Biochemical Pharmacology, Vol. 45, No. 1, pp. 217–221, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CHANNEL CATFISH LIVER MONOOXYGENASES

IMMUNOLOGICAL CHARACTERIZATION OF CONSTITUTIVE CYTOCHROMES P450 AND THE ABSENCE OF ACTIVE FLAVIN-CONTAINING MONOOXYGENASES

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(*Received* 16 *June* 1992; *accepted* 19 *August* 1992)

Abstract—Multiple drugs and pesticides are used in the aquaculture of channel catfish in the Southeastern United States. However, little is known regarding the enzymatic metabolism of these chemicals in the fish. Western blots, utilizing polyclonal antibodies raised against five purified rainbow trout liver cytochrome P450 enzymes, revealed at least two protein bands that were approximately 50 kDa (CATL-1) and 53 kDa (CATL-2). Anti-trout LMC3 and LMC4 only hybridized with the 53 kDa protein, whereas anti-trout LMC1, LMC2, and LMC5 recognized both proteins. Cytochrome P450-catalyzed activities (testosterone and progesterone hydroxylases) associated with LMC1 and LMC5 were also found in catfish liver microsomes. These data suggest that at least two constitutive forms of cytochrome P450 are present in the liver of juvenile channel catfish. Western blots utilizing antibodies raised against rabbit-lung flavin-containing monooxygenases (FMO) showed hybridization with two proteins from rainbow trout liver microsomes, but no cross-reaction with microsomes from catfish liver. *N,N*,-Dimethylaniline *N*-oxidase and methimazole oxidase were observed in microsomes from trout, but were absent in catfish liver microsomes prepared in three different laboratories. Consequently, FMO do not appear to be present in liver microsomes from channel catfish or they are rapidly degraded during tissue homogenization.

The culturing of channel catfish in the Southeastern United States is a multi-million dollar industry. The aquaculture process utilizes a vast array of drugs and pesticides to combat infection and parasitic infestation within catfish ponds [1]. Although many of these chemicals have been banned by the U.S. Food and Drug Administration, they are still used widely due to the lack of efficacious alternative treatments. Regarding the limited number of USFDA-approved therapeutic agents, several groups have examined the absorption, bioavailability and distribution of these agents in catfish [2–5]. However, little work has focused upon the potential enzymatic systems in catfish that may be involved in xenobiotic biotransformation.

In mammals, two major classes of monooxygenases have been shown to be responsible for the majority of chemical biotransformations that occur in the cell: the cytochrome P450 and flavin-containing monooxygenases (FMO)‖. Although the presence of cytochrome P450 activity in the liver of channel catfish has been observed [6, 7], the number of isoforms of this polymorphic enzyme in catfish is unknown. Ronis *et al.* [8] have shown that there appears to be a CYP IA-like form that is inducible after exposure to polychlorinated biphenyls and dioxins. Polyclonal antibodies raised to purified P450s from fish and mammals have been used effectively by several groups to show various similarities in structure and regulation between different species of fish [9, 10] as well as invertebrates [11, 12]. While there has been an increasing amount of research regarding P450 in other fish species [13, 14], the identification and characterization of constitutive isoforms in catfish have yet to be performed.

FMO have been observed and partially characterized in several fish species [15–17]. FMO have been shown to play a significant role in the biotransformation of pesticides in rainbow trout [18]. In addition, polyclonal antibodies raised against rabbit lung and pig liver FMO have been shown to hybridize with two proteins in liver microsomes of trout indicative of FMO [19]. However, the occurrence of FMO and their role in xenobiotic biotransformation have not been examined thoroughly in catfish.

Utilizing polyclonal antibodies raised against the purified enzymes (P450 and FMO) as well as enzyme-specific activities, the purpose of this study was to identify and characterize various monooxygenases in catfish liver microsomes that may be involved in chemical metabolism.

MATERIALS AND METHODS

Chemicals. *N,N*,-Dimethylaniline (DMA) hydro-

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‖ Abbreviations: FMO, flavin-containing monooxygenase(s); and DMA, *N,N*-dimethylaniline.

chloride was recrystallized as described previously [12]. Organic solvents were purchased from the Baker Chemical Co. (Phillipsburg, NJ). Unless otherwise stated, all remaining chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animal maintenance. Juvenile channel catfish (*Ictalurus punctatus*) (6-month-old) were maintained at the U.S. Fish and Wildlife Fish Farming Experimental Laboratory in Stuttgart, AR. Fish kept in free-flowing tanks with well-water at ambient temperature (approximately 25°) were maintained on a diet of 32% protein ARKAT (Dumas, AR). Five fish of approximately 300 g were removed and killed by a blow to the head. Livers were removed, frozen immediately in liquid nitrogen, and stored at −80° until homogenization.

Microsomal preparation. Microsomes were prepared utilizing a standard procedure [15]. Thawed livers were pooled, minced and homogenized in 4 vol. of ice-cold buffer (0.1 M Tris-acetate, pH 7.4, 0.1 M KCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). A microsomal pellet was obtained by sequential centrifugation at 20,000 *g* for 20 min and at 100,00 *g* for 90 min. Following a second spin at 100,000 *g*, the resulting microsomes were resuspended in phosphate buffer (0.1 M potassium phosphate, pH 7.25, 20% glycerol and 1 mM EDTA) to a protein concentration of approximately 20 mg/mL, and frozen in small aliquots in liquid nitrogen and stored at −80° until used. Protein concentrations were determined using the Pierce colorimetric protein determination kit.

Assays. Cytochrome P450 content was determined utilizing a dithionite difference spectrum of carbon monoxide-treated samples on a Shimadzu UV160U dual beam spectrophotometer (ε = 100,000 M^{-1} cm^{-1}) [20].

Testosterone and progesterone hydroxylase activities were determined utilizing established methods [21, 22]. Briefly, incubations of 1.0 mL consisted of 1–2 mg microsomal protein, 0.1 mM NADPH and were initiated with the addition of substrate (0.26 mM testosterone and 0.1 mM progesterone). After 45 min, the reactions were stopped with the addition of methylene chloride and extracted twice. The pooled organic layer was dried with nitrogen gas, resuspended in methanol, and injected onto a Beckman System Gold HPLC system utilizing a Beckman Ultrasphere ODS column (4.6 mm × 15 cm). Metabolites were identified by coelution with standards. Samples were run over a gradient of 5–100% methanol:water in 30 min at flow rate of 1 mL/min.

FMO-catalyzed methimazole oxidation was measured by the spectrophotometric assay of Dixit and Roche [23]. Assay mixtures contained 0.1 M Tris–HCl (pH 8.0 to 8.8), 0.06 mM 5,5′-dithiobis(2-nitrobenzoate), 0.025 mM dithiothreitol, 0.10 mM NADPH, 0.5 to 1.5 mg protein, and 1.0 mM methimazole in a final volume of 1.0 mL. Enzyme activity was initiated by the addition of methimazole to one cuvette, and reaction rates were measured on a Shimadzu UV160U dual beam spectrophotometer as the rate of decrease in absorbance at 412 nm between identical assay mixtures with and without methimazole (ε = 28,200 M^{-1} cm^{-1}).

Table 1. Cytochrome P450 content and steroid hydroxylase activities in liver microsomes from channel catfish

Enzyme	Activity
Cytochrome P450*	0.26
Testosterone hydroxylase†	1.62
6β-OH	1.28
2β-OH	0.43
Progesterone hydroxylase†	0.20
6β-OH	0.11

* Average of two assay replications of five pooled individuals. Values are given in nmol/mg microsomal protein.

† Average of two assay replications of five pooled individuals. Values are given in nmol/min/mg microsomal protein. Key: 6β-OH = 6β-hydroxy derivative; 2β-OH = 2β-hydroxy derivative.

DMA N-oxidation was determined as described previously [15]. Assay mixtures contained 0.01 to 0.1 M potassium phosphate (pH 8.0 to 8.4), or 0.01 to 0.1 M Tris–HCl (pH 8.4 to 9.0), 0.1 mM NADPH, and 0.5 to 3 mg protein in a final volume of 1.0 mL. The reaction was started with the addition of DMA (1–4 mM) and then incubated for up to 1.0 hr. *N*-Oxidase activity was measured at 420 nm (ε = 8200 M^{-1} cm^{-1}).

Electrophoresis and Western transfers. Electrophoresis of microsomal proteins was performed using 8.0% separating and 3.0% stacking polyacrylamide gels in the presence of sodium dodecyl sulfate [24]. Following electrophoresis, the separated proteins were transferred via electroblot to nitrocellulose sheets following a modification of the method of Towbin *et al.* [25] and stained by a modification of the method of Burnette [26]. Transfers were incubated for 90 min with FMO or P450 antibody (20 μg/mL) in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and then for 60 min in a PBS solution containing [^{125}I]protein A at 2 × 105 cpm/mL. The nitrocellulose sheets were exposed to Kodak XAR-5 X-ray film for 1–24 hr at −80°.

RESULTS

Cytochrome P450 levels in liver microsomes from catfish were 0.26 nmol/mg (Table 1). Total progesterone and testosterone hydroxylase activities were 0.20 and 1.62 nmol/min/mg. The primary metabolite of both substrates was the 6β-hydroxy derivative with lesser quantities of 2β-hydroxy-testosterone in testosterone incubations. Other minor metabolites were present, but the peak areas of these compounds were less than 1% of total metabolism.

Antibodies raised against five purified trout liver P450s hybridized at various degrees with at least two proteins in the P450 molecular weight region (50–

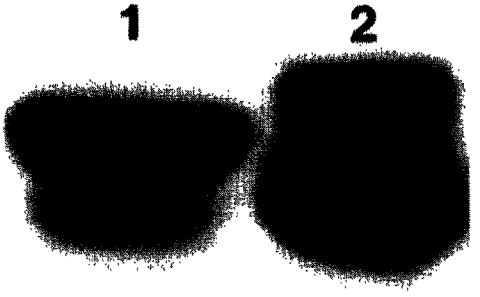

Fig. 1. Western blot of liver microsomes from catfish and trout probed with anti-LMC1 antibody and detected by [^{125}I]protein A. Lane 1, 20 μg of trout liver microsomes; Lane 2, 20 μg of catfish liver microsomes.

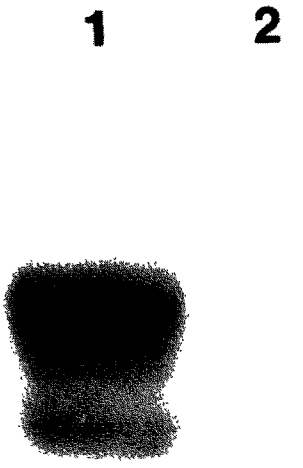

Fig. 2. Western blot of liver microsomes from catfish and trout probed with anti-rabbit lung FMO antibody and detected by [^{125}I]protein A. Lane 1, 20 μg of trout liver microsomes; Lane 2, 20 μg of catfish liver microsomes.

Table 2. Binding of anti-trout P450 antibodies with different catfish liver proteins in a Western blot*

	Anti-trout				
	LMC1	LMC2	LMC3	LMC4	LMC5
CATL-1	+	+	−	−	+
CATL-2	++	++	+	+	+

* Key: (+) moderate hybridization (approximately 6 hr of incubation at −80°); (++) strong hybridization (approximately 1 hr of incubation at −80°); and (−) no visible hybridization after 24 hr of incubation at −80°. Relative intensities were quantitated using laser densitometry.

60 K). Figure 1 demonstrates the hybridization of catfish proteins with anti-LMC1. The approximate molecular weights of the catfish proteins were CATL-1 = 50,000 and CATL-2 = 53,000. Anti-trout LMC3 and LMC4 only reacted with a single protein (CATL-2), whereas anti-trout LMC1, LMC2, and LMC5 reacted with both proteins. The reactions are summarized in Table 2.

FMO activity was not observed in microsomes from catfish liver prepared in this laboratory (which possessed P450) or in microsomes obtained from two other laboratories (see acknowledgements). However, FMO activity was observed in liver microsomes from rainbow trout (0.7 nmol DMA and methimazole oxidase/min/mg). Likewise, Western transfer analysis revealed that only liver microsomes from trout and not catfish possessed proteins that hybridized with anti-rabbit lung FMO in the 50–60 kDa region (Fig. 2).

DISCUSSION

Channel catfish are an economically important fishery in the Southeastern United States. A plethora of therapeutic agents are necessary to effectively culture these organisms. Consequently, a better understanding of how catfish metabolize xenobiotics is required in order to evaluate the toxicity and subsequent regulation of these agents in aquaculture.

The occurrence of cytochrome P450 and related activities (steroid hydroxylases) in liver microsomes from channel catfish is consistent with earlier studies [6, 8, 27]. However, this is the first report of multiple constitutive forms of P450 in catfish. The occurrence of multiple constitutive P450s was based on the molecular weight and hybridization of each band of microsomal protein from catfish liver (CATL-1 and CATL-2) with polyclonal antibodies raised against five purified constitutive P450s from the liver of rainbow trout [22]. Although two forms were recognized by anti-trout P450 antibodies, it is entirely possible that other P450s may also be present possessing unique epitopic sites that were not recognized by the anti-trout antibodies.

Since each P450 in catfish liver microsomes was shown to hybridize strongly with the various antibodies raised against constitutive forms from the trout, it is unlikely that either protein is a CYP IA-like form (i.e. induced by polycyclic hydrocarbons or other planar molecules). Utilizing antibodies raised against trout LM_{4b} (CYP IA1) as well as ethoxyresorufin dealkylase activities (EROD), Ronis *et al.* [8] have shown that a CYP IA-like form of approximately 56,000 is present in polychlorinated biphenyl- and dioxin-induced catfish liver. In addition, an increase in EROD was also observed in liver microsomes of catfish treated with β-naphthoflavone [6]. These data suggest the possibility

of a third inducible form in catfish liver possibly in the CYP IA family.

The hybridization of catfish P450s with multiple trout antibodies appears to be due to similarity in epitopic structure between each form. CATL-2 appeared to have the most common structural similarities, since it hybridized with each trout P450 antibody and showed the stronger cross-reaction when CATL-1 and CATL-2 were present (i.e. anti-trout LMC1, LMC2, and LMC5). In positive controls (trout microsomes), multiple trout P450s hybridized with antibodies raised against a single purified P450 (see Fig. 1). Based on ELISA and Western blot analysis, Miranda *et al.* [13] suggested that some sequence homology exists between LMC1 and LMC2 and between LMC3 and LMC4. Anti-trout P450 LMC2 and LMC1 have been shown to recognize mammalian [13] as well as invertebrate forms (LMC2-only) [11].

The recognition of CATL-1 and CATL-2 by anti-trout LMC1, LMC2, and LMC5, indicated that these two catfish P450s may possibly be involved in steroidogenesis. Hydroxylations at 6β of testosterone and progesterone as well as 2β of testosterone support this speculation. In the trout, 6β-hydroxylation of testosterone was shown to be mediated by LMC1 and LMC5 while 16β-hydroxylation was catalyzed by LMC2 [22]. LMC5 and LMC2 have also been shown to hydroxylate progesterone at the 6β- and 16α-positions, respectively [22]. Further immunologic characterization and comparison with mammalian P450s have indicated that LMC5 is probably in the CYP 3A family [28], for it is this family of P450s which carries out 2β- and 6β-hydroxylations of testosterone in mammals [29].

Hybridization of CATL-1 and CATL-2 with anti-trout LMC1 suggests some structural similarities with LMC1. In the trout, the hydroxylation of lauric acid was shown to be catalyzed by LMC1, suggesting perhaps a CYP 4A classification (Miranda CL, unpublished data). However, neither LMC1 nor LMC2 was induced after treatment with the CYP 4A inducing agent clofibrate (Miranda CL, unpublished data). Purification of both of these catfish forms to perform reconstituted studies is necessary to determine the endogenous substrates for these enzymes.

Although catfish liver microsomes appear to have levels of P450 relatively similar to those of trout, there does not appear to be a homologous form of flavin-containing monooxygenase in catfish. FMO activity was not observed in microsomes prepared utilizing similar procedures from three laboratories. Assay buffers, pHs, temperatures, and incubation times were all varied to try to optimize conditions. In addition, polyclonal antibodies raised against the rabbit lung FMO did not hybridize to microsomal protein from catfish livers, but did recognize FMO from trout. Anti-rabbit lung FMO have been shown recently to hybridize with two microsomal proteins that correlate with FMO activities in trout liver [19]. These same antibodies also have been shown to recognize FMO from liver microsomes of the smooth dogfish shark (Schlenk D, unpublished data).

It appears that FMO are responsible for the metabolism of trimethylamine to the osmolyte trimethylamine *N*-oxide, thus playing a role in osmoregulation in sharks and marine fish [16, 17]. Various strains of rainbow trout in coastal streams migrate to the open ocean to feed and then return to those streams to spawn. However, channel catfish only survive in fresh water and consequently may not require an enzyme necessary for osmoregulation. In addition, channel catfish have been shown to be relatively resistant to the effects of many thioether pesticides [30]. FMO have been shown to bioactivate several thioether pesticides in trout [18] and striped bass [31] which are significantly more sensitive to these compounds than channel catfish [30]. Perhaps the sensitivity to thioether pesticides in various fish is directly related to the presence or absence of bioactivation pathways in these fish. Clearly, more land-locked freshwater fish species need to be examined to test these hypotheses.

In summary, there appear to be at least three forms of cytochrome P450 in the liver of channel catfish. Two of these forms are structurally related to five constitutive trout P450s and a third form is related to CYP IA1 [8]. Regioselective hydroxylations of steroids by these microsomal proteins is consistent with earlier work in trout suggesting the presence of constitutive P450s. There does not appear to be a catalytically active nor structurally homologous FMO form in catfish liver. Studies examining the relevance of these enzyme systems regarding chemical bioactivation and detoxification should lead to a better understanding of how fish bred in aquaculture cope with the many agents used in this industry.

Acknowledgements—We wish to thank Dr. Bill Griffin at the U.S. Fish and Wildlife Fish Farming Experimental Laboratory, Stuttgart, AR, for his valuable help and expertise in maintaining and harvesting the catfish used in this study. We also wish to thank Drs. Evan Gallagher and Richard DiGiulio for help in providing catfish liver microsomes. Polyclonal antibodies to rabbit lung FMO were provided by Dr. David Williams. We also wish to thank Mr. Steven Hennes for his help in the laboratory. This study was supported in part by NIH Grant ES-03850 (C.L.M. and D.R.B.).

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