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Characterization of cholinesterases in marbled sole, *Limanda yokohamae*, and their inhibition *in vitro* by the fungicide iprobenfos

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Abstract

Cholinesterases (ChEs) have been characterized in marbled sole (*Limanda yokohamae*) for use as a possible biomarker of pollution exposure. In brain, ChEs existed almost exclusively (>95%) as acetylcholinesterase (AChE) whereas in muscle, about 20–30% of ChE activity was in the form of butyrylcholinesterase (BChE; pseudocholinesterase). Acetylthiocholine and butyrylthiocholine (identified in mammalian studies as diagnostic substrates for AChE and BChE respectively) were hydrolyzed mainly, but not exclusively, by these enzymes. The inhibitors BW284C51 and *iso*-OMPA (identified in mammalian studies as diagnostic inhibitors of AChE and BChE respectively) were not specific for these enzymes in marbled sole. Brain AChE and muscle AChE and BChE were characterized in terms of their kinetic properties (K_M etc.) and optimal conditions (substrate concentration, protein concentration, pH etc.) were established to allow routine assays of ChE activity to proceed under pseudo-first order conditions. The sensitivity of ChEs to a locally significant pesticide, iprobenfos (IBP; kitazin) was established in terms of IC_{50} concentrations. Brain AChE was relatively insensitive to IBP, but muscle AChE and BChE were sensitive to IBP concentrations in the high nM range. However, ambient IBP concentrations in Korean coastal waters are usually not high enough to cause detectable ChE inhibition in this species.

Keywords: Acetylcholinesterase (AChE); Butyrylcholinesterase (BCchE); Marbled sole; *Limanda yokohamae*; Iprobenfos; IBP; Kitazin

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1. Introduction

Cholinesterases (ChEs) are enzymes whose main function appears to be to hydrolyze acetylcholine after it has functioned as a neuro-transmitter. They are inhibited by two main groups of pesticides, the organophosphates and the carbamates, which combine covalently with specific amino-acid residues to inactivate the enzyme(s). ChE activity in fish brain was used fairly widely during the 1960's and 1970's as a biomarker to assess the zone of impact during large scale pesticide spraying operations, but its use declined somewhat during the 1980's, perhaps as analytical chemistry methods for organophosphates and carbamates became more accessible. During the 1990's there was a resurgence of interest in the use of ChE as a biomarker as evidence accumulated that ChE activity in fish was inhibited at sites not obviously contaminated by organophosphate or carbamate pesticides (e.g., Galgani et al., 1992; Payne et al., 1996), and although the chemical cause of such inhibition is still unidentified, ChE is being used as a bioassay to identify potentially contaminated sites which can be examined in more detail using the approaches of analytical chemistry (e.g., Kirby et al., 2000).

The ChEs fall into two main groups, the acetylcholinesterases (AChE) and the pseudo- or butyryl-cholinesterases (BChE). Both groups are identified by their activity towards "diagnostic" substrates (acetylthiocholine and butyrylthiocholine, respectively) and their response to diagnostic inhibitors (1,5,*bis*-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide [BW284C51] and tetraisopropyl pyrophosphoramide [*iso*-OMPA] respectively); in addition, the inhibition of both ChEs by the alkaloid eserine identifies them as cholinesterases, as opposed to other or non-specific esterases. AChE provides the primary control of acetylcholine, but the role of BChE is less clear. Fish brain tissue usually contains only AChE activity, but axial muscle has both AChE and BChE activity. Therefore, a careful characterization is essential for the use of fish ChEs in monitoring (Sturm et al., 1999).

Marbled sole, *Limanda yokohamae*, is a demersal marine fish indigenous to the coastal waters of Korea and is commercially important. Sole (in general) feed on benthic organisms in the sediment such as small clams, marine worms, small crabs etc. (Hart, 1973). The species is also an important ecosystem component in some Korean polluted areas such as Masan Bay (Shim et al., 2003; Hong et al., 2003). In this study, we have characterized AChE and BChE in marbled sole for potential use as a biomarker of some pollution effects. Specifically we have examined the inhibition *in vitro* of ChEs by the pesticide iprobenfos (IBP; Kitazin) which is used fairly widely in Asia to control the rice blast fungus. Like most organophosphorus pesticides it can be expected to phosphorylate the –OH group on the serine residue in the active (esteratic) site of ChEs (e.g., Giacobini, 2000) and so inhibit both AChE and BChE activity. IBP has been reported as a contaminant in inshore waters, particularly following applications in late summer (Yu et al., 2001). The model ChE inhibitor paraoxon (a powerful ChE inhibitor derived from the insecticide parathion) was also investigated for comparison.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide, S-butyrythiocholine iodide, *iso*-OMPA, eserine sulphate, BW284C51 and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma