and one month after immunization (immune sera). Blood was collected from a tail vein and allowed to clot at room temperature for 2 hours or overnight at 4°C. After clotting, the blood was spun at 14,000g in an Eppendorf 5415 C centrifuge (Brinkmann, Westbury, NY) for 10 min. at 4°C and the sera was removed and stored at -80°C. The antibody response to the antigen was monitored (see below) in each mouse using the collected sera.

Mice exhibiting an immune response were challenged at 7 weeks post immunization with an intravenous dose of purified GST (5μg protein) in sterile phosphate buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.2-7.4). After 4 days, the mouse was killed by cervical dislocation and the spleen aseptically

Figure 5. Monoclonal antibody production. A flow-chart illustrating the steps and time-frame for production of monoclonal antibodies

DAY	
0	Collect pre-immunization sera & Immunize w/ pure AW GST in FCA
	
	⇓
30	Collect post-immunization sera
	⇓
49	Challenge w/ pure AW GST in PBS
	⇓
53	Harvest & fuse spleen cells with myeloma cells
	⇓
54-62	Select for hybridoma cells
	⇓
62-63	Screen & clone cells from positive wells
	⇓
72-73	Screen & clone cells from positive wells
	⇓
82-83	Screen & clone cells from positive wells
	⇓
100-140	Produce & collect ascites ⇓ SAS precipitate MAb & Titer

removed and splenocytes harvested. Splenocytes were fused with SP 2/0 mouse myeloma cells (Shulman *et al.*, 1978) using 50% polyethylene glycol. Fused cells were selected using medium containing azaserine-hypoxanthine (AH) (Foung *et al.*, 1982) and were grown in 96 well tissue culture plates (Corning, Cambridge, MA). After 9 & 10 days, the supernatants were screened for anti-GST antibodies using an ELISA (see below). Cells from positive wells were cloned by limiting dilution as well as expanded in 24-well plates, followed by $25cm^2$ flasks, and then frozen in freezing medium (90% fetal bovine serum, 10% dimethylsulfoxide) at -80°C. Positive cells were then stored in liquid nitrogen. Positive cells went through at least one more round of cloning in order to be certain that the antibody producing cells were monoclonal.

Large quantities of antibody were collected as ascites fluid. Mice were primed with an ip injection of pristane (Sigma, St. Louis, MO) one month prior to injection of hybridoma cells. Hybridoma cells were grown in $75cm^2$ flasks until confluent. Approximately $5x10^5$ cells suspended in PBS were injected ip into mice. When the mouse belly was fully distended with ascites fluid (after 2.5-8 weeks), the mouse was killed by cervical dislocation. The ascites was aspirated from the peritoneal cavity, then rinsed with 5ml sterile PBS. All fluid was once again removed. The ascites was spun at 2271g for 30 min in a Sorvall RT 6000D centrifuge (Du Pont, Wilmington, DE). Any pellet formed was discarded. In some instances, due to time constraints, the ascites was allowed to sit overnight at 4°C before precipitation. The ascites fluid was sterile filtered and the antibody precipitated using saturated ammonium sulfate (SAS) (100g ammonium sulfate, 100ml milliQ water [Millipore, Bedford, MA], pH 6.7) (Harlow & Lane, 1988).

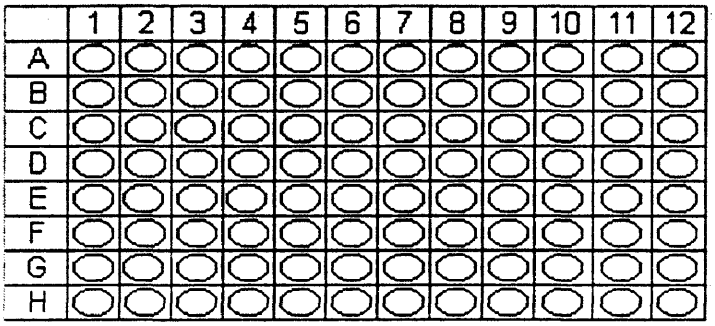
While gently swirling the ascites on ice, a one-half volume of SAS was added over 30 min. The ascites was spun at 3000g at 4°C for 30 min. The supernatant was removed and another volume of SAS equal to one-half the original volume of ascites was slowly added to the supernatant while gently swirling on ice. The sample was spun at 3000g for 30 min, the supernatant discarded and the pellet resuspended in a volume of sterile PBS equal to 1/10 the original volume of ascites. The antibody was then dialyzed overnight in PBS, titered (see below), aliquoted and stored in cryovials (Corning, Cambridge, MA) at -20°C.

Screening Assays

An enzyme-linked immunosorbent assay (ELISA) (Engvall & Perlman, 1971) was used as the primary method for screening mouse sera and hybridomas for anti-GST antibodies (Fig. 6). The assay was optimized for the least amount of coating antigen required to yield a significant reading for screening hybridomas. Briefly, a 96-well ELISA plate was coated with 0.25μg purified AW GST in 100μl coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) per well overnight at 4°C. The plate was then washed 3x with TTBS using a Titertek Microplate Washer/20 (ICN, Costa Mesa, CA), blocked with 250μl 1% BSA/TTBS, and then incubated for one hour at 37°C with either 100μl serum dilution or 10μl cell culture supernatants in 100μl 1% BSA/TTBS. Unbound antibody was washed away and the plate incubated with a secondary goat anti-mouse horseradish peroxidase labeled antibody (G∝M-HRPO). A substrate solution of 18mM ABTS (azino-bis(ethylbenzthiazoline-6-sulfonic acid)) (Sigma, St. Louis, MO) and 0.15% hydrogen peroxide in citric acid buffer (0.2% w/v citric acid, pH 4.0) was then

Figure 6. ELISA screening assay. A flow-chart illustrating the steps for performing an enzyme-linked immunosorbent assay (ELISA).

96-well ELISA plate



Coat w/ 0.25 μg pure AW GST in 100 μl coating buffer
(overnight, 4°C)

⇓

Wash 3x, TTBS

⇓

Block w/ 250 μl 1% BSA/TTBS
(1 hour, 37°C)

⇓

Wash 3x, TTBS

⇓

Incubate w/ 100 μl serum or 10 μl cell culture supernatant
(1 hour, 37°C)

⇓

Wash 3x, TTBS

⇓

Incubate w/ 100 μl 1:10,000 G$\propto$M-HRPO
(1 hour, 37°C)

⇓

Wash 6x, TTBS

⇓

100 μl color development solution
(~20 min.)

⇓

Read at 405nm

added to the plates. In the presence of HRPO, a chromogenic reaction occurs which was measured at 405 nm on a Titertek Multiscan MCC/340 (ICN, Costa Mesa, CA) plate reader. Plates which were not coated with antigen served as negative controls for nonspecific binding of the antibody. Serum titers are expressed in terms of units of antibody activity per volume of serum (units/μl) (Arkoosh & Kaattari, 1990). One unit equals the volume of serum giving the 50% maximum OD (optical density).

Western blotting was used as a second screening method in order to determine which isoform(s) were being recognized by each antibody. After the isoforms were separated by IEF and transferred to membranes, strips of membrane were incubated with hybridoma supernatants. Detection of antibody bound to protein bands was performed using enhanced chemiluminescence as previously described.

N-terminal Amino Acid Sequencing & Amino Acid Analysis

Before the isoform was isolated for protein analysis, it was necessary to determine if it was a homodimer or heterodimer. Homodimers consist of two identical proteins (subunits) and heterodimers consist of two different polypeptides. It was necessary to know if the sample was pure (in the case of a homodimer) or unpure (in the case of a heterodimer). Sequencing and amino acid analysis can only be performed on pure samples. The method of Killick (1991) was used to determine if the elevated isoform was a homo- or heterodimer. Purified AW cytosolic proteins were separated by urea-IEF. Gels were stained for protein to visualize the number and intensity of the bands. Proteins were also transferred to PVDF membranes for western blotting to determine the number of bands which were recognized by the MAb. It was assumed that if the protein were a

heterodimer, two bands would be visualized during western blotting if the antibody recognized the same epitope on each subunit. If the antibody did not recognize the same epitope, and only one band was visualized during western blot, then a second band of equal staining intensity should appear in the protein stain. If the protein were a homodimer, protein stain would reveal a single major protein band that would be visualized during western blotting.

Sample preparation for N-terminal sequencing was performed according to Hsieh *et al.* (1988). Briefly, purified AW GST isoforms were separated on a native IEF gel using ampholytes with pH ranges of 3-10 and 7-9. The gel was soaked in 200ml of 3.5% perchloric acid for 1 hour with a change of solution every 20 min. The ampholytes were then removed using a Southern blot apparatus for 3 hours using 2% acetic acid as a buffer. Proteins were transferred to Immobilon-P in 0.5% NP-40, 1.0% acetic acid buffer at 100V for 1 hour at 4°C. The membrane was stained briefly (less than 1 min.) with Coomassie blue, destained briefly (less than 1 min.), and rinsed several times in milliQ water. The target bands were cut from the membrane, dried, and shipped to the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE) for N-terminal sequencing and amino acid analysis.

A comparison of amino acid compositions was conducted using the fractional content of each amino acid. The sum of the absolute values of the differences of each amino acid was multiplied by 50 to give the difference index (DI) (Metzger *et al.*, 1968). Two proteins with the same amino acid composition have a DI of 0 and two proteins with no amino acids in common have a DI of 100. Amino acid composition for the

mammalian isoforms was calculated using PC/GENE from sequences obtained from the SWISS-PROT database.

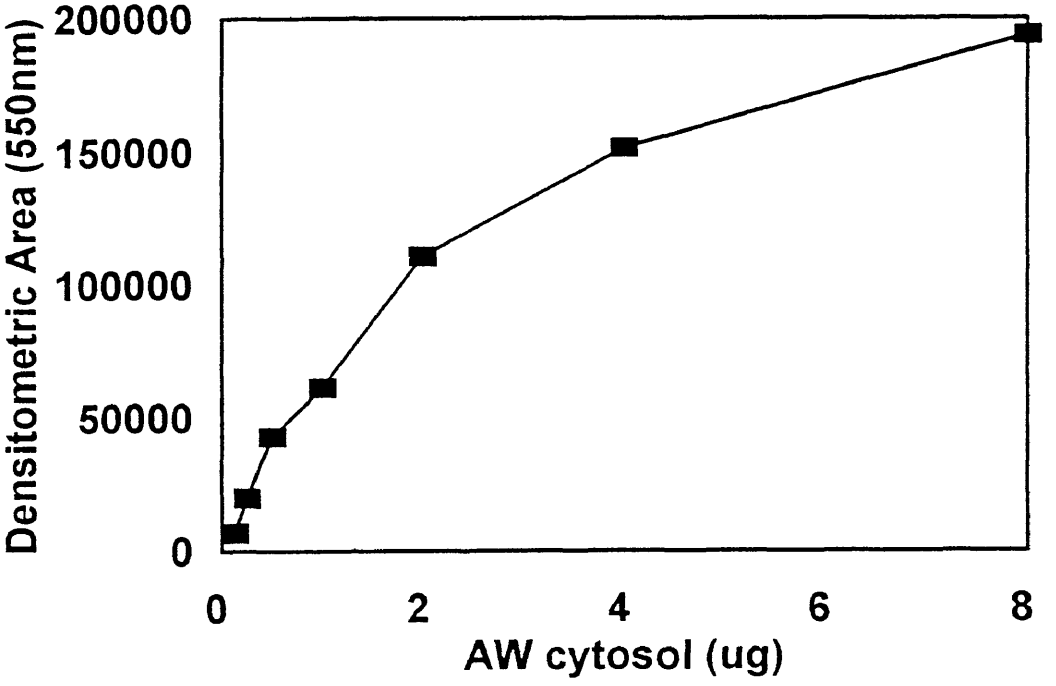
Field Study

The field study was a comparison of the GST activity and the relative amounts of the GST isoform in livers of individual mummichog from the three sample sites. GST activity was determined using CDNB as a substrate. Western blotting was used to determine the relative amounts of the isoform. The isoform was not purified so that the concentration of isoform in a given sample could not be determined.

To determine the relative amounts of isoform, a standard curve was developed for this study using pooled AW cytosolic proteins. The curve consisted of μg amounts of AW cytosolic proteins which, when blotted with the MAb and scanned, would give a linear increase in densitometric area with an increase in amount of protein. AW cytosolic proteins were titrated using MAb GST B2 C4 (see results). The results in Fig. 7 show a linear region ($R^2 = 0.98$) from 0.125 to 2μg cytosol. A standard curve was therefore used that consisted of 0.125, 0.25, 0.5, 1, 2μg pooled cytosolic proteins from AW fish.

The amount of protein used for each sample was 1-12μg for KC, 1-2μg for SC, and 0.5-1μg for AW. Membranes were blotted using a 1:5000 dilution of monoclonal antibody GST B2 C4 (2.45μg/ml protein) as the primary antibody. Samples and standards were analyzed using scanning densitometry with a CS-930 Chromato-Scanner (Shimadzu, Kyoto, Japan). Sample values were determined using the standard curve. Each μg of AW cytosol equals one GST unit. Each sample was then normalized by dividing the GST unit value by the μg of sample which was loaded in the lane.

Figure 7. Titration of AW cytosol using MAb GST B2 C4 (1:5000). Pooled AW hepatic cytosolic proteins were separated using SDS-PAGE and then blotted. The GST band which was recognized by the antibody was scanned at 550nm to determine the densitometric area. The area of each band was then plotted against the amount (μg) of protein loaded. The region from 0.125-2 μg is linear ($R^2 = 0.98$). A standard curve was therefore developed using 0.125, 0.25, 0.5, 1 and 2 μg AW cytosolic proteins. This standard curve was used to calculate the relative amounts of GST isoform in livers of mummichog from the three sample sites.


Densitometric Area (550nm)
200000
150000
100000
50000
0
0
2
4
6
8
AW cytosol (ug)

Sample values are expressed as GST units/μg protein (units/μg). Statistical significance between sample sites was determined by a Kruskal-Wallis analysis of variance because of the non-homogeneity of variances of the samples.

RESULTS

GST Purification

Affinity purification of GST from hepatic cytosols of AW and KC fish resulted in approximately 28-fold and 45-fold GST purification, respectively. Purification of GSTs from Atlantic Wood fish was performed three times with 40%, 41% and 65% recovery of GST activity (Table 2A-C). Purification of GSTs from King Creek fish was performed once with 7.7% recovery of GST activity (Table 2D). At least 70% and 50% of GST activity adsorbed to the column and was recovered in the eluant during AW and KC purifications respectively. The remainder of the activity did not adsorb to the column and appeared in the load fractions. After concentration, 60-70% of the GST activity was recovered for AW while only 14% of the activity was recovered for KC. This low recovery for KC was probably due to leakage through a faulty concentrator membrane. In each purification, GST activity was recovered as a single peak of activity and protein during elution (Fig. 8A & 8B). The purified protein consisted mainly of a band of approximately 27.2 kD (Fig. 9), which is in the molecular weight range of GSTs.

Native isoelectric focusing of the purified enzyme revealed four major bands at the basic end with apparent pIs of 8.8, 8.7, 8.3, and 8.1 along with some minor bands at the acidic end (Fig. 10). The protein with pI of 8.1 appeared to be the major form and was possibly elevated in purified AW GST when compared with purified KC GST and was therefore targeted for monoclonal antibody production.

Table 2. Purification of hepatic glutathione S-transferases from Atlantic Wood and King Creek mummichog.

A. Atlantic Wood

Purification Step	Protein (mg)	GST Activity with CDNB (units)	GST Activity Recovered (%)
Cytosol	98.6	317.8	
Dialyzed Cytosol	101.3	281.7	88.6
Sample Applied to Column	91.6	254.7	
Pooled Fractions	2.4	196.3	77.1
Concentrated Sample	1.2	115.2	58.7
	% Total Activity Recovered		40.1
	Purification		30x

B. Atlantic Wood

Purification Step	Protein (mg)	GST Activity with CDNB (units)	GST Activity Recovered (%)
Cytosol	91.6	285.6	
Dialyzed Cytosol	89.2	159.9	55.9
Sample Applied to Column	83.4	149.6	
Pooled Fractions	ND	171*	114.3
Concentrated Sample	1.5	123.1	73.4
	% Total Activity Recovered		41
	Purification		26.5x

C. Atlantic Wood

Purification Step	Protein (mg)	GST Activity with CDNB (units)	GST Activity Recovered (%)
Cytosol	114.9	438	
Dialyzed Cytosol	125.8	444	101.4
Sample Applied to Column	115.9	409	
Pooled Fractions	ND	452*	110.5
Concentrated Sample	2.6	286.6	
	% Total Activity Recovered		65.4
	Purification		29x

D. King Creek

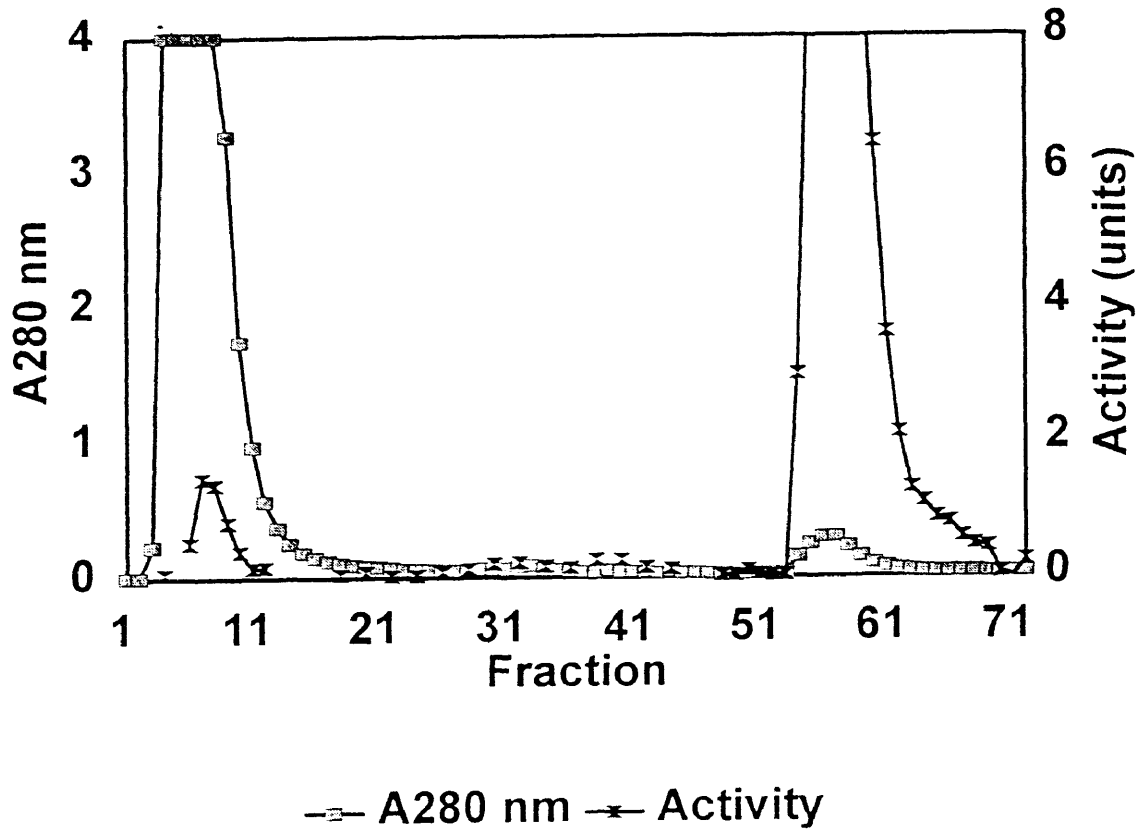
Purification Step	Protein (mg)	GST Activity with CDNB (units)	GST Activity Recovered (%)
Cytosol	71	74.3	
Dialyzed Cytosol	58.1	86	115.7
Sample Applied to Column	54.5	80.6	
Pooled Fractions	ND	39.3*	48.8
Concentrated Sample	0.1	5.1	13.6
	% Total Activity Recovered		7.7
	Purification		44.5x

*Indicates value was determined using sample volume instead of mg protein

ND = <0.1mg/ml

Figure 8. S-hexylglutathione affinity purification of hepatic GSTs from Atlantic Wood (A) and King Creek (B) mummichog, *Fundulus heteroclitus*. A and B were loaded with 83 mg and 55 mg protein, respectively. Fractions (2.0 ml) were collected and analyzed for protein (A280 nm) and GST activity with CDNB as a substrate. Load = fractions 1-25, wash = 26-47, elute = 48-72.

A


A280 nm
Activity (units)
Fraction
0
1
2
3
4
0
2
4
6
8
1
11
21
31
41
51
61
71
A280 nm
Activity

B

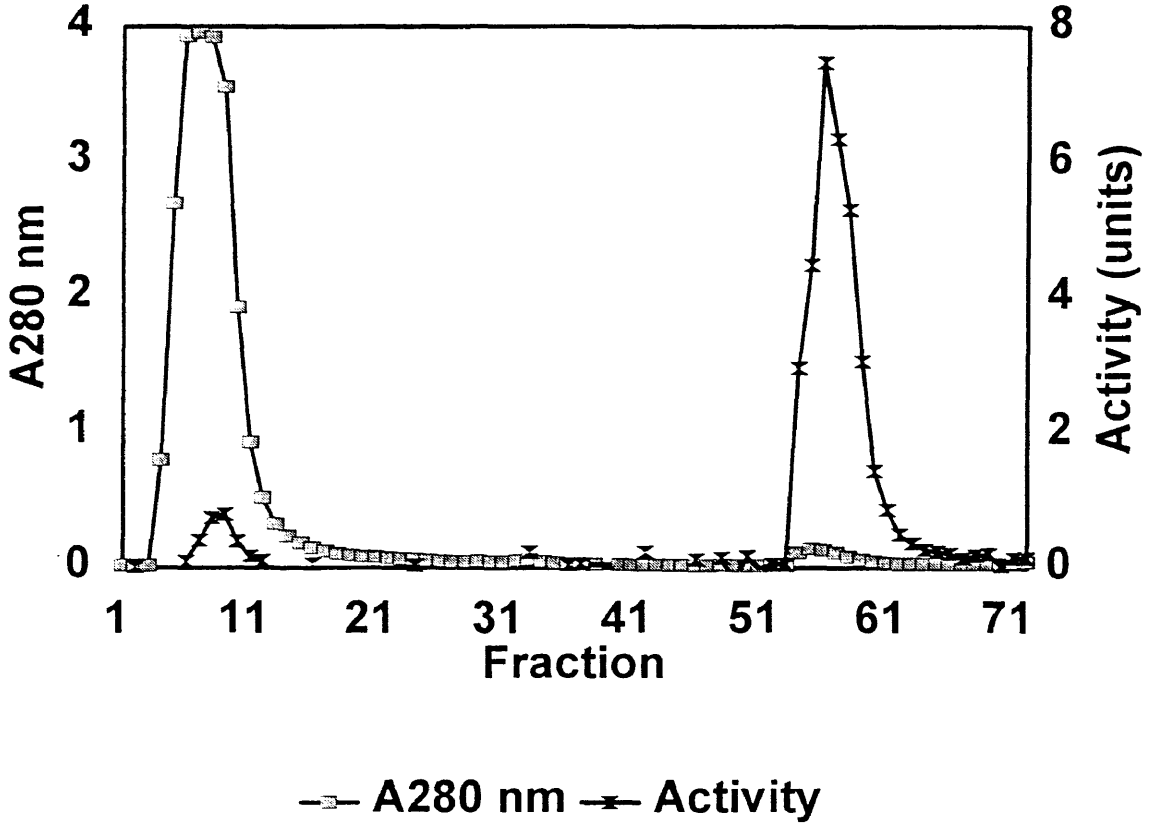

A280 nm
Activity (units)
Fraction
0
1
2
3
4
0
2
4
6
8
1
11
21
31
41
51
61
71
A280 nm
Activity

Figure 9. SDS-PAGE analysis of purified mummichog, *Fundulus heteroclitus*, GSTs. Lane 1, molecular weight standards; lane 2, rat liver GST standards which consist of an Alpha class subunit (28 kD), a Mu class subunit (26.5 kD) and an Alpha class subunit (25 kD); lane 3, AW hepatic cytosol (1μg); lane 4, S-hexylglutathione agarose affinity purified hepatic GST (0.1μg) from AW mummichog. The purified protein consists of a band that is approximately 27.2 kD without any contaminating proteins. Protein bands are visualized with silver stain.

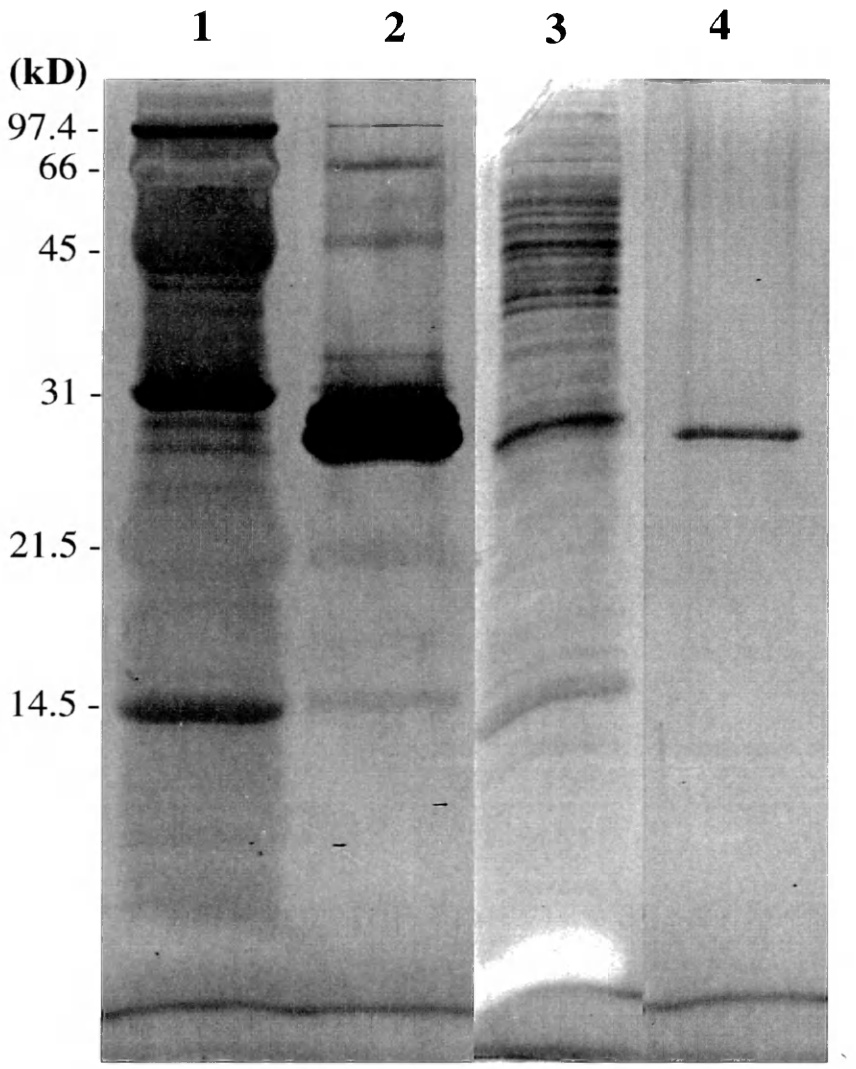
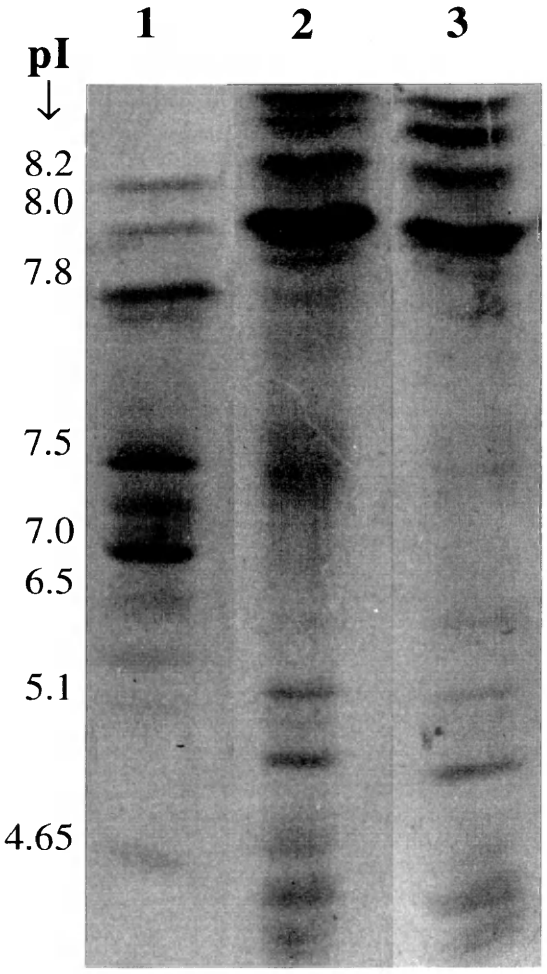

1
2
3
4
(kD)
97.4 -
66 -
45 -
31 -
21.5 -
14.5 -

Figure 10. IEF analysis of purified hepatic GSTs from AW and KC mummichog, *Fundulus heteroclitus*. Lane 1, IEF standards; lane 2, S-hexylglutathione agarose affinity purified GSTs from livers of AW mummichog, and lane 3, KC mummichog. The gel consisted of 3-10 ampholytes. Purified GSTs were loaded at 0.95μg protein/lane. Protein bands were visualized with silver stain. There are four bands at the basic end with a few minor bands at the acidic end. The major band at the basic end has a pI of approximately 8.1 and appears to be elevated in the AW sample when compared with the KC sample.


1
2
3
pI
↓
8.2
8.0
7.8
7.5
7.0
6.5
5.1
4.65

Monoclonal Antibody Production

Four mice (B, C, D, E) were immunized with purified AW GST. After one month, titers for each mouse were as follows: B= 67 units/μl, C= 180 units/μl, D= 100 units/μl, E= 250 units/μl (Fig. 11). Western blot with serum from mouse B against purified AW GST and AW cytosolic proteins indicated that there was no cross-reactivity of the antibody with other cytosolic proteins (Fig. 12). A fusion was performed with mouse B which had a titer of 50 units/μl (Fig. 13) at the time of fusion.

The fused cells were distributed into four master 96-well cell culture plates (labeled GST1-4). The master plates were screened on day 9 and four wells (labeled GST1 D11, GST2 A5, GST2 B6, GST4 H4) had a signal which was 2x the background. These wells were cloned and the master plates were rescreened for slow growing cells on day 11. Nine more wells were cloned for a total of 13 wells which were cloned during the first round. The first round clones were screened on day 10 and only one of 13 clone plates (GST1 D11) had positive wells. Four wells on plate GST1 D11 came up positive (GST A2, GST B2, GST F2, GST A5), all were expanded and frozen, and three (A2, B2, F2) went through a second round of cloning. Supernatants from the first round clones were screened by western blot and all four clones recognized the target protein with pI of 8.1 (Fig. 14). Western blot of SDS-PAGE separated proteins using first round clone supernatants indicated that the antibodies recognized a GST isoform which is elevated in cytosol and purified GSTs from AW fish when compared with cytosol and purified GSTs from KC fish (Fig. 15).

The second round clone plates were screened on day 6 and four positive wells

Figure 11. Titration curves for pre-bleed and immune sera. Four mice (B,C,D,E) were immunized with purified AW GST (40μg protein/mouse). At one month, the titers for each mouse were as follows: B= 67 units/μl, C= 180 units/μl, D= 100 units/μl, E= 250 units/μl. The horizontal line represents the 50% maximum OD.

ELISA Screening of Pre-bleed & Immune Sera

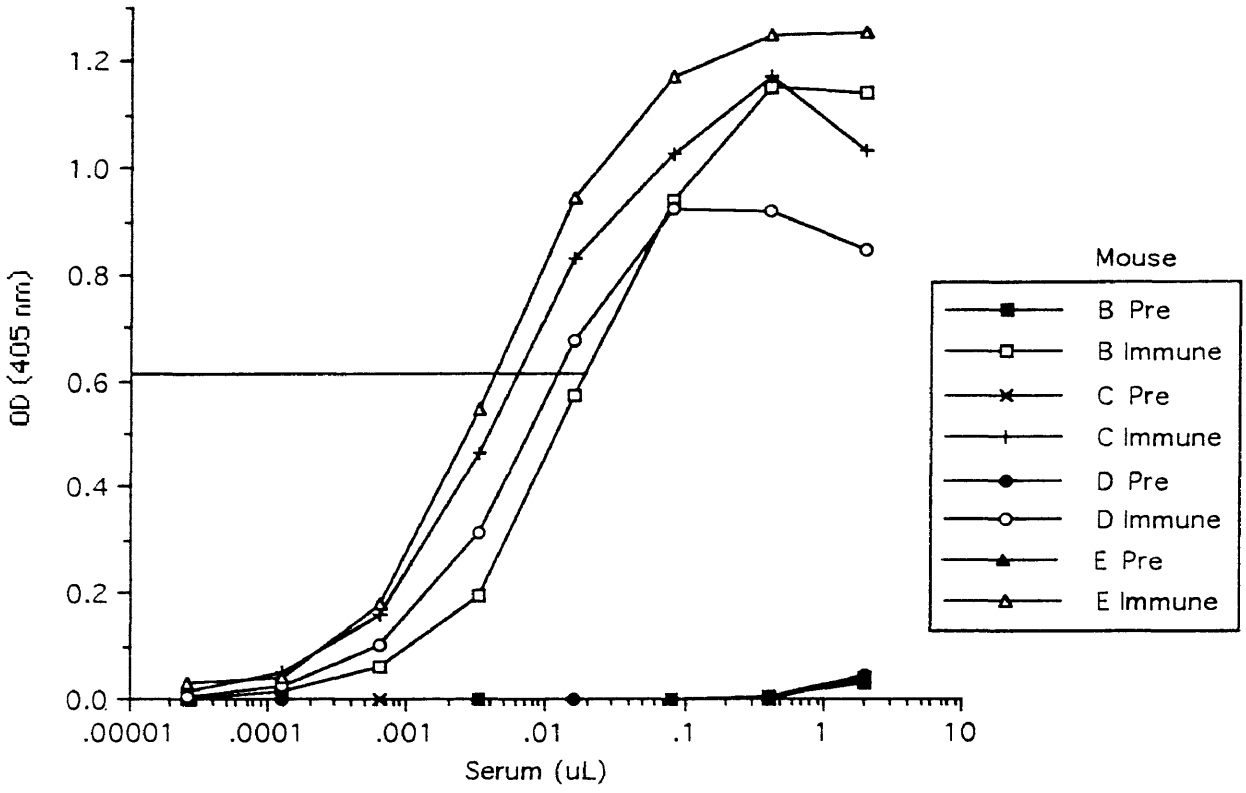


Figure 12. Western blot analysis of polyclonal antibody specificity for purified mummichog, *Fundulus heteroclitus*, hepatic GSTs. Lane 1, 1μg AW cytosolic proteins; lane 2, 0.1μg affinity purified GSTs from AW mummichog. Protein is visualized with colloidal gold stain. Lanes 3 and 4 are western blots of lanes 1 and 2, respectively. Membrane was incubated with a 1:2000 dilution of mouse B immune serum. The polyclonal antibody recognizes the 27.2 kD GST band and does not recognize other proteins in the cytosol.

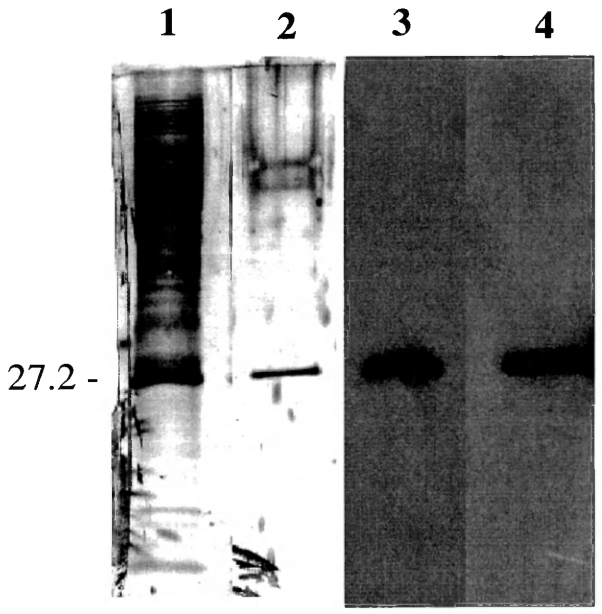

1
2
3
4
27.2 -

Figure 13. Titration curve for mouse B serum collected at time of fusion. Mouse B had a titer of 50 units/μl. Lines represent 50% maximum OD.

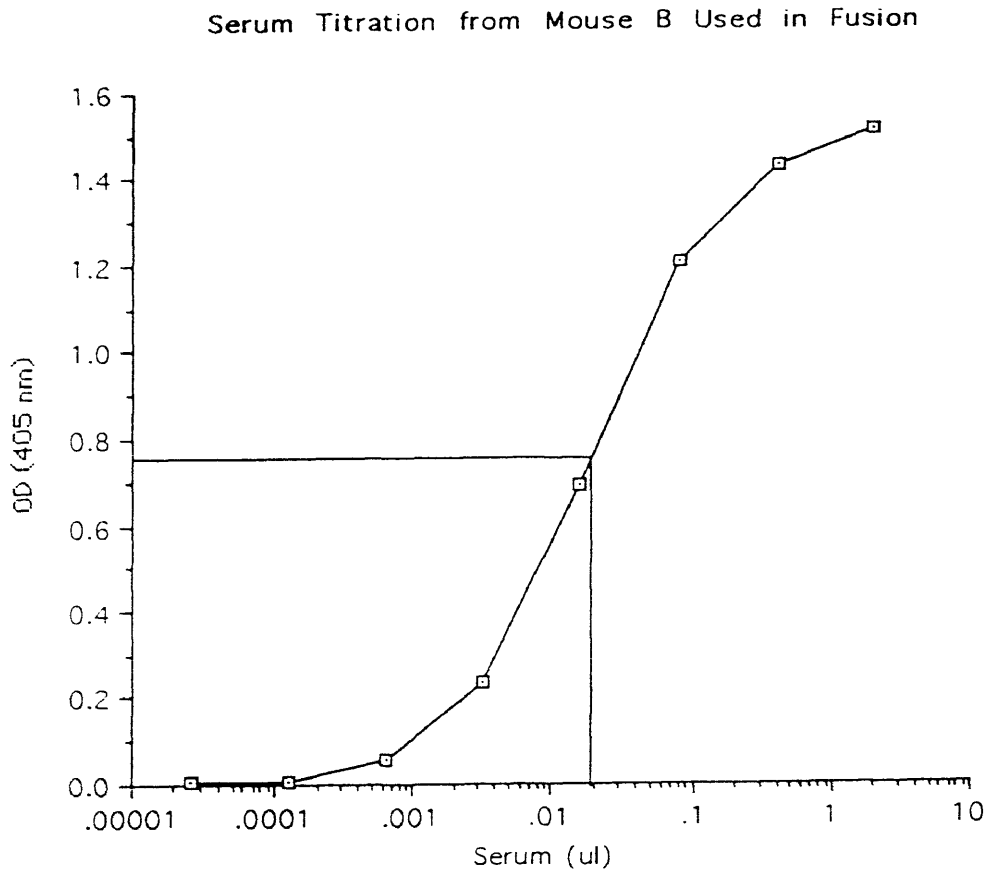

Serum Titration from Mouse B Used in Fusion
OD (405 nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
.00001
.0001
.001
.01
.1
1
10
Serum (ul)

Figure 14. Western blot screening during monoclonal antibody production. Affinity purified AW mummichog, *Fundulus heteroclitus*, hepatic GSTs were separated by native-IEF. Lane 1 is colloidal gold protein stain showing the four bands at the basic end with their pI values. Lane 2 was incubated with 1:20,000 polyclonal serum from mouse B. The polyclonal antibody recognizes the 8.1. 8.3 and 8.7 pI bands but not the 8.8 pI band. Lanes 3-6 were incubated with tissue culture supernatant from first round clones: GST A2 (lane 3), GST A5 (lane 4), GST B2 (lane 5), GST F2 (lane 6). All first round clones recognize the major 8.1 pI band. Lane 7 was incubated with GST B2 C4 MAb. The MAb recognizes both the major 8.1 pI band and the 8.3 pI band. Lanes 1-6 contain 4μg protein, lane 7 contains 6μg protein.

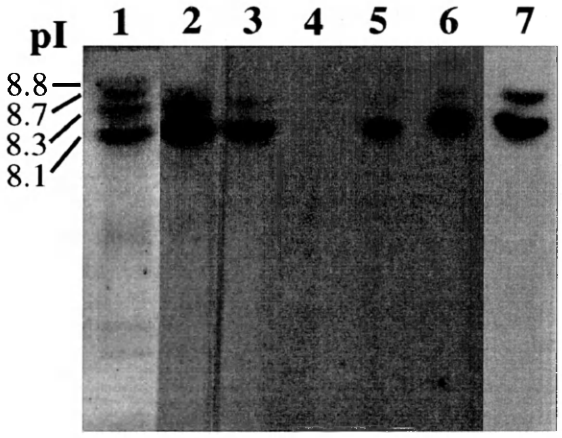
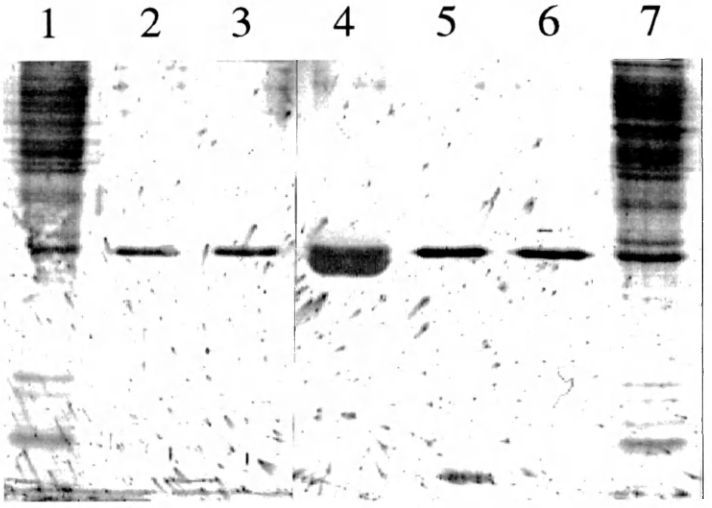
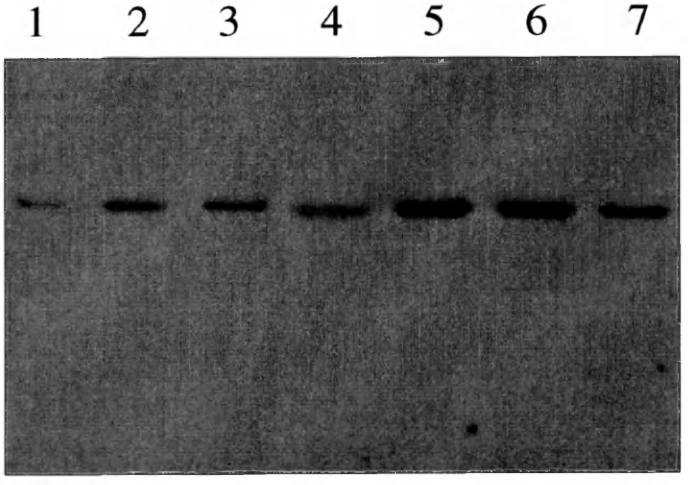

pI
1
2
3
4
5
6
7
8.8
8.7
8.3
8.1

Figure 15. Western blot analysis of hepatic cytosol and affinity purified GSTs from AW and KC mummichog, *Fundulus heteroclitus*. Proteins are separated by SDS-PAGE. A. Lane 1, KC cytosol; lanes 2 & 3, purified KC GSTs; lane 4, rat liver GST standards which consist of an Alpha class subunit (28 kD), a Mu class subunit (26.5 kD) and an Alpha class subunit (25 kD); lanes 5 & 6, purified AW GSTs; lane 7, AW cytosol. Protein loaded at 1μg of cytosol, 0.1μg pure GSTs. Protein is visualized with colloidal gold stain. B. Western blot of A. Membrane incubated with first round clone tissue culture supernatant from GST A2. The antibody recognizes a GST isoform which is elevated in AW cytosol and purified GSTs.

Ⓐ


1
2
3
4
5
6
7

Ⓑ


1
2
3
4
5
6
7

from each plate were expanded and frozen. Cells from each second round clone (GST B2 C4, GST A2 B9, GST F2 D8) as well as one first round clone (GST A5) were used for ascites production. Ascites titers from these clones were as follows: GST B2 C4 = 7 x 10^2 units/μl, GST A2 B9 = 1.1 x 10^3 units/μl, GST F2 D8 = 1.1 x 10^3 units/μl, GST A5 = 2.3 x 10^3 units/μl (Fig. 16). Although all four cell lines used in ascites production arose from the same well, they produce different antibodies as indicated by differences in their electrophoretic patterns (Fig. 17). GST A2 and F2 have the same electrophoretic pattern while GST B2 has a different pattern. GST A5 is probably a third antibody, which was not recognized by the G∝M, IgG, IgM antibody although protein stain does indicate that protein was loaded in the lane (not shown).

Field Study

Analysis of the 27.2 kD protein band corresponding to GSTs by scanning densitometry demonstrated an elevation of the protein in AW and SC fish compared with KC fish (Fig. 18) and represents a major protein in AW hepatic cytosol. There was a significant difference ($p<.0001$) in GST activity with CDNB between all three sample sites (Fig. 19) with 4-fold and 2.2-fold greater activity in AW and SC respectively than KC. There was also a significant difference ($p<.0001$) in GST isoform concentrations between all three sample sites (Fig. 19) with a 5.8-fold and 2.2-fold greater values for AW and SC than KC. Fig. 20 shows a comparison of the western blot results for an individual fish from the three sample sites. There was a strong correlation ($r = .834$, $p<.0001$) between GST activity with CDNB and isoform concentration (Fig. 21). The strong correlation between enzyme activity and isoform concentration indicates that the

Figure 16. Titration curves for ascites. Line represents the 50% maximum OD. The titers for the MAbs are as follows: GST A2 B9 = 1.1 x 10^3 units/μl, GST A5 = 2.3 x 10^3 units/μl, GST B2 C4 = 7 x 10^2 units/μl, GST F2 D8 = 1.1 x 10^3 units/μl.

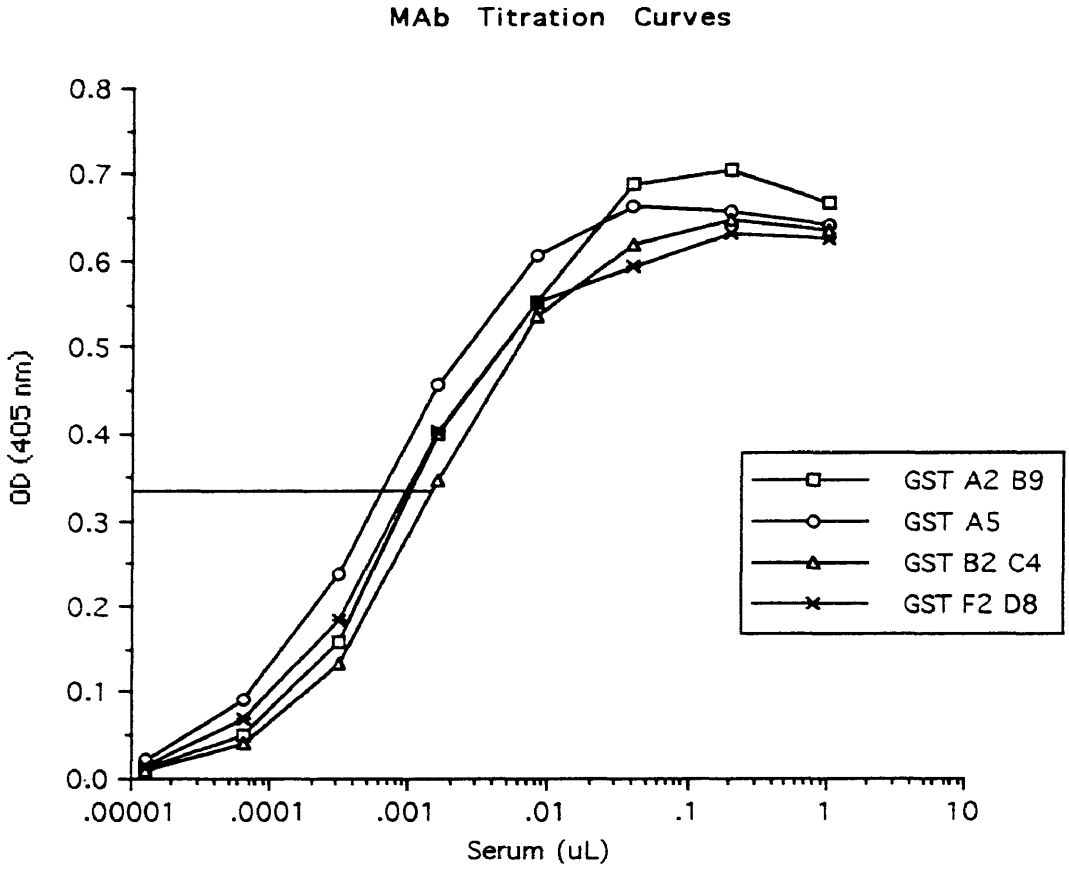

MAb Titration Curves
OD (405 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
.00001
.0001
.001
.01
.1
1
10
Serum (uL)
GST A2 B9
GST A5
GST B2 C4
GST F2 D8

Figure 17. Electrophoretic patterns of MAbs. Tissue culture supernatant (1.5μg protein) from each MAb was separated by native-IEF and then blotted with G∝M (IgG, IgM, H+L). Lane 1, GST B2 C4; lane 2, GST A2 B9; lane 3, GST F2 D8; lane 4, GST A5. GST A2 B9 and GST F2 D8 have the same electrophoretic pattern. GST B2 C4 has a unique pattern and GST A5 is not recognized by the G∝M antibody although protein was loaded in the lane (not shown).

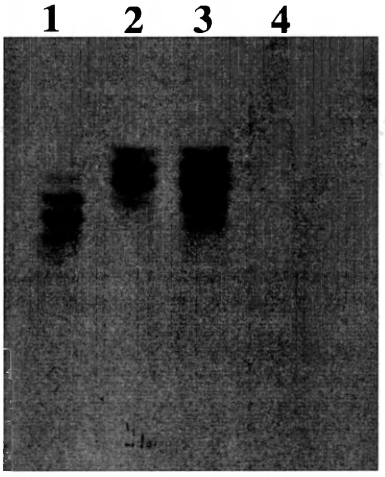
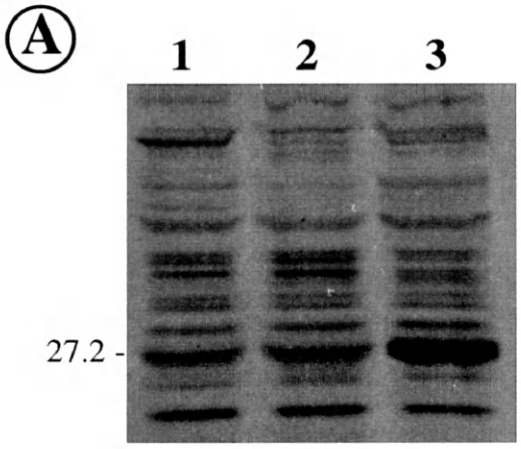

1
2
3
4

Figure 18. Comparison of intensity of GST band in KC, SC and AW mummichog, *Fundulus heteroclitus,* hepatic cytosol separated by SDS-PAGE. A. Silver stained cytosolic proteins from KC (lane 1), SC (lane 2), and AW (lane 3) mummichog. Each lane contains 2μg protein. B. Densitometric tracing of lanes in A.


A
1
2
3
27.2 -

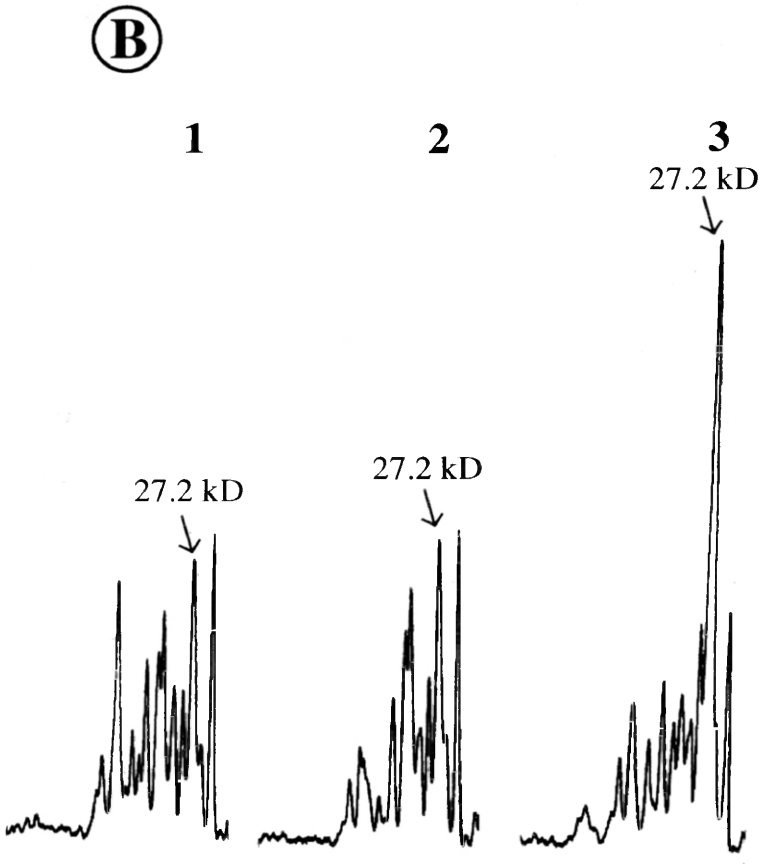

B
1
2
3
27.2 kD
27.2 kD
27.2 kD

Figure 19. Comparison of GST activity and relative isoform concentrations in mummichog, *Fundulus heteroclitus*, from three sample sites. Isoform concentration is expressed in units/μg. GST activity is expressed in units/mg. There is a significant difference in isoform concentrations between all sample sites ($p<.0001$, $n=11$) and a significant difference in activity between all sample sites ($p<.0001$, $n=11$). Bars represent means ± standard error.

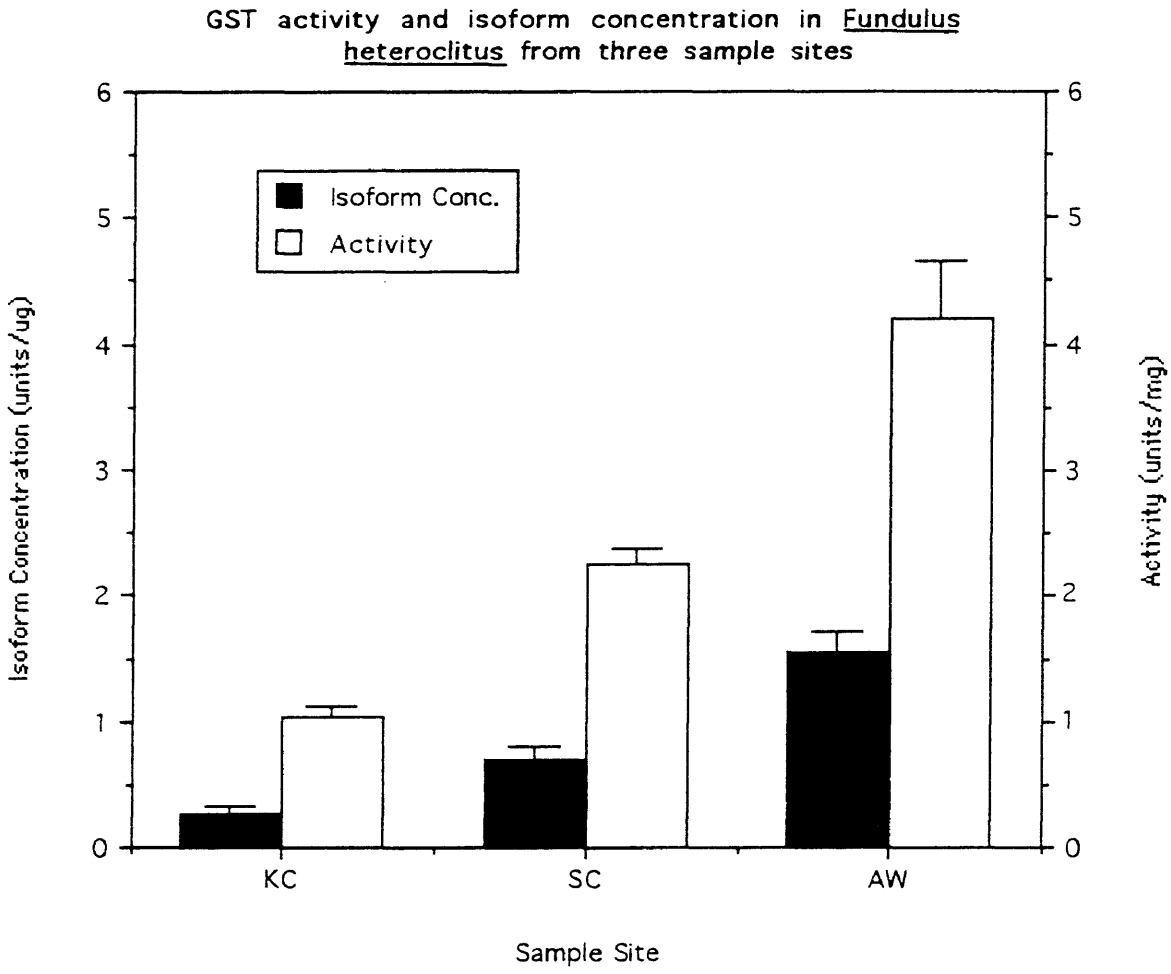

GST activity and isoform concentration in Fundulus heteroclitus from three sample sites
Isoform Conc.
Activity
Isoform Concentration (units/ug)
Activity (units/mg)
0
1
2
3
4
5
6
KC
SC
AW
Sample Site

Figure 20. Comparison of intensity of the GST isoform in western blot analysis of KC, SC and AW mummichog, *Fundulus heteroclitus*, hepatic cytosol. A. Western blot of SDS-PAGE separated proteins. Lane 1, purified GSTs; lane 2, cytosol from KC mummichog; lane 3, cytosol from SC mummichog; lane 4, cytosol from AW mummichog. All lanes contain 2 μg protein. Membrane was incubated with 1:5000 GST B2 C4 antibody. B. Densitometric tracings of bands in A.

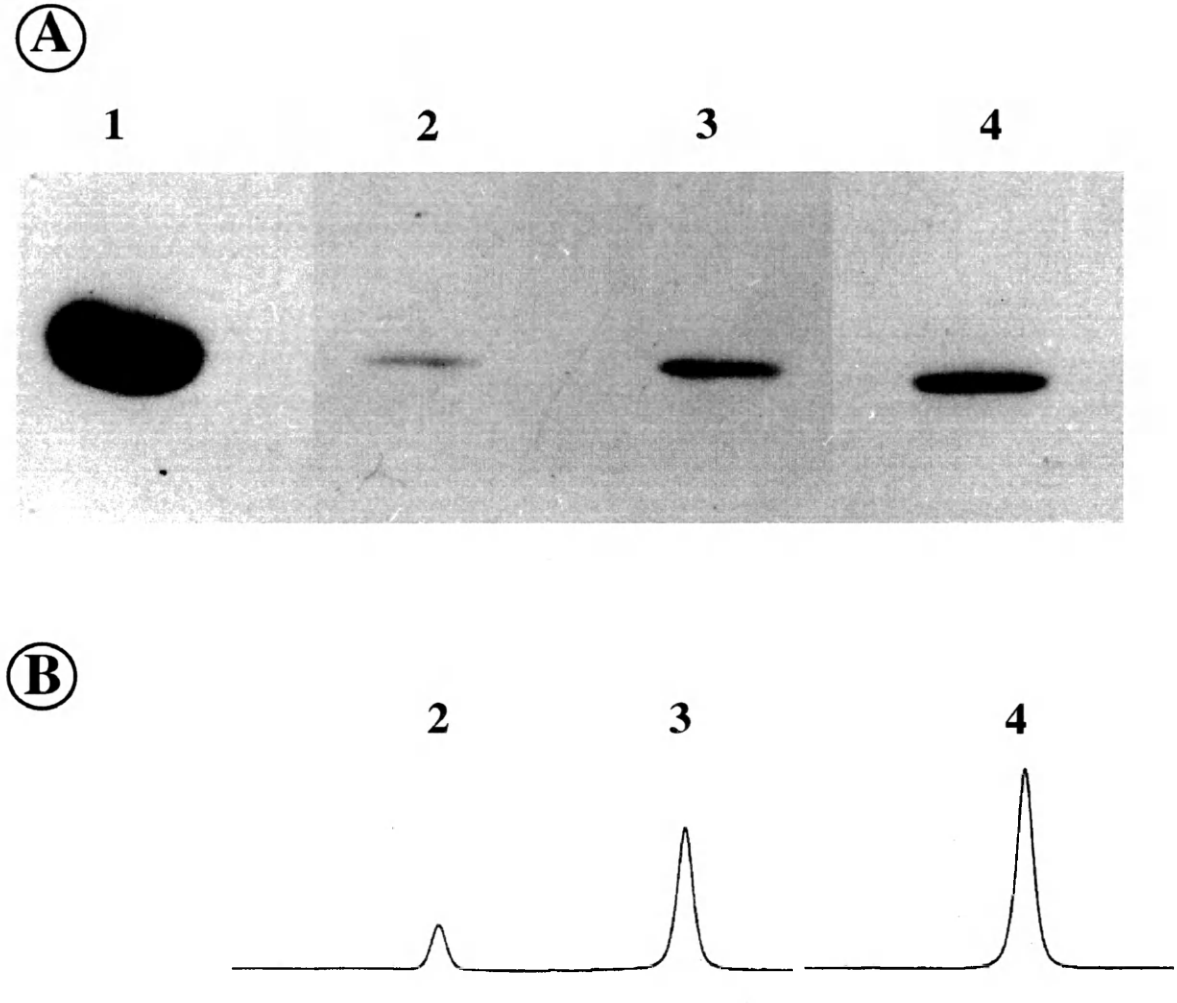
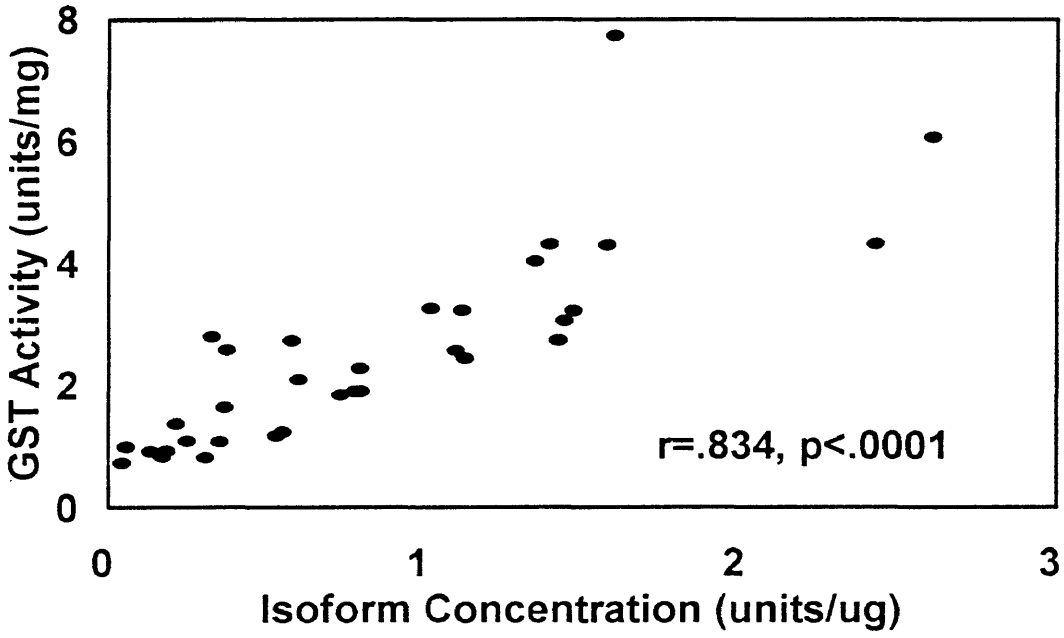

A
1
2
3
4
B
2
3
4

Figure 21. Correlation between GST isoform concentration and GST activity in mummichog, *Fundulus heteroclitus*, from three sample sites. n= 33.


GST Activity (units/mg)
8
6
4
2
0
0
1
2
3
Isoform Concentration (units/ug)
r=.834, p<.0001

enzyme is CDNB reactive.

Dimeric Composition of Elevated Isoform

Protein stain of AW cytosolic proteins separated in an urea-IEF gel revealed a single major band with pI of approximately 8.3 and a few minor bands at the basic and acidic ends (Fig. 22). Western blot showed that the MAbs only recognize the major band. This suggests that the elevated isoform is a homodimer. If the protein were a heterodimer, the other subunit would have a different pI and two bands of similar size would appear with the protein stain and on the blot.

N-terminal Amino Acid Sequencing & Amino Acid Analysis

GSTs are characterized using substrate specificity, immunological cross-reactivity, and protein sequence. The most effective method for characterization, other than sequencing the entire protein, is by determining the N-terminal sequence. The N-terminal region is highly conserved and has been used in identifying GSTs from other organisms. For the elevated isoform in mummichog, the N-terminal amino acid sequence could not be determined because the N-terminus is blocked. Blocking occurs due to post-translational modification of the N-terminal amino group. The modification masks the N-terminus and the protein cannot be sequenced using the conventional methods of Edman degradation.

The amino acid composition was determined (Table 3) and is shown in comparison with the amino acid composition of mammalian isoforms in each class. A comparison of the amino acid composition of the mummichog GST with those of mammalian forms from rat and mouse as well as plaice, salmon, blue mussel and thorny-

Figure 22. Western blot analysis of urea-IEF separated affinity purified GSTs. Lane 1, 4μg protein visualized with colloidal gold stain. Western blot of lane 1 in lanes 2-5. Lanes were blotted with 1:5000 dilution of MAb: lane 2, GST B2 C4; lane 3, GST F2 D8; lane 4, GST A5; lane 5, GST A2 B9. All MAbs recognize the major protein band with approximate pI of 8.3.

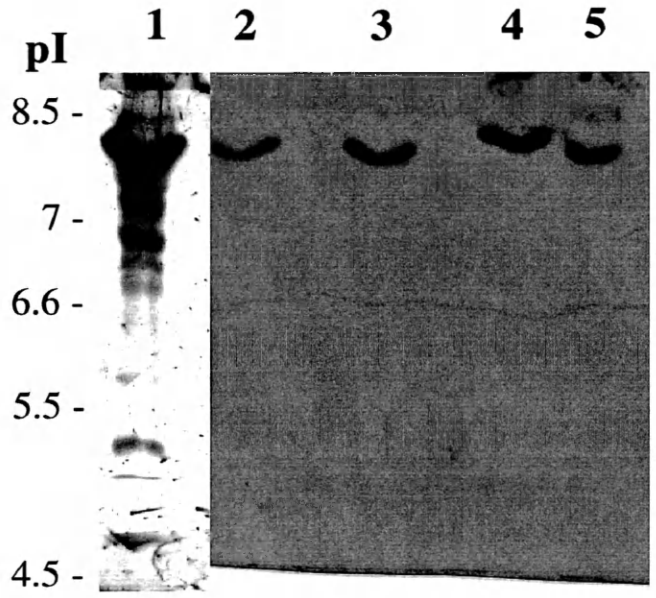

pI
1
2
3
4
5
8.5 -
7 -
6.6 -
5.5 -
4.5 -

Table 3. Amino acid composition of selected glutathione S-transferases including the isoform elevated in Atlantic Wood mummichog liver.

	Composition (%)					
Amino Acid	*Fundulus heteroclitus*	Thorny-back shark*	Alpha Rat GSTA1**	Mu Rat GSTM1	Pi Rat GSTP1	Theta Rat GSTT2
Ala	7	7.1	7.2	5	6.1	9
Arg	4.2	3.1	4.9	5.9	4.2	5.7
Asx	10.6	9.9	9.4	11	11.8	6.4
Cys	0	0	0.9	1.3	1.9	1.2
Glx	12.4	14.3	11.2	10.1	9	13
Gly	11.8	12.4	4.5	4.1	8.5	5.7
His	2.5	2.5	1.3	1.8	1.9	2.4
Ile	3.2	3.1	5.8	5.9	3.8	4.5
Leu	10.8	7.8	13.5	11.5	14.7	14.7
Lys	5.2	4.7	10.8	8.7	6.1	4.9
Met	1.8	2.8	4	3.6	1.4	2.4
Phe	5.4	3.7	4.9	5.5	3.3	3.6
Pro	5.6	5	4.5	5.5	5.2	5.7
Ser	9.2	9.6	4	5.5	5.2	4.9
Thr	4	3.7	3.6	3.2	3.8	3.2
Trp	ND	2.5	0.4	1.8	0.9	1.6
Tyr	4	2.5	3.6	6.4	5.2	2.4
Val	2.4	5.3	4.5	2.3	6.1	7.7

*From Sugiyama et al. (1981).

**Rat isoforms calculated using PC/GENE from sequences obtained from SWISS-PROT database.

ND=Not determined.

back shark are listed in Table 4. There is very little relation between the mummichog GST with any of the mammalian forms but it appears to be similar to the GST isolated from thorny-back shark and salmon. The MAb GST A2 B9 reacts only with the rat Mu subunit in the standards and not with the Alpha or Pi subunits (Fig. 23). The GST B2 C4 MAb does not react with any of the rat subunits.

Dimeric Composition of a Minor Isoform

A comparison of western blots of IEF separated isoforms with polyclonal serum and MAb GST B2 C4 is illustrated in Fig. 14. The polyclonal antibody recognizes three of the four major basic bands. The MAb only recognizes the 8.1 (major) and 8.3 (minor) pI bands. The minor isoform may either be a heterodimer consisting of one subunit in the major form, or the same homodimeric protein as the major isoform but with a slightly different pI due to post-translational modification or to being the product of a different allele of a polymorphic protein. There is some evidence of post-translational modification of some isoforms (Siegel *et al.*, 1990) which would result in the same protein having a slightly different pI. The enzyme was purified from a group of fish in a wild population whose GST genotypes are unknown.

Table 4. Difference index values for comparison of the amino acid composition of the Fundulus heteroclitus GST with GSTs from other organisms.

	Fundulus heteroclitus	Thorny-back shark	Rat GSTM1
RAT GSTA1**	18*	21.3	10.9
RAT GSTA2	18.4	20.9	10.4
RAT GSTA3	17.7	20.4	12.3
RAT GSTA5	18	20.6	11.2
Mouse GSTA1	18	20.5	12.6
Mouse GSTA2	17.7	20.7	11
Mouse GSTA3	17.2	20.2	12.3
Rat GSTM1	16.9	23.2	0
Rat GSTM2	16.5	21.9	3.7
Rat GSTM3	17.5	23.2	6.4
Mouse GSTM1	17.3	23.6	3.8
Mouse GSTM2	16.4	22.3	3.2
Mouse GSTM3	16.6	21.9	6.4
Mouse GSTM5	20.3	25.9	8.7
Rat GSTP1	14.8	18.2	14.6
Mouse GSTP1	13.8	17	12.8
Mouse GSTP2	12.9	16.1	12.7
Pig GSTP	13.3	16.9	12.2
Rat GSTT2	18.7	17.8	18
Mytilus edulis°	12.6	15.5	16.4
Thorny-back shark[1]	9.5	0	23.2
Salt-water salmon (Feb A)[2]	10.7	11.7	16.4
Plaice[3]	16.1	16.1	17.7

*Difference index calculated according to Metzger et al. (1968).

**Mammalian isoforms calculated using PC/GENE from sequences obtained from SWISS-PROT database.

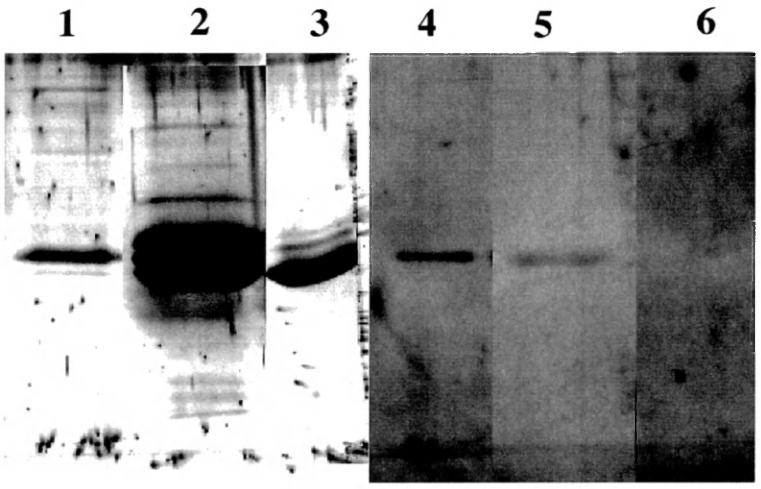
°Fitzpatrick et al. (1995).

[1]Sugiyama et al. (1981).

[2]Ramage et al. (1986).

[3]Leaver et al. (1993).

Figure 23. Western blot analysis of GST A2 B9 monoclonal antibody specificity for rat liver GST subunits. Lane 1, 1μg affinity purified hepatic GST from AW mummichog, *Fundulus heteroclitus*; lane 2, rat Alpha (28 kD), Mu (26.5 kD), and Alpha (25 kD) GST subunits; lane 3, 1.7μg rat Pi GST subunit. Lanes 4-6 are western blot of lanes 1-3, respectively. The MAb recognizes the band corresponding to the rat Mu class GST subunit.


1
2
3
4
5
6

DISCUSSION

In this study I have produced a monoclonal antibody to an elevated GST isoform in the liver of creosote-resistant mummichog. The antibody was used to measure GST isoform concentrations in fish from a clean site and moderately and highly polluted sites. There is a moderate (2.2-fold) and high (5.8-fold) increase in GST isoform concentrations in fish from the moderately and highly polluted sites relative to clean site fish. This is the first study to measure a change in GST isoform concentrations in fish from polluted sites. There was no apparent change in the pattern of isoforms expressed between AW and KC mummichog, indicating that the change in GST expression is due to a constitutively expressed isoform. GST activity was also measured in mummichog from the clean site, and moderately and highly polluted sites. There was a moderate (2-fold) and high (4-fold) increase in activity in fish from the moderately and highly contaminated sites respectively. These results agree with those of Van Veld *et al.* (1991). Other studies have reported either no increase (Collier *et al.*, 1992) or modest (2-fold) increases (Collier & Varanasi, 1984; Monod *et al.*, 1988) in activity in fish from polluted sites. This is the first report of such a large (4-fold) increase in activity in fishes from polluted sites.

In mammals, GST isoforms are grouped into Alpha, Mu, Pi and Theta classes based upon protein sequence, immunological cross-reactivity and substrate specificity. The Mu class is highly reactive with epoxides (Mannervik *et al.*, 1985), including BPDE, the ultimate carcinogen of BaP. While Pi is more efficient at catalyzing the conjugation

of BPDE and is highly selective toward the (+)-enantiomer (Funk *et al.*, 1995; Robertson *et al.*, 1986b), Mu is reactive towards both the (+)- and (-)-enantiomers (Robertson *et al.*, 1986a). Mu isoforms are induced by phenobarbital, 3-methylcholanthrene, *trans*-stilbene oxide (Ding and Pickett, 1985) and BaP (Soni *et al.*, 1995). Mu enzymes conjugate DNA hydroperoxides, cumene hydroperoxide and *trans*-stilbene oxide, products of oxidative stress (Hayes & Strange, 1995). Mu is also believed to play a role in susceptibility to lung cancer. Smokers who lack the gene encoding one of the Mu isoforms are more susceptible to lung cancer than smokers who express the gene (Nakajima *et al.*, 1995).

The elevated isoform in the mummichog is most closely related to the mammalian Mu class enzymes. The isoform was characterized using immunological reactivity, physicochemical properties and amino acid composition. A monoclonal antibody which recognizes the elevated mummichog isoform also recognizes the Mu class subunit in rat liver. Immunological cross-reactivity of fish GSTs with mammalian Mu class subunits has also been observed in cod and sea trout (George *et al.*, 1989). Physicochemical properties of the enzyme include a basic isoelectric point (pI 8.1), similar to Mu class enzymes in mice and rats (Mannervik & Danielson, 1988). CDNB is a substrate for Mu class GSTs. A strong correlation between isoform concentration and GST activity with CDNB indicates that the mummichog GST isoform is also CDNB reactive. The N-terminus of the mummichog GST is also blocked, similar to basic GSTs in mammals (Mannervik *et al.*, 1985).

While one of the monoclonal antibodies produced recognizes a rat Mu isoform,

the amino acid analysis does not place the mummichog GST close to any mammalian GSTs. Similar results were observed with a GST isolated from mussel (Fitzpatrick *et al.*, 1995). The amino acid analysis did not place the mussel GST close to any mammalian forms although the enzyme cross-reacted with, and had an N-terminal sequence that was close to, the mammalian Pi enzyme. Amino acid analysis places the mummichog GST relatively close to a GST in thorny-back shark (Sugiyama *et al.*, 1981) and salmon (Ramage *et al.*, 1986). The significance of this is unclear. The amino acid composition of the elevated isoform in the mummichog was not close to an isoform sequenced from plaice liver that is similar to mammalian Theta (Leaver *et al.*, 1993). Amino acid composition has not been determined for any other fish species. It is my observation that amino acid analysis for the identification of proteins works well when comparing proteins from mammals but is not useful when comparing non-mammalian proteins with mammalian proteins, at least in identifying GSTs.

GSTs play a major role in detoxification of lipophilic xenobiotic compounds in vertebrates and invertebrates. GSTs provide protection in cells against electrophilic metabolites that may bind to critical cellular macromolecules, including DNA, and result in production of mutant gene products and carcinogenesis. GST catalyzed conjugation of BaP metabolites with glutathione reduces the formation of DNA adducts (Hesse *et al.*, 1990) and aids in excretion of the toxic compounds. In addition to their role in conjugation of xenobiotic metabolites, glutathione S-transferases detoxify the products of oxidative stress (Hayes & Strange, 1995; Di Giulio *et al.*, 1995). Oxidative stress occurs due to the production of oxyradicals during aerobic metabolism of endogenous and

xenobiotic compounds and results in oxidative damage to membrane lipids and DNA. In mammals, GSTs protect cells from oxyradical production and the products of oxidative stress through conjugation and peroxidase activity.

Fish exhibit signs of oxidative stress, which include an increase in lipid peroxidation and antioxidant activity, when exposed to PAH contaminated sediments (Di Giulio *et al.*, 1993; Roberts *et al.*, 1987). Hasspieler *et al.* (1994a) found differences in susceptibility to oxidative stress in two fish species that exhibited differences in susceptibility to pollutant-mediated neoplasia. Oxidative stress induced in rat liver cells resulted in down regulation of cytochrome P450 mRNA expression (Barker *et al.*, 1994). This down-regulation may be an adaptive response to carcinogen exposure to minimize cell damage. A similar decrease in cytochrome P450 levels has been observed in AW mummichog (Van Veld & Westbrook, 1995), indicating that these fish may be undergoing oxidative stress due to exposure to creosote-laden sediments. While antioxidant enzyme activity and lipid peroxidation has not been measured in AW mummichog, oxidative stress may play a role in elevation of GST activity and tumor formation in these fish.

This study indicates a relationship between pollution level and GST concentrations and activity in mummichog liver. The relationship suggests that the elevated GST isoform may play a role in acute toxicity resistance of the mummichog to creosote-contaminated sediments. This result is not surprising because conjugation of PAH metabolites by GST is a major pathway of detoxification in fish from polluted and non-polluted sites (Kirby *et al.*, 1990a). Increases in GST expression and activity have

been associated with pesticide resistance in insects (Ku *et al.*, 1994; Lagadic *et al.*, 1993) and drug resistance in cancer cells (Tew, 1994; Mannervik *et al.*, 1990). Differences in GST isoform expression have been associated with susceptibility to DNA damage (van Poppel *et al.*, 1992; Wiencke *et al.*, 1990) and development of cancer in mammals (Soni *et al.*, 1995; Nakajima *et al.*, 1995). Variations in GST activity (Hasspieler *et al.*, 1994b; Collier *et al.*, 1992) and isoform expression (Collier *et al.*, 1992) are also believed to play a role in susceptibility to pollutant-mediated carcinogenesis seen in fishes from PAH-contaminated sites.

The site-dependent differences in GST levels in mummichog may result from physiological acclimation or genetic adaptation. While a dose-response relationship suggests that the differences in GST isoform levels and activity seen in the three populations studied is due to physiological acclimation, there is evidence to indicate that the response may also be genetic. Williams (1995) established that AW mummichog exhibit a genetic resistance to creosote laden sediments. Weis & Weis (1989) also observed a genetic resistance in mummichog exposed to metal contaminated sediments. Luoma (1977) suggested that resistance reflects the degree of contamination and greater resistance to a toxicant in a population from one location than a population from another location is direct evidence that the toxicant is exerting selective pressure. The Elizabeth River is a heavily industrialized area and the AW site has been polluted for years with several major creosote spills in the 1960s (Bieri *et al.*, 1986). The mummichog is the most genetically variable species examined by Smith & Fujio (1982). High genetic variability within a population allows for some individuals to withstand the pollution and

develop a resistant population.

Although the AW mummichog are resistant to the acute toxicity of the contaminated sediments (Vogelbein & Van Veld, unpublished), they are susceptible to the chronic effects. AW mummichog exhibit a high incidence of hepatic lesions including neoplasia while fish from SC and a control site do not exhibit these lesions (Vogelbein *et al.*, 1990), indicating an association between exposure and formation of neoplasms. A similar pollutant associated formation of neoplasms has been observed in other fish species as well (Landahl *et al.*, 1990). Altered expression of GST isoforms may occur in chemically induced tumors. Chemically induced hepatocellular carcinomas in rat expressed an increase in expression of Pi, Alpha and one Mu class enzyme, and a decrease in expression of another Mu class enzyme while the carcinomas expressed an overall increase in GST activity (Stalker *et al.*, 1994). According to Farber (1991), the elevated expression of phase II enzymes may contribute to resistance in mammalian preneoplastic nodules to acute toxic injury.

Although over-expression of GST is commonly observed in mammalian tumors, over-expression of GST has not been reported in fish tumors. In fact, Stalker *et al.* (1991) found that advanced neoplasms in white suckers from polluted sites exhibited a loss in GST expression compared with surrounding normal liver, instead of the increase which is observed in mammals. A decrease in GST expression was also observed in neoplasms induced by aflatoxin B_1 in rainbow trout (Kirby *et al.*, 1990b). While GST induced foci were observed, they did not progress to neoplasms. The development of carcinomas in these fish may be due to the repeated exposure to carcinogens. Repeated

exposure to carcinogens produces multiple genetic alterations and results in a higher rate of malignant conversion (Hennings *et al.*, 1983). The loss of GST expression would make a cell more susceptible to damage. A similar effect may be occurring in AW mummichog. While Van Veld *et al.* (1991) did not detect any changes in GST activity in grossly visible neoplastic tissue, a MAb may be a more sensitive tool to determine if there are changes in GST expression in neoplastic versus non-neoplastic tissues.

Previous studies with fishes have focused primarily on the changes in GST activity upon exposure to xenobiotics. It is apparent that there is a difference in expression of constitutive GST isoforms between fish species, indicating that individual species will respond differently to toxicant exposure. Studies directed at the expression and properties of individual isoforms in fishes may help explain the present inconsistencies seen in different species upon exposure to contaminants. This study has attempted to gain an understanding of the role which GSTs may play in resistance of the mummichog by looking at changes in a major isoform in fish from creosote-contaminated sites, and partial classification of the isoform. Other factors, including cellular glutathione levels and additional enzymes involved in the xenobiotic metabolic process (Hasspieler *et al.*, 1994b; Stein *et al.*, 1992), may play a role in resistance in these fish. Further investigation into the biochemical mechanisms governing metabolism in the mummichog may render the fish an alternative as a vertebrate model to mammals for studying carcinogenesis (Calabrese *et al.*, 1992) in addition to rainbow trout (Bailey *et al.*, 1992).

CONCLUSIONS

This study has shown a relationship between elevated expression of a GST isoform in mummichog liver and increasing environmental contamination. A similar relationship is observed with GST enzyme activity. This relationship suggests that GSTs, and more specifically, the elevated isoform, play a role in resistance of mummichog to creosote-contaminated sediments. Monoclonal antibodies were produced to the elevated isoform and have been shown to be a useful tool for estimation of relative amounts of the protein in mummichog hepatic cytosol using western blot analysis. This isoform has a blocked N-terminus but is most closely related to the rat Mu isoform based upon immunological cross-reactivity with the rat Mu subunit. Mummichog liver contains several GST isoforms that may be purified using affinity chromatography. The major isoforms have basic pIs although there are some minor acidic isoforms.

FURTHER RESEARCH

The ability of the mummichog to develop a genetic resistance suggests that the change in GST activity may be genetic, although there is no evidence to support this theory. In order to address this question, future studies need to 1) determine if GST levels decline and if tolerance to contaminated sediment changes when AW and SC fish are moved to an uncontaminated environment, and 2) conduct LD_{50} studies to see if AW fish are more resistant to toxic compounds than KC and SC fish. Also, further investigation into the specific properties of the elevated isoform in AW fish is required to determine the role it plays in creosote resistance. By conducting substrate studies, it will be possible to see if the components of creosote are in fact substrates for the elevated isoform. It is also necessary to look at changes in expression of the other isoforms, for elevation or depression, and to characterize them. Since the N-terminus of the elevated isoform is blocked, the most effective way to classify the protein will be to determine the cDNA sequence. The antibodies will be useful for immunohistochemical studies with fish from the field and laboratory exposed fish. Immunohistochemistry may be able to detect changes in isoform expression in liver and liver lesions which cannot be measured by enzyme activity assays.

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