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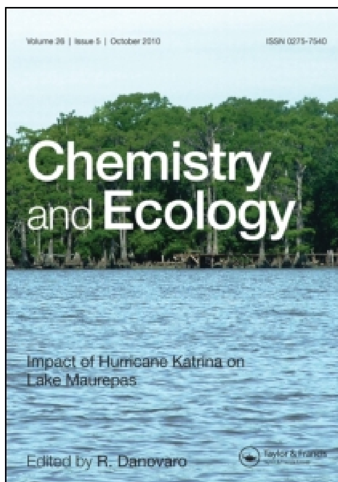

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Preliminary investigation on cholinesterases activity in *Adamussium colbecki* from Terra Nova Bay: Field and laboratory study

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PRELIMINARY INVESTIGATION ON CHOLINESTERASES ACTIVITY IN *ADAMUSSIUM COLBECKI* FROM TERRA NOVA BAY: FIELD AND LABORATORY STUDY

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While the inhibition of cholinesterase (ChE) activity as a biomarker of exposure to neurotoxic insecticides is well established in aquatic invertebrates of temperate areas, little is known about organisms from polar regions including Antarctica. Cholinesterase activity was investigated in specimens of the Antarctic scallop, *Adamussium colbecki*, collected in winter 2000 at Campo Icaro (Ross Sea, Antarctica) for preliminary characterization of a potentially new biomarker. Characterization of various ChE enzymes using specific substrates including an acetylthiocholine iodide (ASCh) and a butyrylthiocholine iodide (BSCh) was performed in gills, digestive gland and adductor muscle of the scallop. The effect of *in vivo* Zn^{2+} exposure in gills and digestive gland of *A. colbecki* was also studied. All the tissues expressed ChE activity (gill > adductor muscle > digestive gland) in accordance with data reported for marine mussels (*Mytilus* sp.) from temperate areas (1.1–13.8 $nmol\,min^{-1}\,mg\,protein^{-1}$). Significant BSCh-dependent ChE inhibition was also measured with a specific inhibitor, Iso-OMPA. Exposure to Zn^{2+} does not seem to affect ChE activity in the scallop although some slight differences were observed in substrate specificities (ASCh and BSCh) between treated and untreated organisms. This preliminary study stresses the need for further investigation on ChE activity in *A. colbecki* as a biomarker for monitoring water contamination in the marine Antarctic environment.

Keywords: *Adamussium colbecki*; Antarctica; Cholinesterase; Biomarker; $ZnCl_2$

1 INTRODUCTION

Antarctica is one of the last areas of the world upon which human activities seem to have minimal direct impact. Although most of the continent is considered to be relatively pristine, the presence of pollutants both of anthropogenic and natural origin like organics (Kennicutt II *et al.*, 1995; Fuoco and Ceccarini, 2001) and heavy metals, respectively (Bargagli, 2000; Sanchez-Hernandez, 2000) has been recorded in diverse environmental matrices as well as in a number of organisms including bivalve mollusks (Berkman and Nigro, 1992). Since marine bivalves have been successfully used as suitable indicators for pollution monitoring

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in temperate areas, there has been a growing interest in studying stress responses in the Antarctic scallop *Adamussium colbecki* as a key species for pollution monitoring of Antarctic coastal environment (Viarengo *et al.*, 1993; Regoli *et al.*, 1997a; 1998; 2002). This scallop accomplishes the requirements of a bioindicator species in terms of both sensitivity to toxic chemicals and accumulation of pollutants and for the wide distribution in marine coastal areas (Berkman and Nigro, 1992).

In an attempt to validate this species as a suitable bioindicator of the Antarctic marine environment, several biological responses to environmental stressors including lysosomal stability, total oxyradical scavenging capacity (TOSC), glutathione and antioxidant enzymes have been characterized in this species (Regoli *et al.*, 1997a; 1998; 2002). Over the past two decades, a wide range of biological responses (biomarkers) had been developed to detect and assess the exposure and effects of toxic chemicals in aquatic organisms (Livingstone *et al.*, 1997). Among them, Cholinesterases (ChEs) have been widely used in bivalves such as mussels (*Mytilus* sp.) as their role in the transmission of nerve impulses is affected by exposure to insecticides, heavy metals and pulp waste (Bocquené *et al.*, 1997; Najimi *et al.*, 1997). The ChEs belong to a large group of highly polymorphic enzymes widely distributed throughout the animal kingdom whose major role is to hydrolyze excess of cholinesters from the synaptic cleft (Habig and Di Giulio, 1991). Their presence and activity have been detected in different tissues and biological fluids of bivalves both in *in vivo* and *in situ* studies (Escartin and Porte, 1997; Galloway *et al.*, 2002). They are classified into two groups, either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE), and although the difference between the two is quite clear in many vertebrate species, it remains largely undetermined in invertebrates (Walker and Thompson, 1991). Cholinesterases that differ in substrate specificity using acetylthiocholine iodide (ASCh) and butyrylthiocholine iodide (BSCh) and susceptibility to inhibitors such as organophosphorus insecticides (OPs) and carbamate compounds (CBs) have been recently well characterized in both freshwater and marine bivalves (Le Bris *et al.*, 1995; Talesa *et al.*, 2001; Escartin and Porte, 1997; Basack, *et al.*, 1998; Doran *et al.*, 2001; Galloway *et al.*, 2002). Recent studies have also provided some evidence that other environmental contaminants could inhibit ChEs activity; these include detergents, heavy metals and components of complex mixtures similar to those present in highly impacted Antarctic regions such as Winter Quarters Bay and Arthur Harbor (Lenihan *et al.*, 1990; Risebrough *et al.*, 1990; McDonald *et al.*, 1995). Moreover, ASCh-dependent ChE activity of the mussel *M. galloprovincialis* has recently been reported to be sensitive to heavy metals which were detected in high concentrations in the tissues of the Antarctic bivalve *A. colbecki* (Mauri *et al.*, 1990; Viarengo *et al.*, 1993; Najimi *et al.*, 1997). A more general use of ChEs as biomarkers to monitor pollution in aquatic environments has therefore been suggested (Guilhermino *et al.*, 1998).

In order to evaluate the usefulness of ChE activity in *A. colbecki* as a potential biomarker of pollution exposure in the marine Antarctic environment, the present study was aimed at (1) characterizing ChE activity in the sentinel species *A. colbecki*, both in terms of substrate specificity (ASCh and BSCh) and tissue distribution and (2) investigating the possible effects of heavy metals on ChE activity in the tissues of scallops exposed to $ZnCl_2$ *in vivo*. The effects of Zn^{2+} exposure on ChE activities was investigated since divalent cations have been shown to inhibit neuromuscular transmission (Forshaw, 1977) and the release of Acetylcholine from the vagus *in vitro* (Hayashi and Takayama, 1978). Zn^{2+} has an important function related to gene expression. Variations of its availability from the diet can influence the rate of gene transcription (Cousin, 1998). It has also been demonstrated that excess Zn causes severe disruption of biochemical processes in aquatic biota including interactions with ion-transport channels in membranes (Hogstrand and Wood, 1995, McGeer *et al.*,

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2000), conformational variations of polypeptide chains (Kellog and Hof, 1996), displacement of other metals from their binding sites on enzymes (Roche and Boge, 1993) and membrane disturbance (Gabryelak *et al.*, 2002). Previous experiments have demonstrated that significant alterations of ChE activities occur in mussel species exposed to Zn^{2+} both *in vitro* and *in vivo* (Bocquené *et al.*, 1990; Najimi *et al.*, 1997).

A validation of ChE activity in this species could provide a new biological tool for pollution monitoring in the marine Antarctic environment.

2 MATERIALS AND METHODS

Acetylthiocholine iodide BSCh, 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB), *tetra* (mono-isopropyl)pyrophosphor-tetramide (*Iso*-OMPA), Tris and Triton X-100 were obtained from Sigma Aldrich Chemie Gmbh. BIORAD Protein was purchased from Bio-Rad Laboratories Gmbh.

Twenty-one specimens of *A. colbecki* of about 7 cm in shell length were collected by SCUBA diving at Campo Icaro (Terra Nova Bay, Ross Sea) near the Italian Antarctic Scientific Station (Lat. 74°41′S, Long. 164°04′E) during the Austral summer of 1999–2000 (Fig. 1). Organisms were immediately sacrificed and gills, digestive glands and adductor muscles were removed. Tissue samples were frozen in liquid nitrogen and stored at −80 °C prior to analyses.

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2.1 *In vivo* Zn^{2+} Exposure

Exposure to Zn^{2+} in the form of $ZnCl_2$ was carried out at the Italian Scientific Station in Terra Nova Bay. The treated group ($n = 7$) was kept in oxygenated seawater at -1 ± 1 °C containing Zn^{2+} as $ZnCl_2$ at the concentration of 400 ppm. The control group ($n = 7$) was maintained in clean oxygenated seawater throughout the experiment. After seven days of treatment, scallops were sacrificed and tissues samples were withdrawn as described previously.

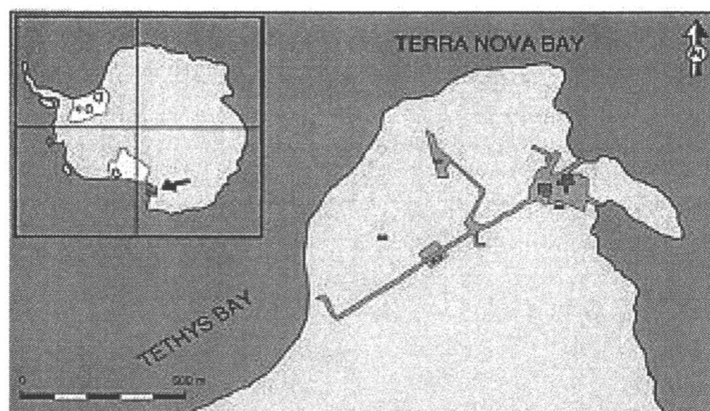

FIGURE 1 Sampling area at Campo Icaro (Terra Nova Bay, Ross Sea) near the Italian Antarctic Base Terra Nova (Lat. 74°41′S, Long. 164°04′E) during the Austral summer 1999–2000.

2.2 Cholinesterase Activity Assay

Aliquots of gills, digestive gland and adductor muscle (0.5 g) were homogenized using a Potter-Elvenjem glass/Teflon homogenizer at 2000 rpm with 16 vols. (w/v) of 100 mM Tris–HCl buffer pH 8.0 containing 0.2% Triton X-100. Homogenates were centrifuged at 2100*g* for 10 min, the resulting pellet containing cellular debris discarded and the supernatant fraction immediately analyzed.

Biochemical measurements were carried out in duplicate on a Shimadzu UV-160A visible recording spectrophotometer. Cholinesterase activity was assayed at 30 °C since no differences were observed in the activity of the enzyme previously measured at 20 °C and 25 °C; however at 30 °C a better linearity of the kinetic curve was obtained during the 5 minutes of the analysis. The method used was Ellman *et al.* (1961), in which ASCh and BSCh derivates are hydrolyzed by ChEs to yield thiocholine. Subsequent combination with DTNB forms the yellow anion 5-thio-2 nitrobenzoic acid that absorbs strongly at 410 nm. Assay components included: 25 mM Tris–HCl buffer (pH 7.6, containing 1 mM $CaCl_2$), DTNB (0.333 μM, final concentration) and 200–250 μl of sample supernatant, in a total volume of 3 ml. The reaction was initiated by adding ASCh or BSCh (277 μM and 261 μM final concentration, respectively) and the progressive increase in absorbance recorded for 5 minutes at 410 nm. Spontaneous hydrolysis was determined in the absence of supernatant. Cholinesterase activity was expressed as nmol substrate hydrolyzed min^{-1} mg $prot^{-1}$. Total protein was measured according to Bradford (1976), using bovine serum albumin as a standard. Values were expressed as mg total protein ml^{-1} supernatant.

To better characterize and distinguish the type of ChEs, samples were pre-incubated for 5 minutes at 30 °C with 3 mM *Iso*-OMPA a selective inhibitor of BChE enzyme, often used to best accomplish the separation of the enzyme AChE from BChE (Sturm *et al.*, 1999). A stock solution was prepared at 100 μM in acetone and diluted to the desired concentration in distilled water. A control vehicle was included for each experimental run and the rate of spontaneous hydrolysis calculated as before.

Data were expressed as mean ± standard deviation (±SD). Data were log-transformed for achieving normal distribution before running ANOVAs. *Post-hoc* Tukey compromise tests were used to determine statistical differences among ChE activity in different sample groups. Correlation was performed using the Pearson correlation coefficient (r). A probability level of less than 0.05 was considered significant. Statistical analyses were performed with Statistica 5.1 (Stat Soft, USA).

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3 RESULTS AND DISCUSSION

3.1 Characterization of Cholinesterase Activity

In order to characterize ChE activity in *A. colbecki*, ASCh and BSCh substrates were tested. Cholinesterase activity in the three tissues was gills > adductor muscle > digestive glands with both substrates (Tab. I). Cholinesterase activity in gills resulted four times higher ($p < 0.05$) than in the other two tissues. Cholinesterase versus BSCh was higher in the gills and in the adductor muscle respect to ASCh; an opposite trend was observed in the digestive gland where ASCh seemed to be the most effective substrate for ChE measurement (Tab. I).

Five-minute *in vitro* incubation of gill homogenates with Iso-OMPA (3 mM) resulted in the following percentage of ChE activity inhibition in the three tissue in which ASCh was used as a substrate: 19%, 21% and 65% in gills, adductor muscle and the digestive gland, respectively. Iso-OMPA incubation with BSCh substrate showed a 29%, 41% and 59% of inhibition

TABLE I Cholinesterase Activity Assayed with Two Substrates ASCh and BSCh (nmol min^{-1} mg $prot^{-1}$) in Gills, Adductor Muscle and Digestive Gland Homogenates of Antarctic Scallop *A. colbecki* from Campo Icaro. Data Expressed as Mean $\pm$ Standard Deviation ($n = 10$).

Campo Icaro	*ChE versus ASCh*	*ChE versus BSCh*
Gills	11.5 ± 7.9	13.8 ± 12.8
Adductor muscle	2.0 ± 0.6	2.3 ± 1.0
Digestive gland	1.5 ± 0.9	1.2 ± 1.0

in the three respective tissues (Fig. 2). Cholinesterase activity in all three tissues was also strongly correlated in each individual: r values ranged 0.79–0.98 ($p < 0.05$) in gills and adductor muscle and 0.46–0.95 ($p < 0.01$) in digestive gland and adductor muscle for ASCh- and BSCh-dependent ChE activity, respectively.

3.2 *In vivo* Zn^{2+} Exposure

Since digestive gland and gills are the most targeted tissues for trace metal accumulation, ChE activity was not assayed in the adductor muscle.

Tissue distribution of ChE activity in both Zn^{2+}-treated and untreated organisms confirmed previous observations in which activity was higher in gills than in digestive glands ($p < 0.01$, Tab. II). In particular, both ASCh and BSCh-dependent ChE activity were three times higher in gills than in digestive gland homogenates (Tab. II). Cholinesterase activities measured in gills of untreated scallops were markedly lower (>50%) than those detected in the same tissue of wild specimens. The percentage of inhibition in gill and digestive gland homogenates incubated *in vitro* with Iso-OMPA (3 mM) fell within a similar range. A different trend was observed in terms of substrates sensitivity in gill homogenates: Zn^{2+}-treated organisms showed significantly higher ASCh-dependent ChE activity than that of BSCh-dependent activity ($p < 0.05$), while no significant differences were observed in substrate sensitivity in the untreated group (Tab. II).

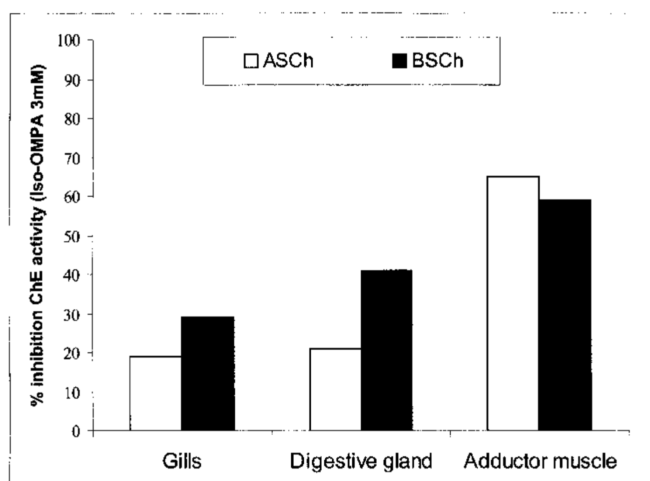

FIGURE 2 Percentage of inhibition of ChE activity with an *in vitro* pre-incubation with Iso-OMPA (3 mM).

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TABLE II Cholinesterase Activity Assayed with Two Substrates ASCh and BSCh (nmol min^{-1} mg $prot^{-1}$) in Gills and Digestive Gland Homogenates of Antarctic Scallop *A. colbecki* from *in vivo* Zn^{2+} Exposure. Data Expressed as Mean ± Standard Deviation ($n = 7$).

In vivo Zn^{2+}exposure	*ChE versus ASCh*	*ChE versus BSCh*
Control group		
Gills	5.4 ± 0.8	5.9 ± 0.8
Digestive Gland	1.6 ± 0.3	1.1 ± 0.2
Treatment group		
Gills	6.4 ± 1.3	5.1 ± 0.6
Digestive gland	1.7 ± 0.4	1.1 ± 0.1

The results of this preliminary investigation on ChE activity in *A. colbecki* show sensitivity for both ASCh and BSCh substrates suggesting the occurrence of more than one ChE as previously described for invertebrate species from temperate areas (Mora *et al.*, 1999). The absence of complete inhibition (from 19% to 59%) observed in all three tissues in which Iso-OMPA was used as a specific butyrylcholinesterase activity inhibitor suggests the occurrence of more than one form of ChE enzyme in *A. colbecki* (Fig. 2). Gills of *A. colbecki* showed the highest expression of ChE activity, followed by adductor muscle and digestive glands as reported for the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis* and the common oyster *Crassostrea gigas* (Bocquené *et al.*, 1997; Escartin and Porte, 1997; Mora *et al.*, 1999). Values of ChE activity in *A. colbecki* were in the same range as those reported for *M. galloprovincialis* from the Mediterranean Sea (ASCh 11.5 ± 7.9 and BSCh 24.2 ± 3.5 nmol min^{-1} mg $prot^{-1}$ in gills and ASCh 1.5 ± 0.9 and BSCh 4.0 ± 0.1 nmol min^{-1} mg $prot^{-1}$ in digestive gland, respectively, Escartin and Porte, 1997).

Exposure to Zn^{2+} does not seem to affect ChE activity in the scallop, although some slight differences in substrate sensitivity (ASCh and BSCh) between treated and untreated organisms suggest a different role of Zn^{2+} in modulating substrate specificity of the enzyme.

Regarding the lower ChE activities observed in scallops exposed in the lab compared to wild specimens, past studies have demonstrated that starving can cause a significant decrease of several enzymatic activities in marine species (Lemaire, 1990) and that variations in diet and food availability can also play a significant role (Kleinow *et al.*, 1987). It has been observed that dietary restrictions, such as those present in aquaria during exposure, have the potential to induce oxidative stress and consequently increase lipid peroxidation (Pascual *et al.*, 2003); it is widely accepted that such phenomenon can cause inhibition of several enzymatic activities in freshwater mussels (Doyotte *et al.*, 1997). We infer that decreased ChE activities and the absence of effects from Zn^{2+} in experimentally exposed scallops could be related to altered environmental and dietary conditions of captivity and to subsequent variations of the specimens' physiological status.

Although slightly evident, the lower BSCh-dependent ChE activity observed in the gills of Zn^{2+}-treated organisms could be related to a potential inhibitory role of Zn^{2+} at the enzyme site as described in other studies (Çokuğraş and Tezcan, 1993; Bhanumanthy and Balasubramanian, 1998; Sarkarati *et al.*, 1999). The mechanism of ChE modulation by metals is still unknown, however two main mechanisms have been proposed for ChE activity inhibition in invertebrates. One is that the enzyme is inactivated by binding to specific groups and the other seems to be an enzyme precipitation as a consequence of high salt concentrations (salting-out) (Najimi *et al.*, 1997; Guilhermino *et al.*, 1998). The slightly higher ASCh-dependent ChE activity observed in gills of Zn^{2+}-treated scallops also suggests a positive

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Zn^{2+} stimulation of ChE activity in regulating the release of acetylcholine (ACh) substrate within the synaptic cleft as reported in the African mussel *Perna perna* after a 4-day Zn treatment (Najimi *et al.*, 1997).

4 CONCLUSIONS

ASCh- and BSCh-dependent ChE activity was present in gills, digestive gland and adductor muscle of wild and experimentally treated specimens of *A. colbecki.* Cholinesterase substrate sensitivity and tissue distribution were consistent with previous investigations conducted in *M. galloprovincialis* and *M. edulis* from temperate areas (Radenac *et al.*, 1998; Guilhermino *et al.*, 1998).

Cholinesterase activity assayed using the Iso-OMPA specific inhibitor suggests that AChE might be present in tissues of *A. colbecki* (Basack *et al.*, 1998; Escartin and Porte, 1997). Cholinesterase versus ASCh activity seems to share similarities with specific AChE activity in marine bivalves from temperate areas reported by other authors (Galloway *et al.*, 2002; Escartin and Porte, 1997; Bocquenè *et al.*, 1997).

Results from the *in vivo* study suggest a different sensitivity to Zn^{2+} exposure of ChE activity respect to the ASCh and BSCh substrates. Further investigations regarding experimental conditions including food availability and diet will be performed in order to identify factors linked to conditions of captivity that may affect ChE activity in the Antarctic scallop.

Enzyme titer and tissue activity must also be investigated in order to better characterize ChE enzyme type. Likewise, other heavy metals and chemical inhibitors will be investigated to validate the use of ChE activity as a biomarker of neurotoxic exposure/effect in *A. colbecki.*

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