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Esterases in Indian major carps – 'Rohu' (*Labeo rohita*) and 'Mrigal' (*Cirrhinus mrigala*) (Teleostei, Cyprinidae)

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ABSTRACT

Soluble esterases of rohu and mrigal were characterised by electrophoresis using different substrates and inhibitors. Four separable regions of esterolytic activity were observed in liver, brain and white muscle extracts of rohu and in three regions in mrigal, with liver of both species exhibiting maximum number of bands. Species-specific differences were observed in some of the regions. Striking substrate-specific reactions were not observed but based on sensitivity to inhibitors, the liver esterases of both species were classified into aryl, carboxyl, choline, ER and Esdp esterases.

Introduction

Esterases comprise a diverse group of enzymes catalysing the hydrolysis of organic esters. In a wide variety of organisms, they have been used as important gene markers (Augustinsson, 1961; Holmes and Whitt, 1970; Choudhury, 1972; Matteo *et al.*, 1973; Vedbrat and Whitt, 1975; Massaro *et al.*, 1975; Peters, 1982). In vertebrates, there is a large and unknown number of loci responsible for this complex group of enzymes and the number of loci expressed is found to vary from taxon to taxon, between tissues and between life-history stages (Holmes and Whitt, 1970; Vedbrat and Whitt, 1975). There is also the possiblity of esterases showing post-translational modifications and formation of hybrid polymers. The band pattern also exhibits profound variation with varying electrophoretic conditions (Richardson *et al.*, 1986). As a consequence of these problems, use of inhibitor techniques and substrate specificity studies becomes inevitable for characterisation and genetic interpretation of esterase zymograms. Esterases have been studied only in few tropical species like skipjack tuna, *Barbus* sp., *Amblypharyngodon mola*, *Puntius sophore* and *Micropogonias furnieri* (McCabe and Dean, 1970; Varma and Frankel, 1980; Lakshmipathi and Reddy, 1989; Matthiensen *et al.*, 1993). The National Bureau of Fish Genetic Resources carries out research on genetic characterisation of Indian fishes and the present work forms the base study for

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the major programme. This paper aims at establishing the biochemical characterization and species comparison of different esterases in two Indian major carps viz., rohu (*Labeo rohita*)and mrigal (*Cirrhinus mrigala*).

Materials and methods

Rohu (n = 124) and mrigal (n = 98) were collected from river Ganga and ponds, located about 20 km from Allahabad and a hatchery located near Lucknow, U.P. Rohu was the target species for indepth study, hence more samples were collected. Specimens having total length of 10-15 cm were transported alive to the laboratory and reared until analyses. Liver (160 mg), brain and white muscle (250 mg each) taken from freshly killed specimens were homogenised in 1 ml of ice-cold extracting solution (10 % sucrose solution for muscle and brain and 0.01 M Tris-HCI buffer containing sucrose 500 mg/ litre and EDTA 320 mg/ litre; pH 7.0, for liver samples). Several other solutions were also tried for homogenising tissues but only the above ones produced sharp bands without trailing. Homogenates were kept over ice for 30 minutes and centrifuged at 16,000 rpm at 4°C for 45 minutes in a refrigerated centrifuge and supernatent used for electrophoresis. Vertical slab gel electrophoresis (1.0 mm thick, 7.5 cm tall) was carried out using 7.5 % polyacrylamide gel (containing 5 mg NAD per 20 ml gel mixture) at 20°C. Tris (500mM) - borate (650 mM) - Na_2 EDTA $2H_2O$ (16 mM) - (TBE), pH 8.6 was used as gel buffer and a 1:9 dilution of the same was used as tank (electrode) buffer. To increase resolution of bands, 35 mg of NAD was dissolved in 150 ml of buffer in upper tank. For loading the gel, 3 µl of liver extract and 6 µl each of muscle and brain homogenates were used. Aqueous bromophenol blue (final concentration 0.05 %) was used as tracking dye. The run was carried out at a constant current of 30 mA (150 V) and terminated after 50 minutes. The gels were stained at room temperature following the procedure of Redfield and Salini (1980). One naphthyl esters of acetate, butyrate, caprylate, laurate and propionate, β - naphthyl oleate and naphthol AS - D acetate were used for substrate-specificity studies. EDTA (100 mM), parahydroxymercuribenzoate (pHMB, 1 mM), phenylmethyl- sulphonylfluoride (PMSF, 50 mM), pheylmercuriacetate (PMA, 1mM) and silver nitrate ($AgNO_3$, 10mM) were used in inhibitor sensitivity studies. The gels were pre-incubated in the buffer containing the above concentrations of inhibitors for half an hour, following which they were stained for esterase activity using α - naphthylcetate as the substrate. To prevent reversal of inhibition, the same concentration of inhibitor that was used for pre-incubation was added to the staining mixture also. Since the target of the major programme was to score genetic polymorphism within the species, initially the tissue samples of rohu and mrigal were electrophoresed in separate gels under identical conditions. But interspecific differences were very less and there are reports of occurrence of viable rohu-mrigal hybrids in hatchery and natural populations. Hence in the further study for the tissue and species-wise comparisons, samples were always run in the same gel and under conditions exactly identical in all respects. In the zymogram, bands were serially numbered with the fastest migrating fraction getting the first number and slowest the last. Taking the Rf value

and proximity of bands into consideration, the enzyme activity areas were broadly categorised into different regions, each region having 2-3 bands.

Results and discussion

The electrophoretic pattern of esterases of both rohu and mrigal in the three tissues, liver (L), brain (B) and white muscle (M) are presented in Fig.1. In rohu among the three tissues, the liver exhibited maximum number of bands (13), which could be grouped into four distinct regions. Maximum number of bands (10) was observed in the liver of mrigal also. In both the species compared to liver, the muscle and brain extracts did not exhibit tissue-specific/ additional bands or loci.

Comparison of band patterns of rohu and mrigal revealed that esterases exhibit differences between the two species. The major difference between these two cyprinids was in regions III and IV esterases. The band 3.3 in rohu exhibited intense esterolytic activity, while in mrigal it was the band 3.2 showing the highest reaction in the region. In addition, the staining intensity of band 2.3 was very much low (less than 1+) in mrigal, compared to that of rohu (3+) and the entire region IV was absent in the former. The Rf values of bands 1.2, 1.3, 1.4, 2.1 and 2.2 also differed slightly between rohu and mrigal (Table 1). An earlier report on rohu (Reddy and Lakshmipathi, 1990) identified as many as 30 esterase bands in various tissues with most bands showing wide variations in Rf values between the two adjacent collection sites of the same river system, indicating a high degree of genetic difference in esterases between neighbouring sites, which is not normally observed. In addition, the tissues shared very few common bands in their report. Their results disagree with the normally observed esterase isozyme pattern with respect to the number of tissue-specific bands in other fishes (Holmes and Whitt, 1970; Hart and Cook, 1976; Varma and Frankel, 1980; Haritos and Salamastrakis, 1982, Matthiensen *et al.*, 1993) as well as this report. Differences have been observed in esterase patterns of surface mucus of rohu and mrigal (Padhi and Khuda-Bukhsh, 1990). Mucus of rohu showed three esterase bands in two regions, while in mrigal a total of four esterase bands were observed in two regions (Padhi and Khuda-Bukhsh, 1990). The three tissues used in the present study exhibited less number of bands/regions in mrigal than rohu; hence the variability observed in the former report can be mucus-specific.

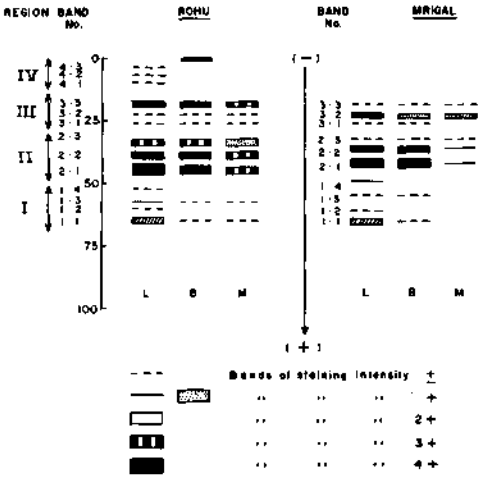

Fig. 1. Electrophoretic patterns of non-specific esterases in three tissues of rohu and mrigal using a- naphthyl acetate as substrate. L = Liver, B = Brain, M = White Muscle. (—) = Origin, (+) = Anode. The scale 0 to 100 indicates relative mobility (Rf) values.

TABLE 1. *Reaction of esterase regions in three tissues of rohu and mrigal with different substrates. Increasing activity is shown on a scale of ± to 4+*

Tissues	Liver													Brain										White muscle								
Regions	I				II			III			IV			I				II			III			I				II			III		
Bands	1.1	1.2	1.3	1.4	2.1	2.2	2.3	3.1	3.2	3.3	4.1	4.2	4.3	1.1	1.2	1.3	1.4	2.1	2.2	2.3	3.1	3.2	3.3	1.1	1.2	1.3	1.4	2.1	2.2	2.3	3.1	3.2	3.3
Relative mobility (Rf) values	67	59*	58*	57*	44*	39*	35	27	24	14	5	4	3	67	-	58*	-	44*	39*	35	27	24	14	67	-	58*	-	44*	39*	35	27	24	14
Labeo rohita																																	
α-naphthyl acetate	2+	±	+	±	4+	4+	3+	±	±	4+	±	±	±	±	-	±	-	4+	4+	3+	±	±	4+	±	-	±	-	3+	3+	+	±	±	3+
α-naphthyl butyrate	2+	±	+	±	4+	4+	3+	±	±	4+	±	±	±	±	-	-	-	4+	4+	3+	±	±	4+	±	-	±	-	3+	3+	+	±	±	3+
α-naphthyl propionate	2+	2+	2+	2+	4+	4+	3+	±	±	3+	±	±	±	+	-	+	-	3+	4+	3+	±	±	4+	±	-	±	-	3+	3+	+	±	±	2+
Naphthol AS-D acetate	-	-	-	-	2+	2+	+	±	±	3+	-	-	-	-	-	-	-	2+	2+	+	±	±	2+	-	-	-	-	3+	3+	+	±	±	2+
α-naphthyl caprylate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-naphthyl-laurate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-naphthyl oleate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cirrhinus mrigala																																	
α-naphthyl acetate	2+	±	±	+	4+	4+	±	±	4+	±				±	-	±	-	4+	4+	±	±	+	±	-	-	-	-	+	+	±	-	+	±
α-naphthyl butyrate	2+	±	±	±	4+	4+	±	±	4+	±				±	-	±	-	4+	4+	±	±	+	±	-	-	-	-	+	+	±	-	+	±
α-naphthyl propionate	2+	+	+	+	4+	4+	±	±	3+	±				+	-	+	-	4+	4+	±	±	+	±	-	-	-	-	+	+	±	-	+	±
Naphthol AS -D acetate	-	-	-	-	2+	2+	±	±	3+	±				-	-	-	-	4+	3+	±	±	+	±	-	-	-	-	+	+	±	-	+	±
α- naphthyl caprylate	-	-	-	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-naphthyl laurate	-	-	-	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-naphthyl oleate	-	-	-	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4+ indicates the highest staining intensity; 3+ strong staining reaction; 2+ medium staining ; + weak staining; ± doubtful/very low reaction; -no staining.
*Rf values of bands 1.2, 1.3, 1.4, 2.1 and 2.2 of mrigal 60, 57, 54, 42 and 37 respectively.

Substrate-specificity : Of the seven substrates tested, only four ; α-naphthyl acetate, α - naphthyl butyrate, α - naphthyl propionate and naphthol AS-D acetate gave sharp staining of esterase zones (Table 1). Among these, α - naphthyl acetate produced strongest staining activity and demonstrated all regions especially the sites of low enzyme activity within a short span of time. No other substrate-specific reaction was observed other than a slightly more intense staining of bands in region I of both rohu and mrigal when propionate was used. Naphthol AS-D acetate did not stain the less-intense bands of zone I of both rohu and mrigal and zone IV of rohu. Oliver *et al.* (1991) reported that naphthol acetates may not be suitable for exhibiting sites of low enzyme activity as these substrates are hydrolysed more slowly than naphthyl acetates. The other three substrates used in the present study *viz.*, caprylate, laurate and oleate, did not exhibit activity for any esterolytic bands, even after overnight incubation of the gels in the staining mixture.

As evident from our studies in rohu and mrigal, esterases display less substrate specificity with the substrates used and support the findings in *Brachydanio* (Hart and Cook, 1976) and *Barbus* (Varma and Frankel, 1980) that utilisation of substrates is an inconclusive means of classification. However, Oliver *et al.* (1991) reported substrates specific for choline esterases like benzoyl choline chloride and n-butyryl choline have been useful in characterising esterases.

Inhibitor sensitivity : Esterases are classified into categories depending on their sensitivity to various inhibitors. Carboxyl esterases (E.C.3.1.1.1.) hydrolyse aliphatic esters and they are inhibited by organophosphate (OP) compounds. Arylesterases (3.1.1.2) preferentially hydrolyse aromatic esters and are inhibited by sulphydryl reagents such as parachloro (hydroxy) mercuribenzoate (pCMB/pHMB). Choline esterases (3.1.1.7 & 3.1.1.8) are sensitive to physostigmine as well as OP compounds. Acetyl esterases on the other hand preferentially hydrolyse acetate esters and are not affected by any of these inhibitors (Metcalf *et al.*, 1972). Three more esterases have been added to the list by Hart and Cook (1976): ER esterases which resist the inhibition by physostigmine, DFP and pCMB/pHMB; Ese esterases susceptible to eserine sulphate alone and Esdp esterases which are inhibited both by pCMB/pHMB and organophosphates.

Due to the consistently low esterase activity observed in skeletal muscles and brain extracts of rohu and mrigal, only liver samples were selected for inhibitor-sensitivity studies. The results are presented in Table 2. All bands in region I and the band 2.1 of both rohu and mrigal and 2.2 of mrigal were classified as aryl esterases based on their sensitivity to pHMB. These are in accordance with the observations in *Barbus* sp. (Varma and Frankel, 1980) that aryl esterases show relatively high electrophoretic mobilities. In region III, all the bands of mrigal and rohu were classified as carboxlesterases because they were inhibited by both PMSF and $AgNO_3$. Band 2.2 of rohu was inhibited by PMSF but not by $AgNO_3$. Hence this band may be either carboxyl or choline esterase. To include this band under either category, its sensitivity with physostigmine must be examined. Band 2.3 of both rohu and mrigal was inhibited by PMSF, pHMB PMA and par-

TABLE 2. *Inhibitor sensitivity of esterase regions in liver of rohu and mrigal, expressed in terms of residual staining activity*

Regions	I				II			III			IV		
Bands	1.1	1.2	1.3	1.4	2.1	2.2	2.3	3.1	3.2	3.3	4.1	4.2	4.3
Relative mobility (R_f) values	67	59*	58*	57*	44*	39*	35	27	24	14	5	4	3
Labeo rohita													
Control (α-naphthyl acetate only)	2+	±	+	±	4+	4+	3+	±	±	4+	±	±	±
PMSF (50mM)	+	±	+	±	3+	-	-	-	-	-	-	-	-
pHMB (1mM)	±	-	±	-	+	3+	+	±	±	2+	-	-	-
PMA (1mM)	+	±	+	±	3+	2+	-	±	±	+	-	-	-
EDTA (100 mM)	±	±	±	±	2+	2+	+	±	±	+	-	-	-
$AgNO_3$(10mM)	-	-	-	-	2+	2+	2+	-	-	-	-	-	-
Classification	Ar.Est.	Ar.Est	Ar.Est	Ar.Est	Ar.Est	C.Est/ Ch.Est.	Esdp	C.Est	C.Est	C.Est	-	-	-
Cirrhinus mrigala													
CONTROL (α-naphthyl acetate only)	2+	±	±	+	4+	4+	±	±	4+	±			
PMSF (50 mM)	+	±	±	±	2+	2+	±	-	±	-			
pHMB (1 mM)	±	-	-	-	-	-	-	±	2+	±			
PMA (1 mM)	-	±	±	±	2+	2+	-	±	+	±			
EDTA (100 mM)	-	-	-	-	+	+	+	±	+	+			
$AgNO_3$ (10 mM)	±	+	-	-	2+	2+	±	-	+	-			
Classification	Ar.Est.	Ar.Est	Ar.Est	Ar.Est	Ar.Est	Ar.Est	Esdp	C.Est	C.Est	C.Est			

Degree of inhibitor sensitivity denoted in terms of residual staining intensity: 4+ no inhibition; 3+ weak inhibition; 2+ medium inhibition; + strong inhibition; ± near total inhibition; - Complete inhibition.

* R_f values of bands 1.2, 1.3, 1.4, 2.1 and 2.2 of mrigal 60, 57, 54, 42 and 37 respectively.

Classification : Ac. Est. Acetyl Esterase; Ar. Est. - Aryl Esterase; C. Est. - Carboxyl esterase; Ch. Est. Choline Esterase; ER - Esterases resistant to OP compounds & pHMB; Esdp - enzyme sensitive to both DFP & pHMB.

tially by AgNO3. Hence this falls under the category of Esdp esterases. All the bands in the region IV of rohu liver showed very low staining intensity and were sensitive to all inhibitors, hence they were not included under any class of esterases. EDTA was shown to partially inhibit the activity of all esterase isozymes of both the species and not any specific band or region. This chelating agent was shown to inhibit esterases of tuna but appeared to have negligible influence on certain cyprinid esterases (Lakshmipathi and Reddy, 1989).

Band 2.2 of rohu and mrigal showed species-specificity. This band of rohu was classified as carboxyl/choline esterase, while the same of mrigal was scored as aryl esterase. Such species-specific differences were also reported in four species of *Barbus* (Varma and Frankel, 1980).

Variation in band pattern between individual samples was observed in liver esterases of rohu. In some samples, band 3.2 exhibited highly intense staining activity in addition to band 3.3. The intense activity of band 3.2 is usually observed in mrigal and not in rohu. Samples expressing both the bands with high esterolytic activity can be intergeneric rohu-mrigal hybrids. Viable rohu-mrigal hybrids are encountered in nature and hatchery populations. They are difficult to be distinguished sometimes at smaller size group from rohu stock. An additional band with 3+ staining intensity is observed between 3.3 and 4.1 in 20 % rohu samples. Further in-depth investigations are being carried out to give requisite interpretation to this variation.

In conclusion, the electrophoretic pattern of soluble esterases of *L. rohita* and *C. mrigala* showed species-specific differences. The basic profile of esterases can be useful in detecting genetic introgression and polymorphism in these cyprinids.

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