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Altered quantities and *in vivo* activities of cholinesterase from *Daphnia magna* in sub-lethal exposure to organophosphorus insecticides

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ABSTRACT

For investigating relationship between activity of cholinesterase (ChE) and ambient concentration of anticholinesterases, *Daphnia magna* had been exposed for 21 day to sub-lethal concentrations, i.e. 1/6 EC_{50}, 1/36 EC_{50}, and 1/216 EC_{50}, of either triazophos or chlorpyrifos. Samples were taken at different points of time for measuring total activity and immunoreactive content of ChE and actual concentrations of the anticholinesterases. A type of antigen formerly developed by immunizing mice with purified ChE was utilized in this study to establish an indirect non-competitive ELISA for measuring immunoreactive content of ChE in *Daphnia*. Studies showed that for apparent activity, i.e. activity that was scaled with total protein, the insecticides caused 5.2–6.9 percent inhibition and 17.0–17.7 percent inductions during the 21 d exposure, whereas for inherent activity, i.e. activity that was scaled with immunoreactive protein, no induction was detected during the exposure. Accompanied by up to 65.9 percent and 68.0 percent promotion in terms of the immunoreactive content, up to 42.8 percent and 44.6 percent inhibition in terms of the inherent activity was indicated, respectively, for triazophos and chlopyrifos. Judged by measured concentrations, the inherent activity recovered faster than the rate of dissipation of the anticholinesterases. Result of the study suggested that the inherent activity was more sensitive than the apparent one in predicting sub-lethal and/or long-term stress of anticholinesterases. It also suggested that apart from promotion in terms of content of the ChE, the *Daphnia* developed capacities to block bio-concentration of anticholinesterases, and these capacities would make it liable to underestimate ambient concentration of anticholinesterases along with the time of exposure.

1. Introduction

Anticholinesterases such as organophosphorus (OPs) and carbamate (CBs) are among the most commonly used pesticides for insect pests management. Despite the high efficiencies, they tend to be toxic to non-target organisms such as fish and many species of aquatic invertebrates. It is known that the principal mechanism by which OPs and CBs elicit their toxicity comes down to the inhibition of cholinesterase (ChE) (Carvalho et al., 2003). In this sense, a number of studies had been performed to urge the enzyme as a biomarker for prediction of anticholinesterases (Sanchez-Hernandez et al., 1998; Stien et al., 1998; Cajaraville et al., 2000; Den Besten et al., 2001; Abdel-Halim et al., 2006).

Anticholinesterases may affect ChE in multi aspects in vivo. It is common to see promotion in terms of activity of the enzyme in exposures (Frawley et al., 1952; Frawley and Fuyat, 1957; Genina, 1974; Hackenberger et al., 2008), a phenomenon that can be regard as a type of hormesis featured by conversion from low-dose stimulation to high-dose inhibition (Calabrese, 2002; Chapman, 2002). This promotion is probably result from acceleration in terms of synthesis of the enzyme in order to rectify functional disable in condition that part of the enzyme was inhibited. The point is that the anticholinesterase- caused promotion oftentimes disturbs the detection of "actual" inhibition of the enzyme so as to bring about difficulty for determining exposure of anticholinesterases in field (Khattab et al., 1994; Khattab and Ali, 2007). It is necessary, therefore, to develop method for measuring relative content of ChE so as to scale the "actual" activity of the enzyme in exposures. Enzyme-linked immunosorbent assay (ELISA), which combined efficiency and specificity, is capable of achieving the task.

Daphnia magna is a species of zooplankton that belongs to Phylum of *Arthropoda*, Order of *Crustacea,* and Family of *Daphniidae*. It consumes algae and other small-sized phytoplankton and in turn serves as prey for freshwater fish and macro-sized aquatic invertebrates. Due to its world-wide distribution and its adaptability to laboratory culture, *Daphnia* has long been employed as representative species for testing of chemicals (OECD (Organization for Economic Co-operation and Development), 2004, 2008; USEPA (U.S. Environmental Protection Agency), 1996a, 1996b). Some papers had

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been published to reveal relationship between in vivo activity of ChE and ambient concentrations of anticholinesterases (Barata et al., 2001; Printes and Callaghan, 2003; Carvalho et al., 2003; Barata et al., 2004; Damásio et al., 2007; Printes et al., 2008). Results of the studies confirmed that, to behave the same as fish and many other aquatic organisms, *Daphnia* had its enzyme obviously inhibited at concentrations adjacent to the lethal ones, conflict results were obtained, however, in case that the *Daphnia* were exposed to concentrations far below the lethal ones.

Triazophos and chlorpyrifos are two kinds of OPs commonly used nowadays in China and southeast of Asia. Considering the physico-chemical properties and application rates, there is large possibility for them to enter water-sediment systems and impose acute and/or chronic hazard to non-target aquatic organisms. A former study indicated a maximal of 76.5 percent and 60.5 percent promotion in terms activity of the ChE, as the *Daphnia* were exposed, in semi-static condition, to either triazophos or chlorpyrifos for 21 day (Li and Tan, 2010). This suggested accelerated synthesis and consequent promotion in quantity of the enzyme.

In a study conducted with Japanese quail *Coturnix coturnix*, a maximal of 40 percent promotion in quantity of immunoreactive ChE was detected in serum in case the bird was orally administered with monocrotophos of 20 mg kg^{-1} (Khattab et al., 1994). Another study conducted with red-legged partridges *Alectoris rufa cross* revealed a maximal of 26.5 percent rise in quantity of immunoreactive ChE in serum in case the bird was orally administered with malathion of 167 mg kg^{-1} (Khattab and Ali, 2007). Up to of 50 percent increase in quantity of the enzyme was detected in brain of *Psudorasbora parva* in case the fish were exposed to sub-lethal concentrations of triazophos (Li et al., 2005). It is logical, therefore, to infer that anticholinesterases such as triazophos and chlorpyrifos could result in promotion in terms of quantity of the ChE in *Daphnia*.

A type of antigen that was formerly developed by purified ChE from *Daphnia* was utilized in this study for measuring content of ChE in *Daphnia* being exposed for 21 d to various concentrations of either of triazophos or chlorpyrifos. The aim of this study was: (1) to clarify extent and pattern of promotion in terms of quantity of ChE in *Dphnia* along with time and concentrations, (2) to explore relationship between in vivo activity of ChE and ambient concentrations of anticholinesterases. Result of the study would help to learn advantages and disadvantages of in vivo ChE in detecting sub-lethal and/or long-term exposure of anticholinesterases and would finally benefit in situ application of the biomarker.

2. Material and methods

2.1. Reagents

Triazophos (with purity of 96.6 percent) and chlorpyrifos (with purity of 99.5 percent) were obtained from the National Standards Company (Beijing, China). Horseradish peroxidase (HRP) labeled goat anti-mouse immunoglobulin G (IgG) with the enzyme of immunoassay grade purity, Coomassie Brilliant Blue G-250, propionylthiocholine iodide, and bovine serum albumin (BSA, with MW of 67,000) were obtained from Sigma-Aldrich® (Steinheim, Germany). Tramethylbenzidine (TMB), which consists of solution A and B, were purchased from Yingchuang Company (Huzhou, China). Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A12217, which contains Amplex Red reagent, dimethylsulfoxide, HRP, hydrogen peroxide, and choline oxidase from *Alcaligenes sp.*, etc.) were bought from Molecular Probes, Inc. (Eugene, Oregen,USA). Defatted milk was purchased from Shanghai Chemical Reagents Company (Shanghai, China). All of the other chemicals were of analytical grade unless stated specially.

2.2. Daphnia magna

Daphnia magna employed in this study was a pure breed of 62 D.M. obtained from Chinese Center for Disease Control and Prevention (Beijing, China). The breed was cultured in semistatic M_4 medium (Elendt and Bias, 1990) and fed with unicellular algae *Scemedesmus subspicatus* from synchronized culture at a density of 2.0–3.0 × 10^5 cells mL^{-1} *Dapnhia*$^{-1}$ day^{-1}. The water temperature, the pH, the light intension, and the light cycle were set as 22 ± 1 °C, 7.8 ± 0.2, 1500–2500 lx, and 16 h light to 8 h dark, respectively.

2.3. Acute immobilization test

It is essential to perform acute immobilization test to set concentrations for 21d exposure. The test was carried out in accordance with the guideline of OECD (2004) and International Standard Oranization (ISO) (1996). The experiment started with neonates of less than 24 h. Individuals were exposed to either triazophos with nominal concentrations from 5.0 to 25.0 μg L^{-1} (i.e. 5.0, 8.0, 12.0, 20.0, 25.0 μg L^{-1}) or chlorpyrifos from 2.5 to 16.0 μg L^{-1} (i.e. 2.5, 4.0, 6.0, 10.0, 16.0 μg L^{-1}). The dilutions were prepared by spiking acetone solution of various concentrations into the M_4 medium of 40 mL. The spiking rate was 45 μL L^{-1} for triazophos and it was 50 μL L^{-1} for chlorpyrifos. The treatment group (including the solvent control) had five replicates and each of the replicate consisted of five daphnids. The temperature, the pH, the light intension, and the light cycle were set as 22 ± 1 °C, 7.8 ± 0.2, 1500–2500 lx, and 16 h light to 8 h dark, respectively. The number and state of the individuals were recorded at 48 h after start of the exposures.

2.4. Chronic exposure

The dilutions were obtained by spiking acetone solutions of various concentrations into the M_4 medium of 400 mL. The spiking rate was 13 μL $L.^{-1}$ Concentration of the dilutions were set to be 1/6, 1/36, and 1/216 of 48 h EC_{50} of two the anticholinesterases, respectively, which supposed to influence activity of the enzyme but caused no mortality to daphnids. Ten replicates were set for each concentration including the solvent control. Each replicate consisted of about 100 *Daphnia* of 14 d old. The *Daphnia* were kept in 22 ± 1 °Cunder a regime of 16 h light to 8 h dark. The feed (i.e. *Scemedesmus subspicatus* from synchronizing culture) were provided once a day during the exposure. The *Daphnia* were taken randomly from each of the ten replications at 2nd, 6th, 10th, 14th, 18th, and 21st d after dosing, respectively. Those that were not able to be analyzed timely were stored at −80 °C. Maximal duration of the storage was 23 d.

2.5. Enzyme activity measurement

2.5.1. Crude enzyme preparation

The collected *Daphnia* were homogenized in 500 μL ice-cold Tris–HCl buffer (pH 7.5) that contained 0.25 percent (V/V) Triton X-100, 1 mM EDTA, and 50 mM Tris. The homogenates were centrifuged for 30 min at 4 °C in acceleration of 10,000 *g*. The supernatants were collected as source of the crude enzyme.

2.5.2. Protein content determination

Protein concentration of crude enzyme was quantified in accordance with the method of Bradford's (1976) using bovine serum albumin (BSA) as the reference. Light absorbencies were measured with an ultraviolet-visible spectrophotometer (UV–vis-7504PC, a product of Xinmao Co., Ltd., Shanghai, China).

2.5.3. Measurement of total activity

The total activity of ChE was measured by a relatively sensitive method previously described by Zhou et al. (2000) using propionylthioncholine iodide as substrate. That was to pipe ChE-containing sample (100 μL) into a well of the polystyrene black fluorescence microplate (High Binding Plates, Catalog no. 3925, Corning, Cambridge, MA, USA). The reaction was started by adding 100 μL working solution of Amplex Red reagent/ HRP/ choline oxidase/ propionylthioncholine iodide that contained 2 U mL^{-1} HRP, 0.2 U mL^{-1}choline oxidase, and 100 μM propionylthioncholine iodide. The microplate was incubated for 30 min in dark at room temperature (about 25 °C). The Reaction Buffer employed to dilute the samples and the working solution of Amplex Red reagent/HRP/choline oxidase/ propionylthioncholine iodide was 50 mM Tris–HCl with pH of 8.0. The fluorescence was measured by a fluorescence microplate reader (Tecan Genios Plus, Tecan Trading Co., Ltd. Shanghai, China) at excitation of 530 nm and emission of 590 nm.

Positive control was prepared by diluting the eel acetylcholinesterase of 100 U mL^{-1} with the Reaction Buffer of 1X to produce a working solution of 0.2 U mL^{-1}. Negative control was prepared using the Reaction Buffer of 1X instead of the enzyme.

Total activity of the enzyme was calculated by follow formula:

$$\text{Total activity} = [(F_x - F_0) \times 0.2]/(F_1 - F_0)$$

where, F_1 was fluorescence of the positive control, F_0 was fluorescence of the negative control, and F_x was fluorescence of a sample.