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ELEVATION OF 7-ETHOXYRESORUFIN *O*-DEETHYLASE (EROD) ACTIVITY IN IMMATURE GROUPER *ANYPERODON LEUCOGRAMMICUS* AFTER EXPOSURE TO BENZO[A]PYRENE : CYTOCHROME P4501A1 LEVELS AS A BIOMARKER OF EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Extensive studies have demonstrated that exposure to specific organic pollutants, initiates an elevation of the hepatic protein cytochrome P4501A1 (CYP1A1). Levels of CYP1A1 may be determined by 7-ethoxyresorufin *O*-deethylase (EROD) activity expressed by the CYP1A1. Juvenile grouper *Anyperodon leucogrammicus* were injected intra peritoneally (20 mg/kg wet weight) with benzo[a]pyrene (B[a]P) or ß-naphthoflavone (model CYP1A1 inducer) dissolved in corn oil, and held for 24 hours in clean sea water. Hepatic EROD activity was determined in the S9 fraction. Enzyme activity in control fish (corn oil alone) was below the detection limits of the experimental assay, however B[a]P and ß-naphthoflavone elevated EROD activity (1043 ± 262 and 450 ± 367 pmol/min/g liver weight respectively). These results suggest that *A. leucogrammicus* may be a suitable sentinel species for assessing the impact of organic pollutants in the field.

INTRODUCTION

The seas and oceans of the world are becoming increasingly contaminated with pollutants of anthropogenic origin. Some pollutants may accumulate and persist in the aquatic environment for many years whilst others readily degrade. Exposure to persistent pollutants or their degradation products, may initiate a wide variety of biological effects in marine ecosystems (Walker & Livingstone, 1992). Responses to pollutants, at the level of the organism however, depends upon the combination of the biochemical pathways capable of metabolising the pollutant. Changes in these pathways, as a result of pollutant exposure, may be used as useful indicators of exposure (Livingstone, 1993).

Environmental contamination of coral reefs by polycyclic aromatic hydrocarbons (PAHs) have been reported in recent years (Loya & Rinkevich; 1980, Guzman, *et al.,* 1991). The impact on the marine environment of PAHs and other organic pollutants may be assessed by biochemical studies of the biotransformation enzymes in sentinel species (Anderson & Förlin, 1992). Monooxygenation is one of the significant processes in the detoxication of PAHs and may be performed by the cytochrome P450 monooxygenase system. Cytochrome P450 is a superfamily of haem proteins that are oxygenases; many isoforms being expressed in fish (Stegeman, 1994). One specific form, cytochrome P4501A1, has been used extensively as a biomarker of exposure to PAHs, planar polychlorobiphenyls and other organic contaminants (Livingstone, 1993). After exposure to these contaminants, hepatic protein levels of cytochrome P4501A1 (CYP1A1) may be elevated.

CYP1A1 has an enzyme activity associated with it (7-ethoxyresorufin *O*-deethylase [EROD] activity) and elevation of this activity may be readily determined *in vitro* by fluorometric analysis. In this study, immature grouper *Anyperodon leucogrammicus* were injected with the PAH benzo[a]pyrene (B[a]P) or the CYP1A1 inducer ß-naphthoflavone to determine the elevation of EROD activity. This species is common to reef environments and may be appropriate as a sentinel species for pollution impact assessment as well as being a fish of commercial value in the coastal districts of Thailand.

MATERIALS AND METHODS

Chemicals

Biochemicals including 7-ethoxyresorufin, resorufin, ß-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), ß-naphthoflavone and B[a]P were ordered from Sigma Chemical Co. (Bangkok). Solvents and buffer reagents were analytical grade from BDH (Bangkok).

Sample preparation

Juvenile *A. leucogrammicus* were obtained from a commercial fish farm and maintained for 24 hours at ambiant sea temperature in continuously aerated flowing sea water. Fish were then injected intra peritoneally with a minimal volume of either corn oil or contaminant dissolved in corn oil (20 mg B[a]P or ß-naphthoflavone per kg fish weight). Fish were kept in separate flowing sea water tanks according to their treatment for 24 hours and then sacrificed. Livers were dissected immediately, weighed and then homogenised in 4 liver volumes of 0.15 M KCl-KOH pH 7.5, 1 mM ethylenediaminetetraacetic acid at 4 °C. Homegenates were centrifuged at 500g for 20 minutes and the supernatants removed and centrifuged at 9000g for 45 minutes (supernatant from 9000g abbreviated to S9). All centrifuge procedures were undertaken at 4 °C.

EROD activity determination

EROD activities were determined using S9 material by the method of Burke & Mayer (1974). Aliquots of supernatant (100 µl) were incubated in duplicate, in a final volume of 1 ml containing 58 mM K2HPO4/KH2PO4 pH 7.4, 225 µM NADPH and 3.74 µM 7-ethoxyresorufin for up to 60 minutes at 30 °C. The incubations were stopped by the addition of 2 ml acetone and the fluorescence was measured (Ex 537 nm; Em 583 nm) against a resorufin standard. Zero-time controls were run for each sample by the addition of acetone to sample followed by buffer and substrates at 30 °C for up to 60 minutes. EROD activity was expressed as pmol resorufin/min/g wet weight.

RESULTS AND DISCUSSION

After 24 hours exposure to corn oil alone, EROD enzyme activity was undetectable in *A. leucogrammicus* hepatic S9 samples. This may be due to low levels of basal EROD activity in the tissue preparation. The specific activity of this enzyme is higher in the microsomal fraction than the S9 and in future studies a microsomal preparation may enable the basal activity to be determined. Fish injected with B[a]P or ß-naphthoflavone (dissolved in corn oil) and kept in clean seawater for 24 hours had elevated EROD enzyme activities compared to controls (Table 1). Elevation of EROD activity in response to exposure to PAHs and planar PCBs has been reported in many fish exposure studies (Livingstone, 1993). In this study, elevation of *A. leucogrammicus* EROD activity was 2 fold higher in fish exposed to B[a]P, compared to ß-naphthoflavone. This response has also been observed in Sunfish hybrids (*Lepomis macrochirus* × *L. cyanellus*) exposed to B[a]P and ß-naphthoflavone (Oikari & Jimenez, 1992).

Table 1. Elevation of 7-ethoxyresorufin *O*-deethylase (EROD) activity in the hepatic S9-fractions of immature grouper (*Anyperodon leucogrammicus*) after 24 hour exposure to benzo[a]pyrene (B[a]P) and ß-naphthoflavone.

Treatment*	Specific activity (pmol/min/g wet weight)
Contrlo	0†
B[a]P	1043 ± 2+62
ß-naphyhoflavone	450 ± 367

* n = 2 (control injected with corn oil, B[a]P and ß -naphthoflavone dissolved in corn oil and injected at 20 mg/kg wet weight fish).
† Rate below the detection limits of the assay.

In this study the incubation temperature for the EROD assay was 30 °C. This approximates to the ambient sea temperature for the region around Phuket, Thailand. The *in vitro* biochemical responses determined in this study, therefore

approach the physiological temperature of the organism in its habitat. In a study of the biotransformation enzymes in the tropical marine fish, Butterfly fish (*Chaetodon capistratus*), the EROD activity was also determined at 30 °C (Vrolijk *et al.*, 1994).

Enzyme analysis of CYP1A, as reported here using EROD activity, may be undertaken using readily available laboratory chemicals and a standard laboratory fluorimeter. The process has been automated enabling rapid sample analysis (Eggens & Galgani, 1992). Other enzymatic markers of CYP1A levels, include the enzyme benzo[a]pyrene hydroxylase (BPH). BPH activity has determined in a selection of organisms from coral reefs including teleosts, elasmobranchs and crustacea from the Florida coast (James *et al.*, 1979).

Other procedures for the determination of CYP1A amount include immunoquantitation by Western blot (Peters & Livingstone, 1993) or ELISA (Goksøyr *et al.*, 1991) using specific antibodies (commercially available). When marine organisms are exposed to both CYP1A inducers and metals *eg* cadmium and organo-tin, CYP1A protein denaturation may occur (George, 1989; Fent & Stegeman, 1993). This denatured protein may be detected using immunoquantitative proceedures. As a result it is reccommended that both enzymic and immunoquantitative methods are performed when assessing the impact of organic pollution in field studies.

In conclusion, the reef fish *Anyperodon leucogrammicus* has a readily inducible CYP1A1 protein when exposed to the model contaminant benzo[a]pyrene. This species may be suitable for the determination of the impact of organic pollutants in reef environments after future studies including field sampling from clean and polluted sites are undertaken.

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