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Cholin- and carboxylesterase activities in developing zebrafish embryos (*Danio rerio*) and their potential use for insecticide hazard assessment

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Abstract

Insecticides are a potential hazard for non-target organisms like fish particularly at run off events. The study of effects to embryos of the zebra fish *Danio rerio* is already an accepted tool in waste water monitoring, but effects of various groups of substances (like most pesticides) to the zebrafish embryo remain to be studied. Enzymes are often taken as biomarkers of exposure and effect. Therefore cholinesterase isozymes and carboxylesterase were examined for their suitability as biomarkers of insecticide exposure. The activities of cholinesterase and of carboxylesterase were monitored in the first 48 h post-fertilization (hpf) of zebrafish development. Significant specific activities in the range of 0.5–25 U could be measured from the sixth somite stage (12 h) up to the Long Pec stage (48 h) for different cholinesterases using acetyl-, acetyl-β-methyl-, butyryl- and propionylthiocholin as substrates. The specific activity of carboxylesterase ranged from 4 to 16 U mg^{-1} protein in the respective developmental stages. Substrate specificity was analysed using specific inhibitors (eserine sulphate, DPDA, BW284c51). The results showed that the observed cholinesterase activities in the whole embryo may be attributed mainly to acetylcholinesterase with a partial capability to use propionylthiocholine as a second substrate. The potential use of cholin- and carboxylesterase as biomarkers was investigated using the organophosphate paraoxon-methyl. A 40% inhibition of enzyme activities was reached by 0.4 μM paraoxon-methyl indicating the possible use of these enzymes as biomarkers of exposure.

Keywords: Cholin-/carboxylesterase inhibition; Zebrafish embryo; Biomarker; Organophosphate insecticide; Paraoxon-methyl

1. Introduction

Several studies carried out with mammals, birds and fish (WHO, 1993) have demonstrated that anticholinesterase pesticides like organophosphates or carbamates pose significant toxicity to nontarget organisms. As fish are an important component of the food chain and are easily obtained, various toxicity tests have been performed within this group to assess the risk of accidential or unintentional intoxication. The toxic effects of pesticides to fish can vary between acute

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lethality and several different sublethal effects. The sublethal effects include differential inhibition of several enzymes, of which the inhibition of cholinesterases is the most obvious and best known (Weiss, 1959).

Cholinesterases (ChE) are the main known sites of action for organophosphorus and carbamate pesticides (Silver, 1974). Acetylcholinesterase (AChE) cleaves the transmitter acetylcholine. An inhibition of the enzyme thereby interferes with neurotransmission in cholinergic synapses and neuromuscular junctions. AChE also is remarkably important for the neuronal and muscular development (Behra et al., 2002; Hanneman, 1992) or the axon outgrowth (Hanneman and Westerfield, 1989) in the embryos of zebrafish.

The detection of inhibition of cholinesterases by pesticides is accepted as a biomarker of exposure (Walker, 1995). Paraoxon-methyl is the metabolically activated metabolite of the parent substance parathion-methyl and was chosen as the model test substance. It is an organophosphate which forms covalent chemical bonds with the isozymes of the cholinesterase type (acetylcholinesterase (EC 3.1.1.7)) thereby inhibiting it.

To be able to use cholinesterases as biomarkers, the different types of cholinesterases were characterized, which may show different sensitivities to organophosphates (Wogram et al., 2001). Several different isozymes of cholinesterases are known in fish and they are differentiated by their substrate preferences. Because the exact composition of the cholinesterases in the zebrafish is not known and because of the small size of the embryos, different substrates and inhibitors were used to characterize the composition of cholinesterases in whole homogenates of the zebrafish embryo (Silver, 1974).

The inhibition of carboxylesterases (CaE) or B-esterases as alternative phosphorylation sites (Watson et al., 1994) by pesticides is faster than that of cholinesterases (Chambers et al., 1994). Consequently, the cholinesterases might be protected stoichiometrically (Maxwell, 1992; Maxwell and Brecht, 2001) by CaE which are therefore considered an organophosphate toxicity buffer enzyme (Clement, 1984). For this reason a CaE assay was included in the enzyme analysis to examine if CaE might be a more sensitive biomarker of exposure to pesticides (paraoxon-methyl as a representative) than the ChE-isozymes.

In the present study the cholinesterases isozymes and carboxylesterases of the zebrafish in the first 48 hpf of development and the effects of paraoxon-methyl on the enzyme activities were analysed.

2. Materials and methods

2.1. Chemicals

The substrates for the esterases, acetylthiocholine-iodide (ASCh), CAS# 1866-15-5, acetyl-β-(methylthio)choline-iodide (AMSCh), CAS# 1866-17-7, *S*-butyrylthiocholine-iodide (BuSCh), CAS# 1866-16-6, propionylthiocholine-iodide (ProSCh), CAS# 1866-73-5 and *S*-phenylthioacetate (PSA), CAS# 934-87-2 were purchased from ICN Biomedicals (Eschwege, Germany). Triton X-100 and sodiumdihydrogenphosphate-monohydrate were obtained from Merck (Darmstadt, Germany). The inhibitors, *N*,*N*′-diisopropylpyrophosphorodiamidic anhydride (DPDA) (Austin and Berry, 1953) also known as tetramonoisopropylpyrophosphortetramide ("*iso*"-OMPA) CAS# 513-00-8, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51) CAS# 402-40-4, 1′-methylpyrrolidino (2:3:2:3)1,3-dimethylindolin-5-yl *N*-methylcarbamate (*physostigmine* also known as *eserine*) CAS# 64-47-1 were from Sigma–Aldrich, Göttingen, Germany. The chromoagent DTNB (5,5′-dithio bis-2-nitrobenzoate) CAS# 69-78-3 was from Serva (Heidelberg, Germany).

DC Protein Assay was purchased from BioRad (München, Germany) while the bovine serum albumine (BSA) was from Serva (Heidelberg, Germany). The pesticide paraoxon-methyl (CAS# 950-35-6) was a certified pure compound (98% purity) from the company Dr. Ehrenstorfer (Augsburg, Germany). All other chemicals were at least *pro analysi* quality.

2.2. Fish culture and embryo collection

Adult fish obtained from West Aquarium (Bad Harzburg, Germany), recommended in the German fish embryo test guideline (Anonymous, 2001), for the analysis of waste water were kept in glass aquaria (120 L) with activated carbon filtered tap water at 27.5 °C with a density of 5 fish/L and a female to male ratio of 2:1 (Westerfield, 2000). The light/dark regime was set to