ABSTRACT

Environmental contamination by pesticide is a worldwide problem and affects a number of non-target organisms other than the target organism. Pesticides are widely used throughout the world to protect the crops from the damage caused by the pests. The excessively used pesticides eventually get accumulated in the aquatic system through surface run off and affect the non-target organisms which are very much sensitive to the pesticide. Cytochrome P450 (CYP 450) is one of the most important phase I enzyme of biotransformation and is considered as an important biomarker for its sensitiveness towards a wide range of xenobiotics. CYP 450 is a very large and diverse superfamily of heme proteins found in all domains of life. Among the diverse family of CYP 450, family 1-3 and to some extent family 4 are of prime importance for the oxidation of xenobiotic compounds, including pesticides. Except for CYP 4501A (CYP1A), information on other CYP 450 families in fish is limited as compared to mammals.

The general aim of the present study was to examine CYP 450 of an economically important air breathing freshwater fish, *Channa punctatus*, *Heteropneustes fossilis* and *Clarias batrachus*. Microsomal isolation was performed based on conventional ultra-centrifugation at 1,00,000 g for 60 minutes. The carbon monoxide reduced CYP 450 showed the spectrum of absorption maximum at 450 nm in liver microsome. The CO-difference spectra of dithionite reduced liver microsomes of *C. punctatus*, *H. fossilis* and *C. batrachus* were studied at 1 minute interval over a period of 5 minutes. The maximum absorbance was noticed at 1 to 2 minutes and then a gradual decrease in absorbance was seen with the increase in time interval.

In the present study, CYP 450 content, EROD (ethoxyresorufin O-deethylase) activity, N, N-dimethylaniline demethylase (N,N-DMA) activity, aniline hydroxylase (AH) activity and erythromycin N-demethylase (ERND) activity have been reported from liver microsome of fish, *H. fossilis* to indicate the presence of multiple forms of CYP 450 in fish. Induction of specific enzyme activity with a significant difference upon treatment with specific inducers such as naphthalene (CYP1A), acetone (CYP2E1) and deflazacort (CYP3A4) was found except for phenobarbitone (CYP2B) which was found to be unresponsive. The results clearly showed that the specific inducers were responsible for the induction of specific CYP 450 isoforms and the presence of multiple CYP 450 isoforms in fish, *H. fossilis*. The results also indicated that CYP1A mediates EROD (ethoxyresorufin O-deethylase) activity, CYP2B mediates N, N-dimethylaniline demethylase (N,N-DMA) activity, CYP2E1 mediates aniline

hydroxylase (AH) activity and CYP3A4 mediates erythromycin N-demethylase (ERND) activity.

β-naphthoflavone (β-NF), a proven inducer of CYP1A was also administered in all the 3 fish species, *C. punctatus*, *H. fossilis* and *C. batrachus* and CYP 450 in hepatic tissue was examined. β-naphthoflavone treatment significantly induced CYP 450 content and EROD activity in all the 3 fish species, *C. punctatus*, *H. fossilis* and *C. batrachus* along with induction of erythromycin N-demethylase activity.

The effects of 3 different classes of pesticide viz. pyrethroid (cypermethrin), organophosphate (ethion) and organochlorine (dicofol) on hepatic CYP 450 isoforms of an economically important freshwater air breathing fish, *C. punctatus*, *H. fossilis* and *C. batrachus* was also studied. The LC_{50} value of all the pesticide was determined and the fish treated with 1/3 sub-lethal concentration of calculated LC_{50} value for a period of 5, 10 and 15 days. The LC_{50} values in *C. punctatus*, *H. fossilis* and *C. batrachus* towards cypermethrin were calculated to be 19.9, 3.7 and 5.6 µg/L respectively, towards ethion 43.9, 54.8 and 48.7 µg/L respectively and towards dicofol 45.8, 36.1 and 51.8 µg/L respectively. Apart from the enzyme activities, liver somatic index (LSI), protein content and CYP 450 content were also examined which revealed a significantly higher value in the pesticide treated fish compared to their respective control group. The 3 fish species also revealed marked interspecies differences in all of the parameters studied when comparing their basal level activities.

Upon treatment with pesticide cypermethrin, CYP1A, CYP2B and CYP2E1 activity were significantly induced. These 3 isoforms were found to be the most important CYP 450, catalyzing cypermethrin metabolism, while CYP3A4 activity was significantly down regulated and did not catalyze the metabolism of cypermethrin in all the 3 fish species studied, *C. punctatus*, *H. fossilis* and *C. batrachus* when compared with their respective control group. However, *C. punctatus* displayed a negligible response in CYP2E1 activity. Upon treatment with ethion, CYP1A, CYP2E1 and CYP3A4 activity were induced and significantly catalyzed the metabolism of the pesticide in fish, *C. punctatus*. In general, CYP1A and CYP3A4 played a crucial and CYP2E1 a lesser role in the metabolism of ethion whereas CYP2B had no role in the metabolism of ethion. In *H. fossilis*, all the activities mediated by CYP1A, CYP2B, CYP2E1 and CYP3A4 were induced and significantly catalyzed the metabolism of ethion. However, CYP1A, CYP2B and CYP3A4 catalyzed ethion in *C. batrachus* while CYP2E1 had no role in the metabolism. Similarly, CYP1A and CYP3A4 activity were induced and catalyzed the metabolism of dicofol in all the 3 fish

species with a significant difference when compared with their respective control group. In *C. punctatus*, CYP2B also played a role in the metabolism of dicofol while CYP2E1 was moderately inhibited. On the other hand, in *H. fossilis* all the activities mediated by CYP1A, CYP2B, CYP2E1 and CYP3A4 catalyze the metabolism of dicofol similar to that of ethion, whereas in *C. batrachus*, CYP2B and 2E1 played no role in the metabolism of dicofol. The results showed that in *C. punctatus, H. fossilis* and *C. batrachus,* all the CYP 450 isoforms viz. CYP1A, CYP2B, CYP2E1 and CYP3A4 play a role in biotransforming the pesticide and thereby ensure its survival.

In vitro kinetics of hepatic phase I biotransformation reactions in 3 air breathing teleost fish, *C. punctatus*, *H. fossilis* and *C. batrachus* were also examined. The fishes were treated with 1/3 sub-lethal concentration of the pesticide cypermethrin, ethion and dicofol for a period of 15 days and enzyme kinetics were studied against the control. Maximal velocity (Vmax), binding affinity (Km) and catalytic efficiency (Vmax/Km) were used as endpoints for comparison. All the 3 fish species displayed marked differences in maximal velocity (Vmax), binding affinity (Km) and catalytic efficiency (Vmax/Km). Intrinsic clearance expressed as Vmax/Km ratio for 7-ethoxyresorufin was seen to be highest followed by erythromycin, N, N-dimethylaniline and aniline in all the fish species studied. The results clearly demonstrated the vital role played by CYP1A and CYP3A4 isoforms and are subsequently considered to be important isoforms in metabolizing the xenobiotics. Marked interspecies differences in the expression and metabolism of pesticides by CYP 450 were also clearly seen.

In the present study, fishes, *C. punctatus*, *C. striatus*, *C. gachua*, *H. fossilis* and *C. batrachus* were collected from 9 different sites in Terai region of North Bengal though all the species were not present in every site. All the fish species displayed a variable range of CYP 450 content and CYP 450 mediated enzyme activities and were attributed to the exposure of fish to different physiological and environmental stress.

The microsomal fraction was also subjected to SDS-PAGE and heme staining by TMPD (N,N,N’N’ -tetramethyl-p-phenylenediamine) which identified the presence of cytochrome P450 in all 3 fish species with a molecular weight ranging from 50-56 kDa. Microsomes from control and pesticide treated fish liver were separated and the bands were visualized in molecular mass ~54 kDa for *C. punctatus,* ~53 kDa for *H. fossilis* and ~51 kDa for *C. batrachus* with increased intensity in all the pesticide treated populations.

The CYP1A genomic sequence for all 3 fish species, *C. punctatus* (2511 bp), *H. fossilis* (534 bp) and *C. batrachus* (509 bp) were obtained and successfully submitted in the GenBank. All the gene sequences were deposited in the GenBank/NCBI data bank except the gene of *H. fossilis* which was deposited in GenBank/EMBL with the accession numbers KP282054, LN736019 and KP336485 for *C. punctatus, H. fossilis* and *C. batrachus,* respectively.

The phylogenetic tree based on the amino acid sequences clearly showed *C. punctatus* and C. *maculata* CYP1A and *H. fossilis* and *C. batrachus* CYP1A to be more closely related to each other than CYP1A subfamily of other fish employed in the current study. *H. fossilis* and *C. batrachus* CYP1A was more closely related with *Peltobagrus fulvidraco* (yellow catfish) another member of the order Siluriformes. Comparing the orders, the order Siluriformes (*Ancistrus sp., Peltobagrus fulvidraco, H. fossilis* and *C. batrachus*) was closely related to order Cypriniformes (*Catla catla* and *Cyprinus carpio*). The order Perciformes (Scup, Nile tilapia, *Channa maculate* and *Channa punctatus*) was closely related to Salmoniformes (Rainbow trout).

The present study clearly established the efficacy of CYP 450 as an important biomarker for monitoring the aquatic pollution. Of all the CYP 450 isoforms (CYP1A, CYP2B, CYP2E1 and CYP3A4), CYP1A was the most responsive isoform towards pesticide metabolism and in accordance with various other studies proved itself to be one of the most reputable biomarker of CYP 450 family.