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Measurements of cholinesterase activity in the tropical freshwater cladoceran *Pseudosida ramosa* and its standardization as a biomarker

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ABSTRACT

The activity of cholinesterases (ChE) has been recognized as a useful tool for assessing the toxicity in the environmental assessment programs. Nevertheless, the prior optimization of the experimental conditions for the appropriate measuring of the ChE activity enables us to get reliable results. Thus, the main objective of this study was to adapt and optimize a microplate assay for measuring the activity of ChE in the tropical cladoceran *Pseudosida ramosa*. The best readings for the reaction rates were obtained with buffers of pH 8.0 and molarity of 0.02 M. The measurements of the reaction rates for the different substrate concentrations showed that the maximum reaction rate (32 mOD min^{-1}) was achieved by the final concentration of 2 mM of substrate. In relation to the enzyme concentration, reaction rates were directly proportional to the protein concentration, which confirmed the linear kinetics for a maximum reaction rate. On the basis of the results of the assays for the effect of the number of individuals and homogenate dilution on the reaction rate of substrate hydrolysis and ChE activity, we recommend using of 30 individuals (3 days-old) in 250 μL of buffer, 20 individuals (7 days-old) in 250 μL of buffer and 15 individuals (both 14 and 21 days-old) in 300 μL of buffer. The limits of quantitation obtained were 1.419 mOD min^{-1} ($\leq$72 h-old), 1.670 mOD min^{-1} (7 days-old), 0.943 mOD min^{-1} (14 days-old) and 0.797 mOD min^{-1} (21 days-old). In conclusion, it was possible to measure the ChE activity in *P. ramosa* with the methodology adapted, thus contributing to the implementation of a biochemical biomarker in freshwater toxicity assessments in tropical regions.

1. Introduction

Widespread concern about the impact of chemical in the aquatic environment has instigated the development of sensitive and ecologically relevant methods to predict and monitor their toxic effects (Rand, 1995). The employment of biochemical biomarkers to assess the risks of exposure to low concentrations of toxicants has evidenced potential for application in this field (Timbrell et al., 1996).

Measurements of cholinesterase (ChE) activity have been successfully used to monitor the effects of anti-cholinesterase toxicants (Day and Scott, 1990; Fisher et al., 2000; Guilhermino et al., 1996a; Printes and Callaghan, 2003; Xuereb et al., 2009). Such toxicants inhibit the enzyme acetylcholinesterase (AChE), which is responsible for the hydrolytic degradation of acetylcholine in the synaptic cleft (Domingues et al., 2010; Jemec et al., 2007; Peakall, 1992; Varó et al., 2002). Once the AChE is inhibited by anti-cholinesterase toxicants, there is an overstimulation of the central and peripheral nervous system, resulting in deleterious effects on the organism, which may even be leading to death (Xuereb et al., 2009).

Freshwater invertebrates play a critical role in aquatic toxicology, due to its ecological importance in the food chains, their high susceptibility to pollutants and their use as test organisms in ecotoxicological assessments (Sturm and Hansen, 1999). In particular, in relation to the cladocerans, *Daphnia* species are among the most common animals currently used in assessment programs of freshwater environments (ABNT, 2009; OECD (Organization for Economic Cooperation and Development), 2004, 2008; U.S. EPA (U.S. Environmental Protection Agency), 2002). In the temperate regions, methods for measuring ChE activity in *Daphnia magna* are

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fairly well established (Day and Scott, 1990; Guilhermino et al., 1996a, 1996b; Printes and Callaghan, 2003; Sturm and Hansen, 1999); however, in tropical regions, there is so far no record in the literature of protocols for measuring the ChE activity in cladocerans species native of this region.

Since the toxic responses of the organisms to the chemicals are often species-specific, the use of temperate species in tropical regions for the ecotoxicological assessments has been considered inappropriate by some authors (Do Hong et al., 2004; Freitas and Rocha, 2012). Thus, the main objective of this study was to adapt and optimize a microplate assay developed by Fisher et al. (2000) and Printes (2003), which were modified from Ellman et al. (1961), for measuring the activity of ChE in tropical cladoceran *Pseudosida ramosa*. The cladoceran *P. ramosa* was shown to be a good substitute to species *D. magna* in standard ecotoxicological assays (i.e., acute and chronic toxicity tests) (Freitas and Rocha, 2011a, 2011b, 2011c, 2012), besides having a wide geographical distribution in the tropical and subtropical regions (Elías-Gutiérrez et al., 2001; Korovchinsky, 1992; Maiphae et al., 2005; Morrone and Coscarón, 1998; Rey and Vasquez, 1986; Roa and Vasquez, 1991; Sanoamuang, 1998), including Brazil (Brandorff et al., 1982; Elmoor-Loureiro, 2007; Freitas and Rocha, 2006; Lansac-Tôha et al., 2009; Neves et al., 2003; Rocha and Güntzel, 1999).

2. Material and methods

2.1. Organisms and culture conditions

P. ramosa was originally collected from the Óleo Lake (21°20′–21°55′S and 47°35′–47°55′W), an oxbow lake in the Mogi-Guaçu River flood plain, Jataí Ecological Station, district of Luis Antônio, São Paulo State, Brazil, and was used to establish a culture that has been maintained in our laboratory since 2004. One ovigerous female was isolated to start a clonal culture of this cladoceran species.

Each *P. ramosa* culture consisted of one single individual in a 50 mL beaker filled with 30 mL of soft reconstituted water (or ASTM medium) as recommended by the American Society for Testing and Materials (ASTM (American Society for Testing and Materials), 2001). The ASTM medium was prepared with 0.03 g L^{-1} of $CaSO_4 \cdot 2H_2O$; 0.061 g L^{-1} of $MgSO_4 \cdot 7H_2O$; 0.048 g L^{-1} of $NaHCO_3$ and 0.002 g L^{-1} of KCl dissolved in 1 L of distilled water and had a total hardness of 40–48 mg $CaCO_3$ L^{-1}, a pH range of 7.0–7.6 and an electrical conductivity around 160 μS cm^{-1}.

The cultures were started with juveniles ($\leq$24 h-old), which were isolated from parthenogenetic mothers. Only juveniles from the 3rd to the 5th brood were utilized for establishing new cultures and for performing the ChE assays. In general, 140 individual cultures were kept simultaneously.

The ASTM medium was renewed three times weekly. The culture conditions were: temperature of 25 ± 2 °C, photoperiod of 12 h L: 12 h D and fed with the green algae *Pseudokirchneriella subcapitata* (1×10^5 cells mL^{-1}) combined with a suspension of 0.05 mg L^{-1} (dry wt) of dried baker's yeast daily, as recommended by the U.S. EPA (U.S. Environmental Protection Agency) (2002).

The algae *P. subcapitata* was cultured in CHU-12 medium (Müller, 1972), which was previously autoclaved at 121 °C and 1 atm for 20 min. The temperature during culturing was 25 ± 2 °C with a photoperiod of 12 h L:12 h D and the culture medium was gently aerated. For food preparation, the algal cells were centrifuged at 352*g* for 10 min at temperature room. The supernatant was discarded and the cells were re-suspended in ASTM medium to remove culture nutrients. The suspensions of algae and dried baking yeast were kept at 4 °C and used within a maximum time interval of one week.

Monthly, the health and sensitivity of the cultures were evaluated by acute toxicity tests with the reference substance sodium chloride. For *P. ramosa*, the range of sensitivity to sodium chloride is between 1280 and 1480 mg L^{-1}, with a mean value of 1380 mg L^{-1} (Freitas and Rocha, 2011b). The 48-h LC_{50} to sodium chloride obtained in our study remained between the upper and lower limits of the range of sensitivity (ranging from 1310 to 1450 mg L^{-1}).

2.2. Treatment of the organisms for their use in the ChE assays

For ChE assays, juveniles of *P. ramosa* (±24 h-old) were obtained from individual cultures. Neonates were separated in 2000 mL beakers filled with 1800 mL of ASTM medium, being the population density of 35 individuals per liter. They were maintained in the same culture conditions (see Section 2.1) until completing 72 h-, 7 days-, 14 days- or 21 days-old, as specified for each particular experiment. The only exception was that no food was provided for the animals with 72 h-old. After the individuals reached the specified ages, they were dried on absorbent paper to remove all the ASTM medium, transferred to 1.5 mL micro-centrifuge tubes and snap-frozen by immersion in liquid nitrogen. The juveniles and adults were stored for up to 3 months at −80 °C before the ChE assays.

2.3. General description of the method of ChE assays

The adapted method for measuring the ChE activity in homogenates of *P. ramosa* was based on Fisher et al. (2000) and Printes (2003), which were modified from Ellman et al. (1961). This method has been successfully used to estimate the ChE activity in cladocerans, especially in *Daphnia* species (Printes and Callaghan, 2003, 2004; Printes et al., 2008).

In this method, an artificial substrate acetylthiocholine iodide (ATCI) is broken down by cholinesterase producing acetyl and thiocholine. The yellow color is produced when thiocholine reacts with 5,5′-dithio bis-2-nitrobenzoate (DTNB). The enzyme activity is measured by spectrophotometric detection. The molar extinction coefficient of this colored product is known and ChE activity can be calculated. To rationalize the activity expression by the normalization of the protein content, the protein concentration in the homogenate is estimated using the bicinchoninic acid (BCA) kinetic protein assay (Pierce, Rockford, IL, USA) (Smith et al., 1985) as described in previous works (Callaghan et al., 2002; Fisher et al., 2000; Printes, 2003; Printes and Callaghan, 2003).

Whole animals of *P. ramosa* (the number of the individuals was specified in each particular experiment) were homogenized in 1.5 mL micro-centrifuge tubes with homogenizing buffer (ice-cold 0.02 M sodium phosphate buffer pH 8.0 (defined in a previous assay) with 1 percent Triton X-100, Sigma). The homogenization was performed manually using a microcentrifuge tube pestle for this purpose (40 cycles, 10 s) and it was carried out on ice in a room at a temperature of 18 °C. To the initial homogenate, ice-cold 0.02 M sodium phosphate buffer pH 8.0 was added, whirlimixed and centrifuged at 14,000*g* and 2–4 °C for 4 min. Supernatants were subsequently transferred to clean pre-cooled micro-centrifuge tubes, whirlimixed, and assayed immediately. Additions to the microtiter plate were made in the following order: 100 μL of 8 mM DTNB (D-8130, Sigma) in sodium phosphate buffer pH 8.0 supplemented with 0.75 mg mL^{-1} of sodium hydrogen carbonate; 50 μL of blank buffer (sodium phosphate buffer pH 8.0 containing 0.1 percent Triton-X-100, Sigma) or 50 μL supernatant; and 50 μL of 16 mM ATCI (A-5751, Sigma) in sodium phosphate buffer pH 8.0. The microtiter plate was inserted into the integral incubator of a Dynex MRX microtiter plate reader (DYNEX Technologies, USA) and incubated at 30 °C for 5 min. This was followed by the measurement of reaction rate (ΔOD min^{-1}) at 405 nm and 30 °C over a 10 min period with intermittent shaking among each reading cycle. Twenty-one cycles of measurement were performed.

The activity of enzyme in μmol min^{-1} g^{-1} protein was calculated by the following equation:

$$\text{Activity} = (\Delta\text{OD min}^{-1})/(\text{MEC} \times C),$$

where ΔOD min^{-1} is the variation of optical density in the time; MEC is 8160, molar extinction coefficient of the colored product at 405 nm; and *C* is the supernatant protein concentration in the assay (g L^{-1}).

The normal reaction rate of the blank assay was up to 3 mOD min^{-1}.

2.4. Protein concentration

The protein standard curve was prepared with a series of bovine serum albumin (BSA) (B14046, Pierce), with standards diluted in blank buffer with pH 8.0. The concentrations of BSA used in this assay were based on Printes (2003) and they were: 5, 25, 50, 125, 250, and 500 μg mL^{-1} for juveniles with 72 h-old; 5, 25, 50, 125, 250, 500, 750, 1000, and 2000 μg mL^{-1} for individuals with 7 days- and 14 days-old; and 5, 25, 50, 125, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and 5000 μg mL^{-1} for adults with 21 days-old. The working reagent was prepared in a 50 mL screw cap tube by mixing 500 μL of Reagent B (23224, Pierce) with 25 mL of Reagent A (23223, Pierce) (Fisher et al., 2000; Printes, 2003; Smith et al., 1985). It was then pre-heated in a water bath at 30 °C for 10 min. Additions to the microtiter plate were made in duplicate in the following order: 20 μL of the BSA standards or 20 μL of supernatants and 200 μL of the working reagent. After the additions, the plate was immediately placed into the Dynex MRX microtiter plate reader and measurements were taken using the kinetics mode. Twenty-one measurements of absorbance at 540 nm were taken at 30 s intervals interspersed by shaking to mix the reaction. Protein content was determined by calculating the average slope of the reaction and relating it to the standard bovine serum albumin protein concentrations. The normal reaction rate of the assay blank was 0.1 to 0.9 mOD min^{-1}.

2.5. ChE assay conditions

2.5.1. Effect of pH and molarity on measured reaction rate in the ChE assays

The effect of pH on the reaction rate of acetylthiocholine iodide (ATCI) hydrolysis (mOD min^{-1}) was evaluated. Forty neonates of *P. ramosa* ($\leq$72 h-old)