Bull. Environ. Contam. Toxicol. (2003) 71:219–225

DOI: 10.1007/s00128-003-0153-7

Bulletin of Environmental Contamination and Toxicology

Characterization of Cholinesterases from *Daphnia magna* Straus and Their Inhibition by Zinc

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Received: 2 November 2002/Accepted: 6 May 2003

Daphnia magna is widely used as test organism in aquatic toxicology (Guilhermino et al. 2000). The activity of cholinesterases (ChE) has been used as a biomarker in toxicity tests with several species (Sturm et al. 2000), including *Daphnia magna* (Guilhermino et al. 1996). However, as far as we know, the biochemical characterization of the ChE present in the soluble fraction of whole body homogenates of *D. magna* has not been performed before. One of the main difficulties of using enzymatic alterations in toxicity studies lies in the need to characterize the enzymes present in the fraction used and to know the variability that is considered "normal" in the selected model (Bocquené et al. 1990; Varó et al. 2002).

ChE have been divided in two types, acetylcholinesterase (AChE) and butyrylcholinesterase or pseudocholinesterase (PChE) (Eto, 1974) according to the characteristics of the mammalian enzymes. AChE and PChE may be distinguished by distinct substrate specificity and different sensitivity toward specific inhibitors. AChE shows a high rate of hydrolysis when acetylcholine is used as substrate, a relatively lower hydrolysis rate when propionylcholine is used and a very low activity with butyrylcholine (Vellom et al. 1993). Furthermore, it is inhibited by high concentrations of substrate (Eto, 1974). Eserine sulphate is a potent inhibitor of ChE (Eto, 1974), iso-OMPA a strong inhibitor of PChE but not of AChE (Barahona and Sanchéz-Fortún, 1999) and BW284C51 a selective inhibitor of AChE (Xu and Bull, 1994).

Zinc is one of the most common metals that occur as a contaminant in industrial effluents (Mance, 1987). Cladocerans are sensitive to this metal and natural populations may be affected by the exposure to relatively low concentrations of this metal in water (Canizares-Villanueva et al. 2000).

The objectives of this study were two-fold: i) to characterize the soluble ChE present in the total body of *D. magna* juveniles using different substrates and selective inhibitors; ii) to evaluate the *in vivo* and *in vitro* effects of zinc on *D. magna* ChE.

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MATERIALS AND METHODS

Parent animals were cultured in ASTM hard water (ASTM, 1980) with an organic additive (Baird et al. 1989), in groups of 10 animals per 1000 ml of medium, and fed with the algae *Chlorella vulgaris* (0.322 mg carbon/daphnia/day). The photoperiod was 16 h light: 8 h dark and the temperature was 20°±1°C.

The homogenates of *D. magna* were prepared as described in Guilhermino et al. (1996).

To characterize the soluble ChE present in total body homogenates of *D. magna*, enzyme substrate specificity were investigated by determining the enzymatic activity at increasing concentrations of acetylthiocholine, butyrylthiocholine and propionylthiocholine (0.005 to 16 mM), in independent experiments. Km and Vmax values were determined by Linewaver-Burk reciprocal plots using the linear part of the curves. Three replications of each experiment were performed. In addition, the sensitivity of the enzyme to specific inhibitors was studied.

Eserine sulphate (0.0125-0.2 mM), iso-OMPA (tetraisopropyl pyrophosphoramide) (0.5-8 mM) and BW284C51 (1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide) (0.0078-0.25 mM) were used in this study as selective inhibitors of ChE, PChE and AChE, respectively. Eserine sulphate was prepared in ultrapure water and both iso-OMPA and BW284C51 in ethanol. In each experiment with the selective inhibitors, homogenate samples (495 µl) were incubated at 20°C with 5 µl of each test solution. Ultra-pure water (5 µl) was added to control. An additional control, incubated with 5 µl of ethanol was included in the experiments with iso-OMPA and BW284C51. Three replicates per treatment were used. The activity of ChE was determined in triplicate, by the Ellman method (Ellman et al. 1961), adapted to microplate (Guilhermino et al. 1996). The protein content of the samples was determined according to the Bradford technique (Bradford, 1976) with the modifications described by Guilhermino et al. (1996). The activity of ChE in each sample was presented as the mean of the three determinations performed and was expressed in Units (U) per milligram of protein (1U=1 nM of substrate hydrolyzed per minute). A Labsystems Multiskan microplate reader was used.

The *in vitro* ChE inhibition test with zinc chloride was performed as described in Diamantino et al. (2000). The incubation procedure, the activity of ChE and the protein content of the samples were performed and determined as described above for the tests with the selective inhibitors. A water control and five nominal concentrations of zinc were used (12.5, 25, 50, 75 and 100 mg/L).

The *in vivo* ChE inhibition test with zinc chloride was carried out according to Guilhermino et al. (1996) with the following modifications: 60 animals were used per treatment, in groups of 20 per 1000 ml of test solution. Neonates were cultured for 48 h. Preparations of homogenates and determinations of ChE activity and protein content were performed as described above.

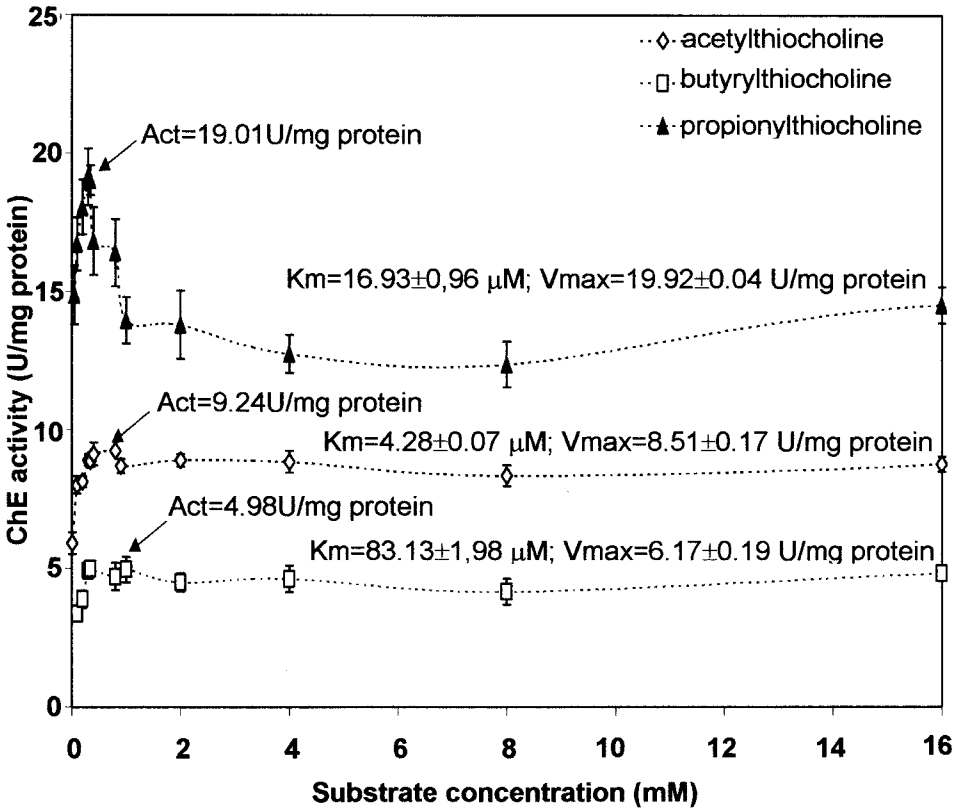

Figure 1. Activity of *D. magna* ChE as a function of increasing acetylthiocholine, butyrylthiocholine and propionylthiocholine concentrations. Results are expressed as the mean ± SE of 3 samples.

Previous experiments (results not shown), indicated that the concentration of zinc chloride in ASTM hard water did not significantly change during 48 hours. Therefore, nominal concentrations were used in this study.

Aceylthiocholine iodide, butyrylthiocholine iodide, propionylthiocholine iodide, iso-OMPA, eserine sulphate, acid dithiobisnitrobenzoate, bovine γ-globulin's were purchased from Sigma. Bradford reagent was from Bio-Rad, and all the other chemicals were from Merck.

The Michaelis-Menten constant (Km) and the maximum velocity of substrate hydrolysis (Vmax) for each substrate were calculated from reciprocal plots of reaction velocity and substrate concentration (Linewaver-Burk representation). The 50% inhibition concentration (IC50) and the 50% effective concentration (EC50) were determined by Probit analysis. Data from ChE activity tests were analyzed using one-way analysis of variance; the no-observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were determined by Dunnett's test. The significance level was 0.05.

Table 1. Effect of selective inhibitors on *D.magna* ChE.

Chemical	NOEC (mM)	LOEC (mM)
Eserine sulphate	<0.0125	0.0125
iso-OMPA	0.5	1
BW284C51	<0.0078	0.0078

NOEC – no observed effect concentration, LOEC – lowest observed effect concentration

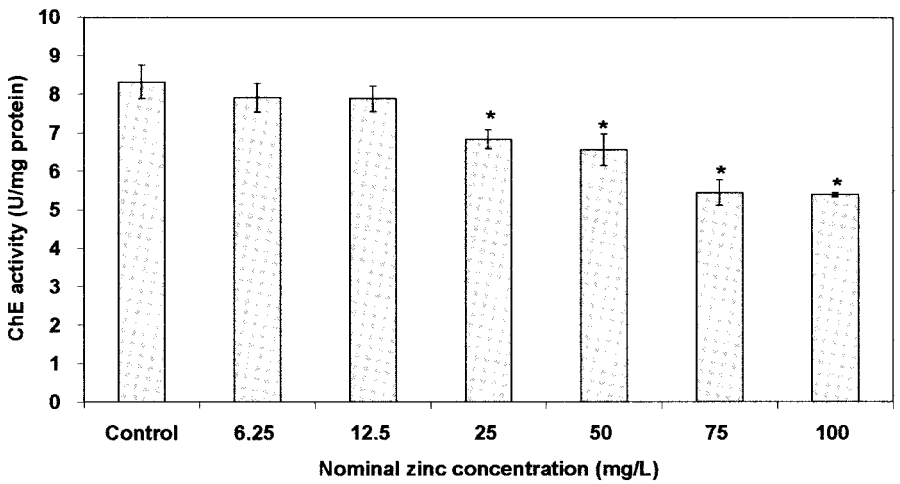

Figure 2. *In vitro* effects of zinc on ChE activity. Results are expressed as the mean ± SE of 3 samples. * Significantly different from control (P<0.05)

RESULTS AND DISCUSSION

D. magna ChE showed preference for propionylthiocholine at all the concentrations of substrate tested (Figure 1). The highest hydrolysis rate was obtained with 0.3 mM of propionylthiocholine. Inhibition at higher substrate concentrations was observed for propionylthiocholine.

The values of Km for the three substrates decreased in the following rank order: butyrylthiocholine, propionylthiocholine and acetylthiocholine. The highest Vmax value was obtained with propionylthiocholine followed by acetylchiocholine and butyrylthicholine. These findings suggest that *D. magna* ChE shows characteristics of a pseudocholinesterase, since it prefers propionylthiocholine to acetylthiocholine. However, like mammalian AChE, it is inhibited by high concentrations of substrate.

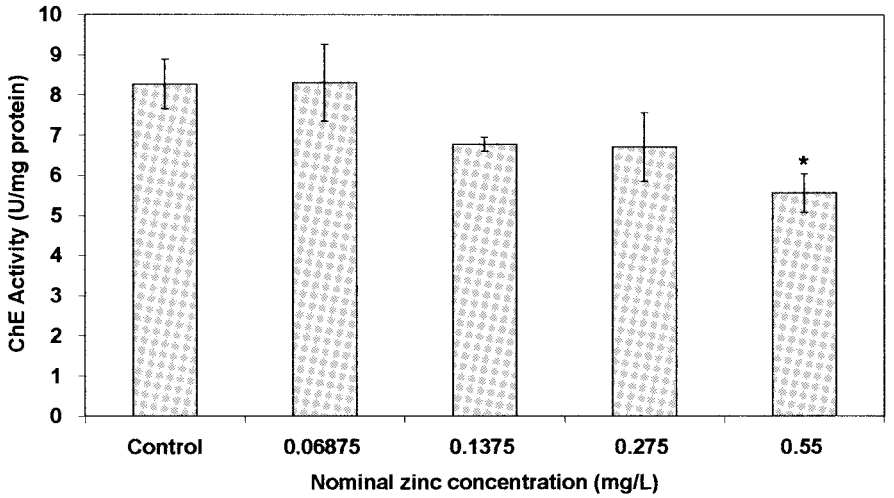

Figure 3. *In vivo* effects of zinc on ChE activity. Results are expressed as the mean ± SE of 3 samples. * Significantly different from control ($P<0.05$)

Eserine sulphate completely inhibited ChE activity ($F=723.8$; d.f. = 5, 12; $P<0.05$; LOEC=0.0125 mM) (Table 1). This result indicates that the enzyme assayed in the experimental conditions used is (are) cholinesterase(s). Both iso-OMPA ($F=9.345$; d.f. = 6, 14; $P<0.05$; NOEC=0.5 mM; LOEC=1 mM) and BW284C51 significantly inhibited the enzymatic activity ($F=415.1$: d.f. = 7, 16; $P<0.05$; LOEC=0.0078 mM).

Therefore, the experiments with selective inhibitors also indicate that *D. magna* ChE shows characteristics of both PChE and AChE, since, like PChE, it is sensitive to iso-OMPA and, like AChE, it is sensitive to BW284C51. Consequently, the results obtained in this study indicate *D. magna* ChE can not be classified as AChE or PChE because it shows characteristics of both enzymes. In the last years, untypical enzymes have been found by different authors in several species of invertebrates and fish (Bocquené et al. 1997; Sturm et al. 2000; Varó et al. 2002).

Zinc significantly inhibited ChE activity in *in vitro* conditions ($F=12.72$; d.f. = 5, 56; $P<0.05$). The NOEC value was 12.5 mg/L and the LOEC was 25 mg/L (Figure 2). Although the concentrations of zinc tested are expected only to occur in heavily polluted environments, these findings clear show that the enzyme is sensitive at these concentrations of this environmental contaminant.

The 24 and 48 h LC50 values for zinc reported in the literature are 2.3 mg/L (95% CL 2.1-2.5) and 0.8 mg/L (95% CL: 0.7-0.9), respectively (Diamantino et al. 2001). These values are similar to the corresponding LC50 reported in the literature (Baird et al. 1991; Guilhermino et al. 1997). *In vivo* exposure to zinc

significantly inhibited ChE activity ($F=3.355$; d.f. = 4, 40; $P<0.05$). The NOEC value for ChE depression was 0.275 mg/L and the LOEC was 0.550 mg/L. EC50 was 1.008 mg/L (95% CL: 1.002-1.013) (Figure 3). Thus, a significant *in vivo* inhibition of ChE was observed at concentrations lower than LC50 values, indicating that this test is suitable and sensitive even for agents that are not considered to be specific ChE inhibitors.

Acknowledgments. This work was partially founded by the European Commission (project TROCA – WET, contract IC18 CT98 0264) and by Fundação para a Ciência e a Tecnologia of Portugal (PRAXIS XXI – BD/2872/94; project CETERA, contract PRAXIS/C/MGS/10200/1998).

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