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Acetylcholinesterase of *Mytilus galloprovincialis* LmK. Hemolymph: A Suitable Environmental Biomarker

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Marine bivalves, such as the mussel *Mytilus galloprovincialis*, are extensively used as biological indicators of marine pollution in biomonitoring programs. As filter feeders, these animals are able to accumulate in their tissues a wide range of chemical contaminants present in seawater. In addition, they are semi-sessile and euryhaline organisms with a wide geographical distribution, characteristics that make them particularly suitable organisms for investigating the biological impact of pollutants (Viarengo and Canesi 1991).

Biomarkers, like the inhibition of the activity of cholinesterases (ChE), have been widely used in monitoring studies of the aquatic environment. Haemolymph is a suitable biological material to be used in biomonitoring programs since it allows the determination of several parameters, including the activity of ChE, in a non-destructive way. Due to the fact that some species of mollusks have polymorphic ChE (Bocquené et al. 1997, Talesa et al. 1993), and distinct forms may show different sensitivity to anticholinesterase agents, their use as a biomarker of exposure to neurotoxic compounds requires the biochemical characterization of the forms present in the species and in the tissue to be studied (Garcia et al. 2000). In order to standardize the conditions for the use of ChE from *Mytilus galloprovincialis* haemolymph as environmental biomarker, the objectives of this work were: (a) to characterize the ChE presented in haemolymph using different substrates (acetylthiocholine, butyrylthiocholine and propionylthiocholine) and specific inhibitors (eserine, BW284C51 and iso-OMPA), (b) to study the influence of the protein content of the samples used for ChE determinations and (c) to determine the normal range of activity in non-exposed individuals.

MATERIALS AND METHODS

Specimens of the marine mussel *Mytilus galloprovincialis*, with a shell length of 30-35 mm, were collected from the intertidal zone of a non-polluted beach, Vila Chã, localized on the northern coast of Portugal. They were maintained in the laboratory for 24 hours prior to the experiments at thermostatic temperature of 20 ± 1°C with continuous aeration. The biological material, haemolymph, was collected with a syringe and diluted (1:4) with ice-cold phosphate buffer (0.1M, pH=7.2), as described in Guilhermino et al. (1998). With exception of the

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experiment where the effect of protein content on ChE activity was investigated, the samples of diluted haemolymph were normalized to 0.5 mg of protein per ml, kept on ice and used on the same day to prevent alterations during storage.

In order to characterize the soluble cholinesterases present in haemolymph, different substrates and specific inhibitors were used. To investigate the substrate preferences of *Mytilus galloprovincialis* haemolymph cholinesterases, the activity of ChE was determined at increasing concentrations (from 0.01 to 20.5 mM) of acetylthiocholine, butyrylthiocholine and propionylthiocholine, in independent experiments. All the other experiments were performed using the substrate acetylthiocholine (0.4 mM) as described in Guilhermino et al. (1996). Eserine sulfate, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one (BW284C51) and *N,N'*-diisopropylphosphorodiamic acid (iso-OMPA) were used in this study as specific inhibitors of ChE, AChE and BuChE, respectively (Barahona and Sánchez-Fortún 1999; Garcia et al. 2000). For each chemical, six concentrations were prepared (ranging from 0.006 to 0.2 mM for eserine and BW284C51 and from 0.25 to 8.0 mM for iso-OMPA) in ultra-pure water or in ethanol, as appropriate. In each experiment, samples of diluted haemolymph (495 µl) were incubated, at 25ºC, with 5 µl of each chemical concentration for 30 minutes. Ultra-pure water (5 µl) was added to controls. An additional control, incubated with 5 µl of ethanol, was included in the experiments with iso-OMPA. ChE activity was determined immediately after the end of the incubation period. In each experiment, three replicates per treatment were performed.

To test the effect of the protein content on ChE determinations, samples containing 0.1 to 0.6 mg of protein per ml were prepared from different mussels and used to measure enzyme activity. The range of ChE activity in non-exposed animals was determined by measuring the activity of the enzyme in 95 samples prepared from different mussels.

The activity of acetylcholinesterase was determined in triplicate, at 25ºC, by the Ellman method (Ellman et al. 1961) adapted to microplate, as described in Guilhermino et al. (1996), using 50 µl of sample and 250 µl of the reaction solution and a wavelength of 414 nm. The reaction solution [phosphate buffer 0.1 M + 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) 10 mM + substrate] was used as blank. The protein content of the samples was determined, in triplicate, by the Bradford method (Bradford 1976) adapted to microplate, using γ- bovine globulin´s as standard and a wavelength of 600 nm. A Labsystem Multiskan EX microplate reader was used. The activity of ChE was expressed in Units (U) per mg of protein (1U is a nmol of substrate hydrolysed per minute).

All the chemicals used in this study were purchased from Sigma (USA), except the Bradford reagent, which was purchased from Bio-Rad (Germany).

Data were analyzed by the Analysis of Variance (ANOVA), one way or nested design (Zar 1996). Tukey multirange test was used in order to determine the no-observed effect concentration (NOEC) and the lowest observed effect

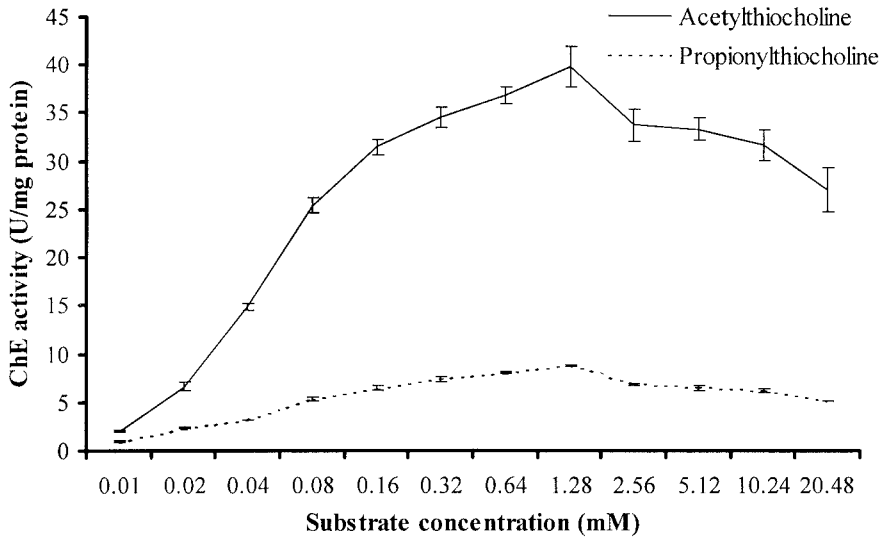

Figure 1. Effect of substrate concentration on ChE activity of *M. galloprovincialis* haemolymph. Results are expressed as the mean ± SE of three replicates (three enzymatic determinations per replicate).

concentration (LOEC) values (Zar 1996). The 50% inhibition concentration (IC_{50}) was estimated using the probit method (Finney 1971). The significance level was 0.05.

RESULTS AND DISCUSSION

In order to investigate the substrate preferences of *Mytilus galloprovincialis* haemolymph ChE, three substrates were assayed: acetylthiocholine, propionylthiocholine and butyrylthiocholine. The activity of ChE of *M. galloprovincialis* haemolymph as a function of increasing concentrations of acetylthiocholine and propionylthiocholine is showed in figure 1. The highest activity (39.9 ± 2.1 SE U/mg protein) was found with 1.28 mM of acetylthiocholine. A decrease of activity was observed when propionylthiocholine was used as substrate (8.8 ± 0.04 SE U/mg protein at 1.28 mM) and no significant activity was found when the substrate used was butyrylthiocholine. Furthermore, a reduction of ChE activity was observed at concentrations higher than 1.28 mM.

The effects of eserine and BW284C51 on ChE activity of *M. galloprovincialis* haemolymph is presented in figure 2. Eserine fully inhibited the enzymatic activity even at the lowest concentration tested (0.006 mM) (Nested ANOVA; $F=1158,9$; d.f.=6, 8; $P<0.05$). BW284C51 significantly inhibited the enzyme activity (Nested ANOVA; $F=74.06$, d.f.=6, 8; $P<0.05$). LOEC value for this compound was 6.3 μM and the IC_{50} was 0.173 μM (95% confidence limits were 0.045-0.659 μM). No significant inhibition of the activity was observed with the compound iso-OMPA up to the highest concentration tested (8 mM) (Nested ANOVA; $F=0.207$; d.f.=7, 9; $P>0.05$).

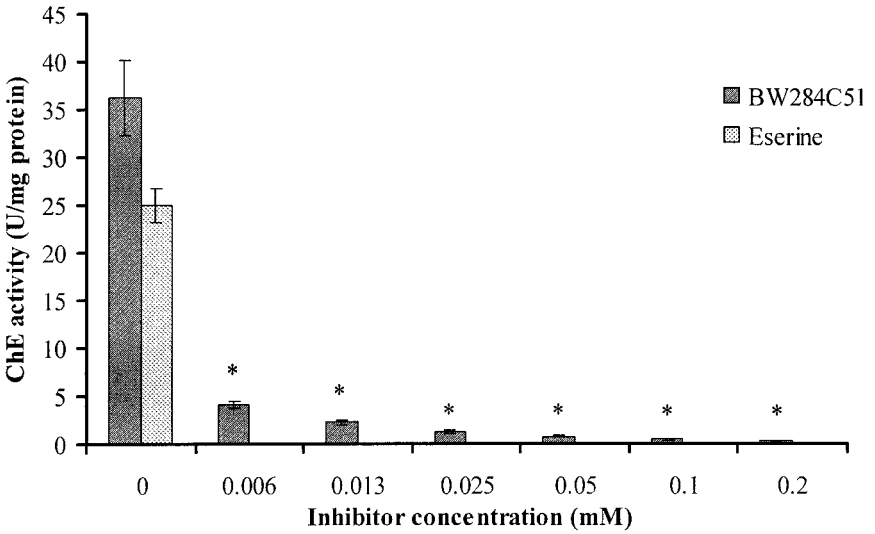

Figure 2. Effect of BW284C51 and eserine on ChE activity of *M. galloprovincialis* haemolymph. Results are expressed as the mean ± SE of three replicates (three enzymatic determinations per replicate; * significantly different from control ($P<0.05$).

Low concentrations (10^{-5} M range) of the carbamate eserine induce the inhibition of ChE (Eto 1974). This fact allows to distinguish ChE from non-specific esterases, which are not inhibited by this compound. In biomarker studies, this knowledge is very important since tissues may contain significant amounts of non-specific esterases, which contribute to the measured activity and may be more or less sensitive than ChE to anti-cholinesterases chemicals (Garcia et al. 2000). In the present study, the enzymatic activity of the mussel haemolymph was completely inhibited by eserine even at the lowest concentration tested, indicating that the enzyme(s) measured is (are) ChE.

ChE present in haemolymph showed a higher activity when acetylthiocholine was used as substrate and an enzymatic inhibition was observed at high concentrations of this substrate. These findings and the results obtained with BW284C51 and iso--OMPA (99% inhibition induced by BW284C51 and insensitivity to iso-OMPA) suggest that the enzyme is AChE (Garcia et al. 2000). In *M. galloprovincialis*, similar results were found in gills (Mora et al. 1999a) and whole body extracts (Mora et al. 1999b), while in digestive gland a significant BuChE activity was also measured (Escartín and Porte 1997). Winners et al. (1978) provided evidence that the main ChE present in *M. edulis* haemolymph is also AChE. Although, in the haemolymph of the bivalves *Cardium edule*, *Mya arenaria* and *Anodonta cygnea* a higher specific activity was found using propionylthiocholine as substrate (Winners et al. 1978). Considering all these results, the characterization of ChE in bivalves used as sentinel species seems to be necessary for a more reliable use of these enzymatic activities as biomarker (Mora et al. 1999a).

Significant differences of ChE activity were found among samples with different protein content (one-way ANOVA, $F=6.46$; g.l.=5, 30; $P<0.05$). Two main groups of samples can be identified according to the level of ChE activity. The first group, which includes the samples with 0.1, 0.2, 0.3 and 0.4 mg of protein per ml present low ChE activity. The second group, which includes the samples with 0.5 and 0.6 mg of protein per ml, has high ChE activity, and no significant differences were found between these samples. Thus, 0.5 mg of protein per ml seems to be the most suitable protein concentration for the determination of the ChE activity, since it allows the attainment of an adequate level of enzymatic activity using the lowest amount of haemolymph possible. The compromise between sensitivity of enzymatic determinations and the amount of biological material required for the analysis is important from both economical and ethical perspectives.

ChE activity of *M. galloprovincialis* haemolymph, determined in non-exposed specimens was 34.0 ± 9.2 SD U/mg protein. A considerable interindividual variability was observed, which may be related with seasonal variations in physiological and biochemical processes of the species (Sheehan and Power 1999). These results are in good agreement with results obtained by Herbert et al. (1995/96) using *M. edulis* haemolymph.

The results of this study indicate that the ChE present in *Mytilus galloprovincialis* haemolymph is AChE, which can be a valuable environmental biomarker, especially if used in a non-destructive way.

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