Bull. Environ. Contam. Toxicol. (1998) 61:378-383

Bulletin of Environmental Contamination and Toxicology

Microsomal Monooxygenase Activity in Milkfish (*Chanos chanos*) from Aquaculture Ponds near Metal Reclamation Facilities

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Received: 10 March 1998/Accepted: 6 July 1998

Cytochrome P-450 (P450) based mixed-function oxidases (MFO) are the primary metabolic system responsible for biotransformation of xenobiotics as well as endobiotics in vertebrates. Specific enzymes (i.e. CYP1A) of this system are known to be induced by lipophilic environmental contaminants, such as co-planar polychlorinated biphenyls (PCBs), dioxins, and polynuclear aromatic hydrocarbons (PAHs), and can be used as a biological indicator or a biomarker. There have been many studies using cytochrome P450 enzyme inducers in aquatic organisms as an indicator of environmental contamination. For instance, Ahokas *et al.(* 1993) correlated increased 7-ethoxyresorufin *O*-deethylase (EROD) activity, a sensitive indicator of CYP1A activity, with the dioxin levels detected in muscle of fish exposed to treated pulp and paper mill effluent. Van der Weiden *et al.* (1993) demonstrated that the CYP1A protein content and EROD activity in mirror carp (*Cyprinus carpio*) were elevated by exposure to dioxins and PCB-contaminated sediment.

In Taiwan, there have been a few investigations on the cytochrome P450 activity in fish species. Ueng et al. (1995) reported that both benzo[*a*]pyrene hydroxylase (B[a]PH) and EROD activities in tilapia (*Tilapia* sp.) were induced by sediment extracts from the Damsui River, a highly polluted river in northern Taiwan. A study showed that EROD and B(*a*)PH activities in tilapia from downstream regions of the Er-Jen River which were heavily contaminated with metals, PCBs, and dioxins, were induced almost ten-fold more than those in tilapia from an unpolluted area (Lia *et al.* 1993).

The Er-Jen River is one of the most heavily polluted rivers in Taiwan. Riverside industries include paper, sugar, electroplating and coating plants, with the metal reclamation business being concentrated in the downstream area, called Wan-Li. Untreated process effluents and wastes dumped into the river resulted in the famous "Green Oyster" event which was due to copper accumulation in the oyster tissue. Other environmental contaminants, such as PCDDs (polychlorinated dibenzo-*p*-dioxins)/PCDFs (polychlorinated dibenzofurans), PCBs and PAHs (polyaromatic hydrocarbons) were also detected in biological, soil, and sediment samples collected from the area (Lu *et al.* 1994a, 1994b, Hong *et al.* 1990, Pan *et al. 1990).* In Lu *et al.*'s report (1994a), PCDDs/PCDFs and co-planar PCBs were detected in

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muscles, guts and livers of milkfish (*Chanos chanos*) taken from aquaculture ponds near the different metal reclamation facilities. The total dioxin toxic equivalent (TEQ) levels including co-planar PCBs were in the range of 1.13~268 pg/g. Therefore, it is possible that these contaminants may alter the cytochrome P450 enzyme levels in the exposed animals. The objective of this study was to determine if there was any difference between the cytochrome P450 levels in milkfish from a contaminated area and from an unpolluted area. In addition, this was the first study examining the cytochrome P450 activity in this fish species.

MATERIALS AND METHODS

Milkfish (*Chanos chanos*) were collected either by trawling or angling. Three to live males were taken from 3 different ponds at different locations in the investigated area. These ponds are adjacent to metal reclamation plants. These plants were still operating at the time of sampling. The reference site was located at Pei-Men, Tainan County. This area has no industrial activity, and the dominant industry is aquaculture, mostly milkfish. After collection, the specimens were kept alive and brought back to our laboratory. Before dissecting, animals were anesthetized with tricainemethanesulfonate and body weights were recorded. Livers were removed and weighed, and rinsed with a cold KC1 solution (1.15%) in a dry ice bath before transferring to a -70°C freezer.

Microsomal fractions were prepared no more than 48 hours after collection. Liver was homogenized in cold KC1 solution (1.15%) and centrifuged at 10,000g for 20 minutes at 4°C. The resultant supernatant was centrifuged at 100,000g for 60 minutes at 4°C. The microsomal pellet was resuspended in KCl solution (1.15%) and centrifuged again at 100,000g for 60 minutes. The final pellet was resuspended in 0.1M phosphate buffer (pH 7.4) for enzyme analysis.

Determination of total cytochrome P450 content was conducted following the method of Omura and Sato (1964). B(a)PH activity was determined using the method of Nerbert and Gelboin (1968) with modification. This method measures the formation of the fluorescent phenolic metabolites. EROD activity was determined by measuring enzymatic formation of a fluorescent compound, resorufin (Pohl and Fouts 1980). Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The total cytochrome P450 content, EROD and B(a)PH activities (expressed as pmol/mg/min) measured in fish livers were compared to those observed in fish collected from the reference pond. Student's t-test (95% significance level) was used to determine statistical differences.

RESULTS AND DISCUSSION

Within Table 1 are summarized the results from this study. This includes fish body and liver weights, cytochrome P450 contents, EROD and B(a)PH activities. Fish taken from different ponds were older than 5 months except fish from Pond 3 (less than 3 months), and had body sizes between 200 and 570g.

Table 1. Cytochrome P450 contents and activities (mean±standard deviation) in milkfish liver.

Pond	1(n=3)	1[a](n=3)	2 (n=3)	3(n=4)	Ref(n=5)
Body Wt.(g)	294.3±95.0	435.1±98.0	568.8±23.5	131.5±16.3	382.4±66.5
Liver Wt.(g)	5.90±1.36	7.50±1.70	8.08±0.41	2.01±0.27	6.1±1.06
P450(nmol/mg protein)	0.063± 0.013	0.153± 0.060*	0.170± 0.059*	0.102± 0.057	0.058± 0.031
EROD(pmol/ min/mg protein)	100.0± 23.8*	195.8± 119.0*	19.85± 5.71*	29.12± 12.76	46.8± 10.3
B(*a*)PH(pmol/ min/mg protein)	139.2± 64.6*	156.7± 71.8*	27.7± 5.7	30.5± 10.7	36.6± 20.5

a. This is the second collection from Pond 1. There is a 20-day time lag between two collections. The reason for the second collection is that the owner of Pond 1 was going to harvest and clean up the pond at that time.

* indicates statistically different from the reference pond (Ref), $p<0.05$.

Comparing cytochrome P450 microsomal contents, we found that fish obtained from the three ponds near the metal reclamation plants had elevated enzyme contents compared to those collected from the reference pond. For the second collection from Pond 1, the increases in cytochrome P450 microsomal contents were up 2-3 fold compared to the contents in the reference fish, but the elevation in the first collection from Pond 1 was not significant. The reason for this discrepancy is unknown. Although the fish from the first collection had less liver weights (5.90±1.36g) than the second collection (7.50±1.17g), we do not think this is the factor that resulted in the variation observed in cytochrome P450 microsomal contents. The reason is that EROD and B(*a*)PH activities in the fish livers from the first collection of Pond 1 were 2~4 times higher than the activities in the reference fish livers. However, it should be noted that total cytochrome P450 content does not necessarily correspond to the measured CYP1A1 activities. Milkfish collected from Pond 3 were relatively small in size (estimated to be less than 3-months old) and they had lower liver weights, but the enzyme contents were higher than the reference fish, but not significantly so.

The results of EROD assay showed that activity was significantly higher in fish from Pond 1 than the activity measured in fish from the reference pond. Comparing the EROD activity of reference fish (46.8±10.3 pmol/min/mg protein) to Pond 1 (two collections) fish, we found that there was up to a two (100.0±23.8 pmol/min/mg protein) and a four (195.8±119 pmol/min/mg protein) fold increase in EROD activity (Table 1). For Pond 2, although fish were fully grown and had higher body and liver weights, the EROD activity was actually significantly lower (19.85±5.71 pmol/min/mg protein). It should be noted that the cytochrome P450 enzyme content in fish from Pond 2 was actually higher than that measured in the reference fish. We did not observe induction in fish from Pond 3. Those fish had smaller body sizes (135.5~142.3g) and liver weights (1.77~2.29g) compared to

others, and were relatively younger than fish from other ponds.

Data from the B(a)PH assay were consistent with those from the EROD assay. It should be mentioned that both catalytic reactions are CYP1A1-specific. B(*a*)PH activities in fish from Pond 1 from the two different collections were 139.2 (±64.6) and 156.6 (±71.8) pmol/min/mg of protein, respectively. These increases were approximately four times higher than the level in reference fish (36.3±20.5 pmol/min/mg protein). There were no statistical differences in B(*a*)PH activity of fish from either Pond 2 (27.7±5.7 pmol/min/mg protein) or Pond 3 (30.5±10.7 pmol/min/mg protein) compared to the activity of the reference fish.

This study showed that cytochrome P450 contents in milkfish from the ponds near metal reclamation plants was elevated compared to those in milkfish from the reference pond, except for those fish from Pond 1 at the first collection. If compared to the reference fish, the monooxygenase activities measured (EROD and B[*a*]PH) were higher in fish from Pond 1, but were decreased in fish from Pond 2 and 3. The cause of the discrepancy between the results of cytochrome P450 contents and enzymatic activities is unknown. For Pond 2 fish, both EROD and B(*a*)PH activities were much lower than those observed in the reference fish. This may result from the presence of enzyme inhibitors in the sample or as contaminants during microsomal preparation, or from other environmental factors, such as food. It should be noted that if the animals receive much higher doses resulting in hepatic toxicity, the cytochrome P450 enzyme activity might be decreased. The decreases in EROD and B(*a*)PH activities in Pond 3 fish may be due the reasons mentioned above or due to their younger ages for which the enzymatic activity is not yet fully developed. There was also a possibility that metals play a role in modification of P450 enzyme activity, since the investigated area could be contaminated by metals. Heavy metals have been reported to have inhibitory effects on cytochrome P4501A induction in fish hepatoma cells (Bruschweiler et al., 1996). Milkfish collected from this area had higher metal contents (Cu, Pb, Cd) than the fish from unpolluted water (Chen *et al.*, 1996), but the effects of metal contamination on the cytochrome P450 activity in milkfish were unclear.

Cytochrome P450 inducers, such as dioxins and PCBs, were detected at high levels in this area. Lu *et al.* (1994a,b) reported different levels of dioxins in fish and sediments collected from aquaculture ponds close to our study area. Preliminary chemical analysis using high resolution GC with high resolution MS indicated that our samples (fillet) from Pond 1 contained dioxins (we did not analyze fish from other ponds). The TEQ levels were in the range of 0.28~0.38 ng/g, while TEQ levels were about 0.05 ng/g in the reference fish (unpublished data). However, we have to point out that other environmental or biotic factors, such as temperature, development status, nutrition, are known to alter the cytochrome P450 enzyme levels. We have noted that there are differences in culturing milkfish between the investigated and the reference area. The reference area is far north from our studied area, so the water temperature is much lower than that in the studied ponds. It is about 2~3°C different. Other differences (studied vs. reference) include feeding patterns (every 4 hours vs. twice a day), saltwater intake (underground vs. seawater)

and salinity (110‰ vs. 20‰), and stock density (500 vs. 700 per pond). There may be other factors not identified. It should be mentioned here that it is unlikely for the reference pond to be polluted, because there is no industrial activity except aquaculture in this area or near the coast.

There are currently no reports on cytochrome P450 activity in milkfish from the investigated area. Lia *et al.* (1993) reported that EROD and B(*a*)PH activities in different fish species (*Tilapia* sp., *Megalops cyprinoides, Channa sp., and Liza macrolepsis*) collected from the Er-Jen River were elevated. In this study, we have detected differences in microsomal monooxygenase activity (EROD and B[a]PH) between milkfish collected from polluted and non-polluted water. Biochemical assays can serve as bioindicators for organisms responding to environmental stress, but the results should be interpreted carefully. Along with chemical analysis, this approach could be very useful in environmental monitoring or in pollution detection.

Acknowledgments
We thank Chia-Nan College of Pharmacy and Science for funding this study, and Dr. Ueng's group members, especially Mr. Wang for his help in the biochemical analyses.

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