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Characterization of type "B" esterases and hepatic CYP450 isoenzimes in Senegalese sole for their further application in monitoring studies

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ABSTRACT

In fish, the role that cholinesterases (ChEs) play in tissues other than those implicated in neural activity, as well as the involvement of carboxylesterases (CbEs) and cytochrome P450 isoenzymes (CYPs) in drug metabolism needs investigation. For that, Senegalese sole (*Solea senegalensis*) specimens were selected for characterization of several type B esterases and hepatic CYPs in order to further use this fish as sentinel. ChEs (acetylcholinesterase (AChE) and pseudocholinesterases (butyrylcholinesterase-BuChE and propionilcholinesterase-PrChE)) and CbEs were measured in brain, plasma, kidney, liver, gonad, muscle and gills. Moreover, seven fluorimetric substrates were selected to study CYP related activities in fish liver. The results showed that AChE was the dominant ChE form in brain whereas pseudocholinesterases were absent in most tissues, as demonstrated by low enzymatic activities using specific substrates and the lack of inhibition by *iso*-OMPA. Plasma exhibited trace activities of all the esterases assayed and no BuChE activity. CbEs were dominant in liver, but they were also present in kidney and brain. For CbE determination, α-naphtyl acetate (αNA) was seen as the most adequate substrate as it displayed higher enzymatic activities and showed more *in vitro* sensitivity to the carbamate eserine and the organophosphate pesticide dichlorvos. Alkoxyresorufin-O-dealkylase (EROD and BFCOD) activities, indicative in mammals of CYP1A and CYP3A subfamilies, respectively, were the highest microsomal CYP-related activities in liver. The results of this preliminary work allow us to select the most adequate esterase substrate, tissue and hepatic CYP substrate for further monitoring studies.

1. Introduction

The Senegalese sole (*Solea senegalensis*, Kaup, 1858) is considered an invasive species in the western Mediterranean Sea (Zenetos et al., 2010). Although the original distribution of the species is Eastern Atlantic from Senegal to La Rochelle (France), now it has expanded with a self-maintaining population in the western Mediterranean Sea. In the Iberian Peninsula has a great commercial interest as a fishery target as well as in aquaculture, due to its good adaptation to captivity conditions (Dinis et al., 1999). In recent years, it has also been extensively selected as sentinel in field pollution monitoring and laboratory toxicity experiments in the Southern Mediterranean region. To name a few of the recent applications: in relation to surfactants exposure (Alvarez-Muñoz et al., 2007, 2009), to metals (Kalman et al., 2010), antifoulants (López-Galindo et al., 2010a, b), in bioassays with contaminated sediments (Costa et al., 2009a, b; 2010, 2011) and in field monitoring (Oliva, 2011; Fonseca et al., 2011a, b).

Cholinesterases (ChEs) and carboxylesterases (CbEs) are type B esterases both strongly inhibited by organophoshorous compounds (OPs). Whereas AChE (acetylcholinesterase) is mainly involved in neurotransmission breaking of acetylcholine in neuromuscular junctions, pseudocholinesterases (propionilcholinesterase-PrChE and butyrylcholinesterase-BuChE) have a less clear (or have multiple) physiological role (Karczmar, 2010). In turn, CbEs are involved in the metabolism of a broad range of endogenous as well as manmade chemicals and pharmaceuticals (Wheelock et al., 2008). Besides to their physiological role(s), a great sensitivity towards an increasingly broader range of compounds, in addition to pesticides, has been reported for ChEs in invertebrates as well as vertebrates (Guilhermino et al., 2000; Alpuche-Gual and Gold-Bouchot, 2008) including *S. senegalensis* (López-Galindo et al., 2010a, b), and for CbEs (Al-Ghais et al., 2000; Wheelock et al., 2008). Moreover, a role in detoxification and protection towards AChE inhibition in neural tissues has been demonstrated by the action of hepatic CbEs (Maxwell, 1992; Laguerre et al., 2009) and plasmatic BuChE (Salles et al., 2006). Thus their combined inclusion in pollution monitoring programs has been highly recommended (Küster, 2005; Wheelock et al., 2008; Laguerre et al., 2009).

ChEs and CbEs have a particular tissue distribution in vertebrates. While AChE (EC 3.1.1.7), is mostly located in brain and muscle, as they are the most innerved tissues, pseudocholinesterases (EC 3.1.1.8) are mainly present in liver and blood but also

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in fish muscle (Solé et al., 2010). CbEs (EC 3.1.1.1) consists of multiple isoenzymes and although they are dominant in the liver, they are also present in many other tissues, including kidney, blood and brain (Wheelock et al., 2008). ChEs tissue distribution determines that, in most pollution monitoring studies, brain (Oliveira et al., 2007; Assis et al., 2010), head in the case of smaller fish (Varó et al., 2008; Moreira et al., 2010) or muscle (Solé et al., 2008b, 2010) were the tissues chosen for neurotoxicity determination using AChE as biomarker. Other tissues/organs such as blood (Barron et al., 1999; Valbonesi et al., 2011), kidney (Üner et al., 2009), gill (Barron et al., 1999; López-Galindo et al., 2010a, b) and gonad (Galgani et al., 1992) are particularly considered in some fish studies. In the present characterisation, they were included for their involvement as a first line defence (blood and gills) and physiological role in the immune (kidney) and reproductive (gonad) systems.

In marine fish muscle, three ChE forms are present being AChE predominant, followed by PrChE and BuChE to different degrees; whereas in brain, only AChE is present. Pseudocholinesterases sensitivity to chemicals was reported in some fish species as to be greater than that of AChE (Sturm et al., 1999, 2000; Kirby et al., 2000). Thus, formerly to esterases inclusion as biomarkers, a characterisation of their tissue location and their relative activities needs to be performed (Varó et al., 2003; Alpuche-Gual and Gold-Bouchot, 2008; Solé et al., 2010). CbEs determinations can be done using several substrates, which differ in their isoenzyme specificity (Wheelock et al., 2008; Laguerre et al., 2009). In this study ρ-nitrophenyl acetate (ρNPA) was selected as a more unspecific substrate, whereas αNA has been recognised to have a higher affinity for OPs (Chanda et al., 1997).

As seen for with type B esterases (ChEs and CbEs), monooxigenases (MO) are a family of enzymes also involved in phase I metabolism. The cytochrome P450 is a superfamily of MO proteins that consist of several isoforms involved in the biotransformation of endogenous and exogenous compounds (detoxification step) either by introduction of a polar group (e.g hydroxylation) or reduction of hydrophilic compounds in order to make them more readily excretable (Van der Oost et al., 2003). In fish, the most studied isoform is the CYP1A-dependent ethoxyresorufin *O*-deethylase (EROD) activity currently associated to dioxin-like chemicals exposure (White et al., 2000). Recent pollution monitoring studies have evidenced the importance of the combined action of anthropogenic chemicals over this enzymatic activity. Thus, mixtures of chemicals in the environment can act over this enzyme (either enhancing or decreasing its activity). Therefore the use of EROD activity as an indicator of anthropogenic impact has to be more carefully examined. In this sense, the application of several fluorimetric substrates to measure CYP activities in fish, adapted from mammalian studies on specific CYP families, will allow a closer insight into the metabolism of xenobiotics and the chemical interactions over the detoxification system of fish (Smith and Wilson, 2010). As suggested for esterases, previous to CYPs inclusion in monitoring, the characterisation of its basal activities is recommended. Moreover the combined use of B esterase activities and CYP1A levels has already been applied in the assessment of pesticide exposure in fish (Wheelock et al., 2005; Fasulo et al., 2010).

The aim of this study was to characterise, and measure, AChE, PrChE, BuChE and CbE activities in different tissues/organs (brain, muscle, liver, kidney, gills, gonad and plasma) of *S. senegalensis* using specific substrates and selective inhibitors. Even though some studies have already applied these biomarkers, a detailed characterization has not yet been published for this specie. Moreover, CYPs distribution in liver of adults was made for its further application in pollution monitoring studies.

2. Material and methods

2.1. Tissue preparation

Juveniles (females: 65.16 ± 51.9 g and males: 62.04 ± 38.4 g) and adult females (384.84 ± 91.2 g) of Senegalese sole were provided by the aquaculture facilities of Institute of Aquaculture Torre la Sal (IATS-CSIC, Castellón, Spain) and IRTA-Generalitat de Catalunya (Sant Carles de la Rápita, Spain), respectively. Fish were anesthetised using 2-phenoxy ethanol before blood was taken using a heparinized syringe and, immediately after, the tissues (gills, brain, liver, kidney, gonad and muscle) were snap frozen in liquid nitrogen. Plasma was obtained after blood centrifugation at 1000*g* × 15 min at 4 °C. Tissues and plasma were stored at −80 °C until analyses were performed.

A portion of tissue (0.05–0.2 g) was used for ChEs and CbEs determinations. Tissues were homogenised in ice-cold buffer phosphate (50 mM pH 7.4) in a 1:5 (w:v) ratio using a polytron® blender. The obtained homogenate was centrifuged at 10,000 g × 30′ at 4 °C and the supernatant (S10) used for enzymatic determinations.

Individual livers of adult females were also selected for CYPs-related activities determinations. Livers (≈ 2 g) were homogenised in ice-cold phosphate buffer (100 mM pH 7.4) containing 150 mM KCl, 1 mM DTT, 0.1 mM PMFS and 1 mM EDTA in a 1:4 ratio (w:v). After a 10,000*g* × 30′ and a 10,000 g × 60′ centrifugation steps at 4 °C, the microsomal pellet obtained was dissolved in the homogenisation buffer containing also 20% glycerol in a 2:1 (w:v) ratio according to the method published by Förlin and Anderson (1985).

2.2. Type B esterases characterization

ChE activities were characterised in several tissues (brain, muscle, liver, kidney, gills, gonad and plasma) using eserine (physostigmine) sulphate, BW253c51 (1,5-bis(4-allyldimethyl-ammonimphenyl)penta-3-one dibromide) and iso-OMPA (tetra-isopropyl pyrophosphoramide) as specific inhibitors of the true ChE, AChE and BuChE, respectively. The inhibitor tested concentrations ranged from 0.64 to 800 μM for eserine sulphate and BW253c51, and from 0.08 to 16 mM for *iso*-OMPA. In all cases, 120 μl of adequately diluted S10 supernatant (depending on the tissues) was incubated for 30 min at room temperature with 5 μl of the inhibitor at a specific concentration within the selected test concentrations. At least three independent replicates of each assay were done. A blank without inhibitor and with either bi-distilled water (in the case of BW284c51) or ethanol (solvent used for eserine sulphate and *iso*-OMPA) was also done. After the incubation, ChEs activities were determined according to the principle of Ellman et al. (1961) with appropriate modifications for microplate as described in Solé et al. (2008a), using 1 mM ASCh (acetylthiocholine iodide) as substrate. Reading was performed at 405 nm in kinetic mode (Magellan v6.0) using a TECAN Infinite 200 microplate reader at 25 °C for 5 min. Enzymatic determinations were always performed in triplicate.

The apparent *V*max and Km values for AChE activity in brain, muscle, kidney and gills, were measured using several ASCh concentrations (0.25, 1, 2.5, 5 and 20 mM) with appropriate S10 fraction dilutions, so linearity of the measure was always achieved, following the ChE assays conditions described above. The values were calculated in this range from Michaelis and Menten equation ($V = V\max [S]/Km + [S]$), using the linearity transformation of Lineweaver–Bruk plot.

Hepatic CbE activity using either αNA or pNPA as substrates was also tested (as described in 2.3 section). Moreover, its inhibition by the carbamate serine (from 0.64 to 800 μM) or the OP pesticide dichlorvos (*O*-(2,2-dichlorovinyl)-*O*, *O*-dimethylphosphate (DDVP) from PESTANAL® CAS 62-73-7) in the range from 0.256 to 320 μM was assessed. Furthermore, the apparent *V*max and Km values for CbE activity were determined in liver and kidney using five concentrations of αNA (0.0625, 0.125, 0.25, 0.5 and 1 mM) as substrate as described above for AChE.

2.3. Type B esterases measurements

For ChEs measurements three different substrates ASCh, S-butyrylthiocholine iodide (BuSCh) and propionylthiocholine iodide (PrSCh) at a concentration of 1 mM were used. In each microplate well, 150 μl of 270 μM DTNB (5,5′-dithio-bis-2-nitrobenzoat) were mixed with 25 μl of sample (undiluted or appropriately diluted) and after 2 min pre-incubation, the reaction was started adding 50 μl of the substrate. ChE activity was determined in triplicate as previously described (point 2.2) and was expressed as nmol/min/mg prot.

CbE activity was measured using either αNA or ρNPA as substrates. αNA was assayed following an adaptation to microplate from an UV method (Mastropaolo and Yourno, 1981), briefly, 25 μl of sample and 200 μl of αNA as substrate (250 μM final concentration in well) were followed up over 5 min at 235 nm. The other assayed substrate, ρNPA, was used as indicated by Hosokawa and Satoh (2001) with some modifications, that is, 25 μl of sample and 200 μl of ρNPA as substrate (1 mM final concentration in well) were followed up for 5 min at 405 nm over ρ-nitrophenol formation using the same kinetic method as for ChEs in a TECAN Infinite 200 microplate reader. CbE activity was also measured in triplicate and expressed as nmol/min/mg prot.