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Bivalve Esterases as Biomarker: Identification and Characterization in European Cockles (*Cerastoderma edule*)

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Abstract This study characterized esterase activity in *Cerastoderma edule* tissues using different substrates and specific inhibitors and identified the tissue distribution of esterases in this species. Synthetic thiocholines and thioacetate esters and specific inhibitors (eserine, BW284C51 and iso-OMPA) were used to identify and quantify cholines and carboxyl esterases. The results demonstrated the presence of a non-specific propionyl thiocholine (PrSCh)-cleaving cholinesterase (ChE) and a large amount of carboxylesterases (CaE). For further studies using *C. edule* esterases as biomarkers, our results suggest that the adductor muscle, with PrSCh (5 mM) as substrate should be used to analyze ChE, and for CaE analyses, phenyl thioacetate should be used in digestive gland extracts (PSA, 5 mM).

Keywords Cholinesterase · Carboxylesterase · Propionyl thiocholine · Phenyl thioacetate

Esterases have been applied as biomarkers for the exposure of mollusks to organophosphorus and carbamate pesticides (Bolton-Warberg et al. 2007; Galloway et al. 2002; Solé et al. 2010; Valbonesi et al. 2003; Wheelock et al. 2008). However, several studies have shown great variation in the types of these enzymes and their substrate preferences, tissue distribution and physiological roles among different mollusk species (Mora et al. 1999; Stefano et al. 2008; Valbonesi et al. 2003). The role of acetylcholinesterases (Meng and Liu 2010) in vertebrates as the main hydrolase in the neuromuscular system is well established. However, in invertebrates, there are a great variety of cholinesterases, and their function is not completely understood (Kozlovskaya et al. 1993). Cholinesterases are known to preferentially hydrolyze specific substrates. AChEs preferentially use acetylthiocholine (AcSCh), and butyrylcholinesterases (BChE) act on butyrylthiocholine (BuSCh), although these trends may fluctuate depending on the species and tissue assayed. Additionally, carboxylesterases (CaE) play an important role in the detoxification of agrochemicals and pharmaceuticals (reviewed by (Wheelock et al. 2008), and their presence has also been detected in some mollusks (Escartín and Porte 1997; Laguerre et al. 2009; Solé et al. 2010). While pesticides are developed mostly to act in insects, fungi and nematodes, studies are needed to evaluate the probable effects of these compounds on non-target species, such as bivalves. Cockles are widespread in coastal areas throughout Europe and are considered of great socio-economic importance. Additionally, they play an important role in ecosystems as filterers, which combined with their burrowing activities, affects nutrient cycling in aquatic ecosystems. Their potential use as sentinel species based on biomarker analysis has been highlighted in few studies (Baudrimont et al. 2003; Cheung et al. 2006; Diaz et al. 2010; Machreki-Ajmi and Hamza-Chaffai 2008; Paul-Pont et al. 2010).

Before applying esterases as a biomarker of exposure in a new species, it is important to identify and characterize the enzymes present, choose the best tissue to be used in these assays and the best conditions under which to perform the measurements. This is important because different

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tissues may contain significant amounts of nonspecific esterases that may contribute to the measured activity but may show different sensitivities toward anticholinesterase agents (Kozlovskaya et al. 1993; Stefano et al. 2008), which could lead to biases in the interpretation of activity determination or inhibition. Therefore, the aim of this study was to characterize the esterases present in different tissues of the common cockle *Cerastoderma edule* using different substrates and specific inhibitors.

Materials and Methods

Acetylthiocholine iodide (AcSCh), butyrylthiocholine iodide (BuSCh), propoinylthiocholine iodide (PrSCh), phenyl thioacetate (PSA), 1,5-bis (4-allyldimethyl-ammonimphenyl) pentan-3-onedibromide (BW284C51), tetraisopropyl pyrophosphoramide (iso -OMPA), eserine sulfate, γ-bovine globulin were purchased from Sigma-Aldrich. All other reagents and solvents were analytical grade.

Cockles (20–30 mm shell length) were collected from a lightly impacted site (40°38′28.71″N 8°44′7.11″W) at Ria de Aveiro, Portugal in August, 2009. Tissues were dissected and stored at −80°C until analyses. All procedures to determine the substrate preference and specific inhibition of the investigated enzymes were carried out using 3 pools of 3 animals (whole soft tissue) each.

Tissues were homogenized in 0.1 M phosphate buffer, pH 7.2 (1:4 w/v) for 1 min. The homogenate was centrifuged at 3,800×*g* at 4°C for 3 min, and the supernatant was used to perform enzymatic analyses following the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). The protein content of each sample was normalized to 0.5–0.8 mg/mL before performing the enzymatic reactions. Total protein was determined by the Bradford method (Bradford 1976), using γ-bovine globulin as a standard (595 nm).

The first step in the characterization of the esterases in cockle tissues was to determine the substrate preferences of the esterases. For this purpose, 3 different substrates were used (AcSCh, PrSCh and BuSCh) at concentrations ranging from 0.005 to 40.96 mM. PSA ranging from 0.08 to 10.24 mM) was also used as a substrate that is preferentially hydrolyzed by CaE. The second step was to determine the specific inhibition of AChE (by BW284C51), of BChE (by iso-OMPA) and of ChE in general (by eserine). Stock solutions were prepared with ultrapure water (BW284C51 and eserine from 6.25 to 200 μM) and ethanol (iso-OMPA from 0.25 to 8 mM). In this approach, 495 μL of tissue extract was incubated in 5 μL of each inhibitor at 25°C for 30 min (Monteiro et al. 2005; Moreira et al. 2001). Then, measurements were performed using AcSCh, BuSCh or PrSCh as a substrate. In parallel, control experiments were carried out using ultrapure water and ethanol.

Subsequently, the ChE and CaE activities were measured in extracts of gills, mantle, digestive gland, foot, adductor muscle and whole body tissues using the substrates and concentrations determined in the previous steps. The esterase activities were expressed in units (U) per mg of protein, where 1 U is 1 nmol of substrate hydrolyzed per minute.

Data were analyzed based on mean ± standard error of the mean (S.E.M.) from three independent experiments. V_{max} and K_{map} values were calculated by the GraphPad Prism program (Intuitive Software for Science, San Diego, CA, USA) fitting experimental curves to Michaelis–Menten equation.

Results and Discussion

There is currently an increasing concern about new tools for biomonitoring programs in coastal areas. However, knowledge about the biology and physiology of potential sentinel organisms is limited to only a few species, such as the bivalves *Mytilus edulis*, *Mytilus galloprovincialis* and *Crassostrea gigas*, mostly due to the economic importance of these species.

The first aim of this study was to determine the substrate preferences of the esterases present in the investigated cockle species. Among the thiocholines used as specific substrates for cholinesterases, PrSCh was associated with the highest activity in the *C. edule* extracts (V_{max} = 4.57 ± 0.15 U/mg protein), while AcSCh and BuSCh were associated with approximately two-fold lower activities under the same conditions (Fig. 1). These results indicated that the enzyme present in this species is a cholinesterase that cleaves PrSCh more efficiently than the other investigated substrates. Most studies in bivalves have found a high rate of hydrolysis of AcSCh by an AChE, mainly in gill extracts (Monserrat et al. 2002; Mora et al. 1999; Valbonesi et al. 2003), but in other mollusks, such as *Corbicula fluminea* and *Anodonta woodiana*, the ChEs present also use PrSCh as their main substrate in several tissues (Corsi et al. 2007; Mora et al. 1999). PSA was found to be the preferentially hydrolyzed substrate for CaE, and our results indicated a V_{max} of 78.55 ± 3.27 U/mg protein (Fig. 1). These levels were even higher than those obtained when using other substrates, thus demonstrating that *C. edule* tissues contain a significant amount of carboxylesterase. The calculated kinetics parameters are reported in Table 1.

Although some enzymatic activity was observed when using AsCh and BuSCh, these substrates were probably hydrolyzed by a AChE and not by BChE because there was

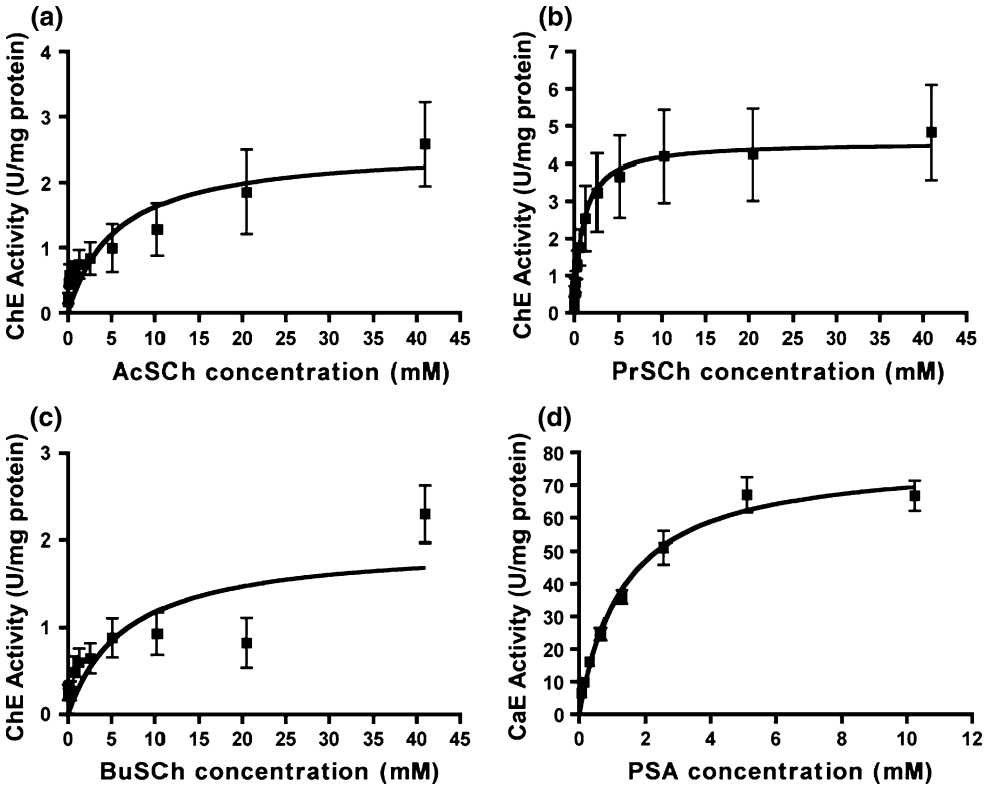

Fig. 1 Cholinesterase (ChE) and carboxylesterase (CaE) activities in a supernatant fraction from whole *Cerastoderma edule* using acetylthiocholine (AcSCh, **a**), propionyl thiocholine (PrSCh, **b**), butyrylthiocholine (BuSCh, **c**) and phenyl thioacetate (PSA, **d**) as substrates. Results are expressed as the mean ± SEM of three pools of three animals

Table 1 Michaelis–Menten constant (K_{map}) and maximal velocity (V_{max}) of ChE and CaE from *Cerastoderma edule* tissues

Substrate	K_{map} (mM)	V_{max} (U/mg of protein)	R^2
AcSCh	5.57 ± 2.68	2.54 ± 0.48	0.80
PrSCh	0.94 ± 0.13	4.57 ± 0.15	0.98
BuSCh	6.64 ± 4.70	1.97 ± 0.40	0.61
PSA	1.34 ± 0.17	78.55 ± 3.27	0.99

Results are expressed as the mean ± S.E.M. of three pools of three animals. R^2 values were included to demonstrate the goodness of fit to Michaelis–Menten equation

a slight inhibition observed when using the inhibitor BW284C51 (32% remaining activity at 200 mM), while no inhibition was observed with iso-OMPA, and these are specific AChE and BChE inhibitors, respectively (Fig. 2). Additionally, other non-specific ChEs were detected in *C. edule* extracts when eserine was used, which is a specific inhibitor of ChE. As shown in Fig. 2, despite the fact that the activity was significantly inhibited at the highest concentration of eserine used (200 mM), the activity measured was not completely inhibited when using the AcSCh (65 ± 7%) and PrSCh (53 ± 2%) substrates. These findings suggest that non-specific esterases may be contributing to the measured enzymatic activity. Different results were obtained by (Valbonesi et al. 2003) in other bivalves, such as *Ostrea edulis* and *M. galloprovincialis*, that presented a high sensitivity to eserine, revealing a predominance of ChE in their tissues.

The enzymatic activities were analyzed in different organs to better understand their distribution and physiological role. Although some studies have demonstrated that gills are the best tissue in which to measure ChE in bivalves, our results suggest the use of the adductor muscles, which presented higher levels of enzymatic activity. As shown in Fig. 3, ChE and CaE activities were detected in all tissues of *C. edule*; however, the adductor muscle extract showed a ten-fold higher ChE activity (54.55 U/mg protein) than the whole-body extract when 5.12 mM PrSCh was used, while the digestive gland was associated with the highest activity of CaE (83.34 U/mg protein) when using 5.12 mM PSA.

This finding reinforced the idea that the movements of the opening and closing of the valves are mediated by cholinergic transmission in cockles (Corsi et al. 2007). Lagerspetz et al. (1970) suggested that the main physiological function of AChE in bivalve gills is related to the movement of the cilia in the gill epithelium, and thus, mollusks such as mussels and oysters that have a greater amount of gill ciliate epithelium may exhibit greater AChE activity in this tissue. Mora et al. (1999) showed differences in ChE activity levels in different tissues of two bivalves: ChE was associated with higher activity in the gill (AcSCh, 2 mM) of *M. galloprovincialis*, and *C. fluminea* exhibited the highest activity in the mantle

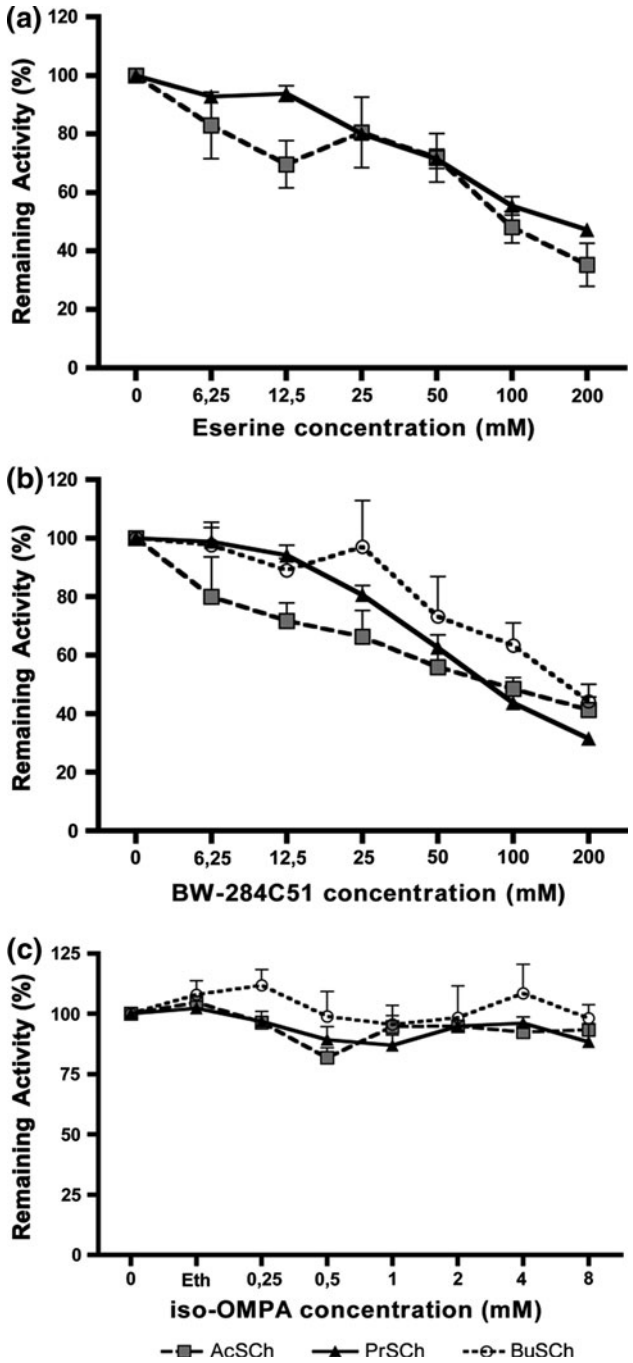

Fig. 2 Effects of specific inhibitors of esterase activity on whole *Cerastoderma edule* extracts using acetylthiocholine (AcSCh), propyionyl thiocholine (PrSCh) and butyrylthiocholine (BuSCh) as substrates. **a** Effects of eserine. **b** Effects of BW284C51. **c** Effects of iso-OMPA. Remaining activities (%) are expressed as the mean ± SEM of three pools of three animals

(PrSCh, 5 mM). Other studies have indicated a metabolic function of CaE in mollusks upon exposure to pharmaceutical products through the increase of hepatic enzyme activity and strong inhibition of CaE activity by

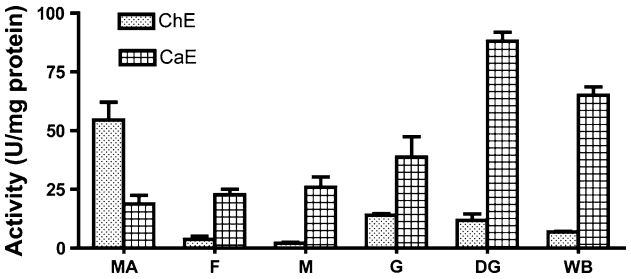

Fig. 3 Cholinesterase (ChE) and carboxylesterase (CaE) activities in the supernatant fraction from five tissues and the whole body of *Cerastoderma edule* using propyionyl thiocholine (PrSCh) and phenyl thioacetate (PSA) as substrates, respectively. *MA* muscle adductor, *F* foot, *M* mantle, *G* gills, *DG* digestive glad, *WB* whole body. Results are expressed as the mean ± SEM of three individual animals

organophosphorus and carbamates (Laguerre et al. 2009; Solé et al. 2010).

The present study demonstrated the presence of a non-specific PrSCh-cleaving ChE and a large amount of CaE, which is likely to be involved in valve closure and metabolism processes in *C. edule*. For future studies using *C. edule* esterases as biomarkers, our results suggested that (a) the adductor muscle should be used to analyze ChE, with propionyl thiocholine (5 mM) as a substrate, and (b) for CaE analyses, digestive gland extracts should be used (PSA, 5 mM). Additionally, further in vivo and in vitro studies are needed to better understand the function and sensitivity of these enzymes and their activity.

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