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Expression of In Situ Biomarkers in Striped Bass

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TECHNICAL COMPLETION REPORT

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ABSTRACT

The proposed project has the following objectives: 1) Identify and sequence the striped bass (**SB**) glutathione S-transferase(s) (GSTs) gene(s); 2) Use the identified sequences as a probe to compare GST mRNA levels in control versus laboratory and/or selected field exposed **SB**.

The goal of this research is to develop a biomonitoring model for specific California surface waters utilizing **SB**, the popular game fish which is present in this environment. An approach based on the increased/decreased expression of GSTs will be used to screen the effect of experimental exposures of **SB** to toxic chemicals including polycyclic aromatic hydrocarbons, rice field herbicides and pesticides for their hepatoxicity. GSTs are a multigene family of enzymes catalyzing the conjugation of numerous electrophiles with reduced glutathione (GSH) by formation of the thioether bond. These reactive electrophiles include metabolites and endogenous compounds, drugs and pollutants. For the majority of substrates, the GSH conjugate is less toxic than the parent compound providing the organism protection against chemical insult. The level of expression of GST isoforms is extremely important in determining exposure to pollutant xenobiotics.

We have already isolated 3 GST proteins from **SB** following standard protein purification techniques and a GSH affinity column. These proteins were digested with CNBr, purified with HPLC and N-terminal sequenced. We plan to utilize the oligos derived from the back translated peptide sequences as well as conserved sequences (obtained from the literature) to screen a **SB** cDNA library for **SB** GSTs. Initially, hepatic mRNA will be isolated from control **SB** and cDNA synthesized using standard protocols. A cDNA library will be prepared in a lambda gt10 bacteriophage (Stratagene gigapack II cloning kit) and probed for the presence of glutathione (GST) clones using the previously described probes. Positive clones will be isolated and the data used to further PCR amplify specific sequences of striped bass GST cDNA of exposed and control fish. It should be possible to

determine biomarkers of host mediated protection (Phase II, GSTs) resulting from specific chemicals.

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PROBLEM AND RESEARCH OBJECTIVES:

We are concerned about exposure of feral fishes to agricultural and related chemical contaminants from water column and sediment and the potential toxicity/disease associated with such exposure as it impacts environmental health. Thus the goal of this research is to develop an *in situ* biomonitoring model for California surface waters utilizing a widespread teleost, Striped Bass (**SB**) of high economic importance.

We have directed our attention to the **SB** expression of glutathione S-transferases (GSTs), a multigene family of enzymes (phase II detoxification enzyme), catalyzing the conjugation of numerous electrophiles with reduced glutathione (GSH) by formation of the thioether bond . These reactive electrophiles include metabolites and endogenous compounds, drugs and pollutants.

For the majority of substrates, the GSH conjugate is less toxic than the parent compound and thus the cell or organism is afforded protection against chemical insult. The GST enzymes catalyze the initial step in mercapturic acid

biosynthesis and are predominantly cytosolic. The level of expression of GST isoforms is therefore extremely important in determining exposure to pollutant chemicals.

Ultimately, we expect this research to result in more rapid-bioassay techniques for detecting ecologically significant concentrations in fish exposed to chemicals (xenobiotics) in water and sediment. Our approach may illustrate a number of enzymatic/toxic mechanisms heretofore not considered vital for survival of the **SB**, which may well be similar in other species of fish. This approach should more accurately define the "defense processes" the **SB** are exhibiting rather than performing multiple enzyme assays which may or may not be the correct assay for a particular chemical or duration of exposure.

METHODOLOGY AND RESULTS

Briefly, **SB** hepatic supernatant was isolated following a 10,000g and 100,000g spin and applied to a GSH affinity column. The affinity resin purified GSTs were subjected to isoelectric focusing, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), reverse phase HPLC and direct mass analysis of transferase subunits using a triple quadrupole mass spectrometer, electrospray ionization (ES+). Isoelectric focusing (pH 4.0-8.0) showed one major band at a position giving it an approximate pI of 6.8. SDS-PAGE revealed the presence of at least 2 subunits having mass of 25,000 and 23,600 as calculated using MW markers. HPLC separation of affinity resin purified GST showed the presence of 3 major protein peaks. These peaks were collected and rerun on SDS-PAGE. The peaks at 6.5 and 10.5 min. banded in exactly the same position (MW 25,000) and the peak at 20 min. banded at a MW of 23,600. Mass spectrometry of affinity resin GST(s) showed only two major peaks at masses 25512 and 25807 (fig 5). The data suggested the presence of one isozyme composed

of subunits differing in mass by only 295. HPLC separations indicated the potential existence of some heterogeneity between the amino acid sequences of, for all practical purposes, homodimers. These results are assuming that the one band seen with isoelectric focusing is not composed of multiple isozymes with identical pIs.

Attempts to N-terminal sequence the reverse phase purified peak eluting at 20 min failed due to blockage of the amino terminal. This is a common problem encountered with cytosolic proteins. We next digested the proteins with cyanogen bromide and separated the resulting peptide fragments by HPLC. A number of the isolated fragments were collected and submitted for sequencing. The sequencing information (via backtranslation) was used to design an appropriate probe (oligo) to screen a **SB** liver library. At that point, we planned to characterize the **SB** GST DNA sequence and also use a conserved fragment as a specific probe to monitor exposure of SB to various levels of toxicants.

A number of oligos were obtained including 1)TCTGAGCACTGAGCTGAAGC; 2)AACGGAAGAGATAAGCCACG; 3) TGTAGTACGCCGTCAGTTGG,
were used to PCR amplify probes for screening the **SB** library. Even with this information, we were unable to isolate any **SB** GST cDNA sequences.

We also examined a protooncogene as they are important in the regulation of growth and are highly conserved among vertebrates. This work also emphasized the molecular biology related to ras protooncogenes in **SB**. Using published primer sequences for rainbow trout and the polymerase chain reaction (PCR), primers were synthesized and used to amplify exon 1 of the Ras gene of **SB**.

As mentioned previously, we prepared a cDNA **SB** liver library (Uni-ZAP^{TM} XR vector, Stratagene) . The cDNA **SB** library was used in an attempt to obtain GST cDNA sequence(s) by probing the library with known sequences (oligos) of GSTs from other species including the only fish (*Pleuronectes plattessa*) for which GST mRNA has been published. A computer program detailing the optimum oligos to screen the **SB** library was used. We also utilized GST oligo information obtained from Evan Gallagher (University of Washington). In spite of the many and varied probes utilized, we were unsuccessful in isolating any GST clones. However, our control ras oncogene probes were successfully used to verify the quality of our **SB** cDNA library.

At the recent Primo 8 meeting held in Monterey, CA., we realized that our lack of success relative to isolating cDNA clones was common amongst molecular biologists studying fish. This accounts in large part for the paucity of fish data relative to their phase I and II detoxifying enzymes. It appears that due to the heterogenity of the oligonucleotide sequences, it is not feasible to PCR amplify probes for screening fish libraries. Thus, investigators have had to screen cDNA fish hepatic expression libraries for the information we were seeking. We fortunately obtained a flatfish GST antibody from colleagues (Stephen George and Mike Leaver) at Stirling University, Scotland and used it to screen a western blot of our isolated **SB** GST proteins. We demonstrated that one of the **SB** GST proteins appears to be similar to the GST of the flatfish, *Pleuronectes plattessa*. At this point, we do not know what class the other two **SB** GST proteins belong to.

We plan to screen our **SB** expression library with the GST flatfish antibody, then isolate and characterize the appropriate cDNA clone(s). This should allow us to characterize at least one of the **SB** GST isomers. In addition,

we will obtain antibodies to our 3 HPLC purified **SB** GST proteins over the next several months and use these antibodies to screen the **SB** library. This should facilitate the isolation and characterization of the **SB** GST clones.

PRINCIPAL FINDINGS AND SIGNIFICANCE

We have shown that the **SB** have potentially 3 GSTs and that they are present in fairly abundant quantities as one would expect. The paucity of data relative to all fish GSTs indicate that this situation definitely needs to be addressed, as the GSTs play such a pivotal role in many of the detoxification processes.

We now have a **SB** library which will facilitate much of our future work. Once antibodies have been produced against each of the three individual GST proteins as well as the mixture of all three, we will proceed to screen our **SB** expression library. This should provide exciting information as the **SB** GSTs may be from different families, thus providing an increased measure of detoxification ability. Our results indicate that one of the **SBs'** GSTs has some homology with the GSTs of the flatfish. However, until **SB** GST clones are isolated and sequenced, we will not know the degree of homology between these fish GST proteins. At present, the data is potentially indicating that the classic substrates used to monitor GSTs via enzymology may be inappropriate for many fish species and thus leading to spurious results.

Finally, we will continue to actively pursue our goal of utilizing the **SB** GST's to monitor the exposure of feral fishes to agricultural and related chemical contaminants from water column and sediment and the potential toxicity/disease associated with such exposure as it impacts environmental health.

THESIS AND DISSERTATION

Investigators supported by these funds were at the post doctoral level.