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Coelomic Fluid Lysozyme Activity Induction in *the Polychaete Eurythoe complanata as* a Biomarker of Heavy Metal Toxicity

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Invertebrate immune assays are being developed for the assessment of sublethal toxicity of xenobiotics. Although direct coelomocyte counts and cell-mediated immunity provide substantial information on the immunotoxic potential of certain compounds, functional and humoral assays could give additional support to hypothesis testing.

Lysozyme activity is a phylogenetically conservative humoral mechanism that has been studied in several classes of invertebrates, reporting basal levels in hemolymph from bivalves, gastropods and echinoderms, as well as in coelomic fluid of annelids (Périn and Jollés 1972; Cheng and Rodrick 1974; McHenery *et al.* 1979; Anderson and Chain 1982; Hirigoyemberry *et al.* 1990; Hawking *et al.* 1993). Goven *et al.* (1994) reported a lysozyme activity test developed for use in assessing immunotoxic effects of copper in the earthworm *Lumbricus terrestris*, showing suppression of the enzyme antibacteriolytic activity in coelomocytes and coelomic fluid.

In recent years there has been increasing concern over the potential deleterious effects of contaminated marine sediments on the biota. Polychaete annelids have been widely recognized as useful sentinels of varying degrees of benthic pollution as they constitute the most abundant number of species and specimens in subtidal marine soft-bottoms (Sanders 1958; Santos and Simon 1974; Reish 1980, 1984, 1986; Reish and LeMay 1989). This paper is aimed at the development of a toxicity test based on coelomic lysozyme measurements for the tropical cosmopolitan polychaete species *Eurythoe complanata*, using copper as a reference contaminant. Considering that lysozyme induction by bacterial inocculation could be sensitive to xenobiotics, it was taken as a sublethal endpoint for testing immunotoxic effects of copper on this organism.

MATERIALS AND METHODS

Sexually inmature polychaetes weighing between 1 and 2 g were collected from

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the shore of the Gulf of Cariaco, Northeastern Venezuela. The worms were maintained indoors in aereated aquaria at 25±1°C, containing gross sand and sea water (salinity: 36‰, pH 7.8) from the collection site, and held for 7-d prior the experimental treatments.

Copper ($CuSO_4.5H_2O$-Merck-) was used in the experiments because it was known to exert immuno-modulatory effects on oligochaetes under similar bioassay conditions (Chen *et al.* 199; Goven *et al.* 1994). Bioassays were run for 7-d at a sublethal nominal concentration of 0.4 mg L^{-1} of Cu^{+2} which represented 30 % of the calculated 96-hr median lethal concentration (LC_{50}: 1.3 mg $Cu^{+2}\,L^{-1}$, 95% confidence limits: 0.4 - 2.0 mg $Cu^{+2}L^{-1}$) for this species, estimated by an LC_{50} computer program developed by Stephan (1977). Two groups of 20 polychaetes were placed in 10-L aquaria containing sea water, 2-cm sand layers (1.2 kg/aquarium), and $CuSO_4.5H_2O$ as contaminant. Another two groups of 20 polychaetes were used as controls. Following the 7-d exposure, copper content in the worm carcasses was determined by flame atomic absorption spectrophotometry using a Varian AA-20 Plus, with a minimal detection limit of 0.003 µg mL^{-1}. Calibration curves were based on a certified copper Baker Instra Analyzed reagent of 1000 µg mL^{-1} (J.T. Baker Inc., Phillipsburg, NJ); while percent recovery was based on an oyster tissue standard reference material from the National Institute of Standards and Technology (NIST, reference code 1566a). The reference standards were subjected to the same digestion procedures as those used for the worm samples. That is, following an acid digestion (application note BI-3 of the CEM Corporation Applications Manual) of dry tissue (1.0 g) in a closed vessel using a pressure controlled microwave heating system (CEM Corporation, model MDS-2000).

Control and copper-exposed worms were injected with 25 µL of a bacterial solution of *Micrococcus lysodeikticus* (Sigma Chemical Co, St Louis, Mo, 8 mg mL^{-1}). At 2 hr after bacterial injection the coelomic fluid was harvested, using a non-invasive extrusion protocol previously developed by Arredondo (1994). Briefly, the worms were bathed in a l0-cm diameter Petri dish containing 40 mL of the extrusion solution (2.5 mg mL^{-1} chloral hydrate, Merck, and 1.0 mg mL^{-1} glyceryl guayacolate ether, Sigma Chemical Co, in sea water 36‰, pH 7.5 - 7.8) for 5-10 min to stimulate the coelomic fluid expulsion. The coelomic fluid was collected directly from the pigidial pore with the aid of a Pasteur pippete and tranferred to 5-mL polyethylene test tubes followed by centrifugation at 200 g and 4°C for 10 min. The supernatant was used as the lysozyme source. In preliminary experiments the pattern of lysozyme activity in un-exposed worms was recorded during 4 hr following bacterial injection (Table 1).

Lysozyme activity was determined by the method of McHenery *et al.* (1979). Forty µL of coelomic fluid were dispensed into 5-mm diameter wells in 1% agarose in 5-cm diameter Petri dishes. The agarose contained 1 M phosphate buffer pH 7.5 - 7.8 and *Micrococcus lysodeikticus* (0.6 mg freeze-dried cell mL^{-1}). After incubation for 48 hr at 27°C, the diameters of zones of lysis were measured

Table 1. Kinetic of lysozyme activity, measured as HEL-equivalent (µg mL^{-1}), after injection of 200 µg M *lysodeikticus* into *E. complanata.* Results are expressed as the mean ± SD (n = 6)

Time (hr)	Lysozyne activity
0	0.49 ± 0.20
1	0.20 ± 0.10
2	2.60 ± 0.34
4	0.48 ± 0.34

and lysozyme concentration was determined by reference to a calibration curve with hen egg-white lysozyme (HEL Sigma Chemical Co). The results are presented as HEL-equivalent (pg mL-1) activity, which was calculated by the following regression model:

$$\text{HEL-equivalent } (\mu g\ mL^{-1}) = \text{antilog}_{10}[a + b\ (\text{diameter, mm})].$$

The incubation period of 48 hr was selected because the bacterial lysis showed a linear response, ranging within 4.2 ± 1.3 and 9.0 ± 1.8 µg mL^{-1} during a time period of 24 - 72 hr.

The differences in lysozyme activities between experimental groups were statistically analysed by one-way ANOVA. Significant differences between pairs of groups were assessed by the Least Signifcant Differences (LSD) Test (Snedecor and Cochran 1971).

RESULTS AND DISCUSSION

In the absence of copper exposure a lysozyme activity of 0.49 µg mL^{-1} was recorded for unsensitized worms, which increased to a maximal value of 2.6 µg mL^{-1} within 2 hr after bacterial sensitization (Fig. 1, ANOVA: F = 20.68; p= 0.0005; LSD Test). These data suggested that the naturally occurring lysozyme activity in the coelomic fluid of *Eurythoe complanata* can be experimentally induced by injection of *Micrococcus lysodeikticus*. Basal levels of this bacteriolytic enzyme possibly reflect a functional mechanism that protects the organism from bacteria living in its environment and controls its natural symbiotic flora. Both basal and experimentally-induced lysozyme activities have been found in the hemolymph and coelomic fluid of many invertebrates (Périn and Jollés 1972; McHenery *et al.* 1979; Hirigoyemberry *et al.* 1990; Hawking *et al.* 1993). In the annelid *Eisenia fetida anderi,* the induction of lysozyme by bacteria was very fast and disappeared in 12 d, (Hirigoyemberry *et al.* 1990). In *Eurythoe complanata,* the sensitization response was very rapid and the basal activity was re-established within 4 hr (Table 1). This induced activity was probably the result of the release of pre-existing proteins into the coelomic fluid which contribute to non-specific immune defense as previously demonstrated in the mollusk

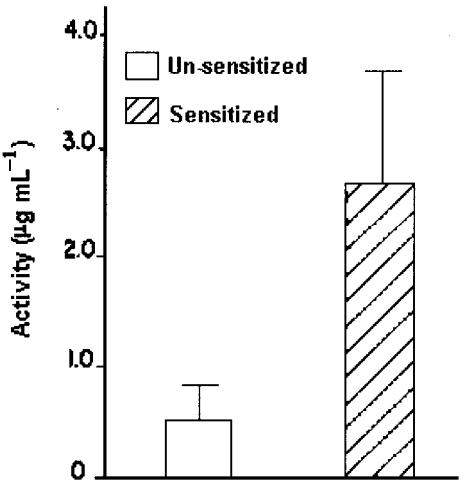

Figure 1. Lysozyme activity in the coelomic fluid of control worms ***(E. complanata).*** Basal activity was measured for worms previously injected with ***M. lysodeikticus*** (sensitized) and a control group (un-sensitized). Each experimental value represents the mean ± SD. (n = 6)

Mercenaria mercenaria (Cheng and Rodrick 1974). Tissue concentration of copper was 5.6 and 42.0 µg g^{-1} dry weight for non-exposed and copper-exposed worms, respectively (the range of estimated % recovery values was 95-105 %, based on five standars). In the presence of copper exposure no significant increase in bacteriolytic enzyme was found in unsensitized worms compared with former basal activity (Fig. 2A). However, when sensitized copper- exposed worms were compared against non-exposed sensitized worms (Fig. 2B), a significant difference was observed with lower activity in the former group (ANOVA: F=20.68; p=0.0005; LSD Test).

We previously observed that a sublethal dose of copper (0.2 mg $Cu^{+2}L^{-1}$) induced immuno-suppression, declining phagocytosis and formation of secretory and erythrocytic rosettes in *Eurythoe complanata,* using mouse erythrocyte as antigen (unpublished data, 14Th Annual Meeting of the Society of Environmental Toxicology and Chemistry, 1993, Houston, Texas). Thus, when the cell-mediated and humoral immune responses had been suppressed by exposure to copper, the lysozyme activity could represent an immune response which would render the organism relatively insensitive to microbial infection. Noteworthy, Ville *et al.* (1995) reported that exposure to polychlorinated biphenyl Aroclor 1254 (PCB) resulted in higher levels of lysozyme activity in the earthworms, *Eisenia hortensis, Eisenia foetida* and *Lumbricus terrestris,* associated with a decrease in other defense mechanisms, including phagocytosis and those related to wound healing.

Interestingly, Goven *et al.* (1994) reported inhibitory effects of sublethal copper concentration on lysozyme activity of coelomic fluid for the earthworm *Lumbricus terrestris* and suggested that copper toxicity was probably related to adverse

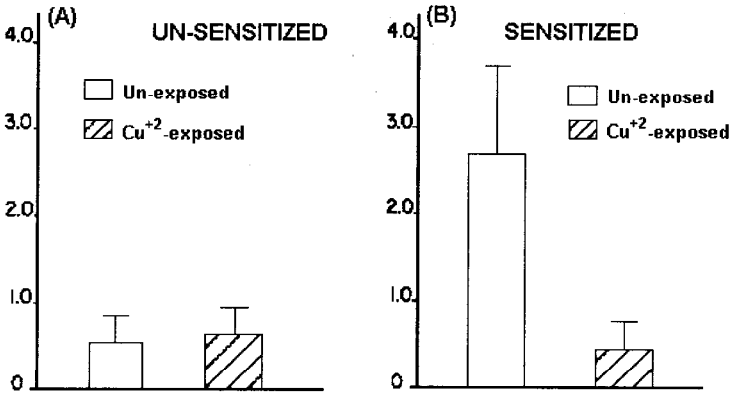

Figure 2. Lysozyme activity in the coelomic fluid of ***E. complanata*** for un-sensitized Cu^{+2}-exposed worms (A) and sensitized Cu^{+2}-exposed worms (B). Each experimental value represents the mean ± SD (n = 6).

action on the functional conformation of the protein. This assumption is consistent with the *in vitro* inactivation of avian lysozyme as described by Feeney *et al.* (1956). For the earthworm, the reduced enzyme activity coincided with copper body loads of 28.5 and 73.1 µg g^{-1} dry weight, showing a tissue concentration-response relationship with inhibition. In comparison with *Eurythoe complanata*, differences in susceptibility to the stressor agent may be envisioned between both species; distinct toxicokinetic mechanisms could emerge among annelids species to control their normal body burden of copper. Copper appears to be potentially immuno-toxic for polychaetes sensitized by bacteria (Fig. 2B); probably the metal produces a distress that disturbs the normal induction of the lysozyme as a protective factor against heavy bacterial infection. Finally, a conclusion from this study is that experimental induction of lysozyme activity might contribute to our ability to assess toxicological risk in a wide variety of species; particularly because this enzyme is highly genetically conserved in the animal kingdom. Moreover, the lysozyme assay in polychaetes could have application in the assessment of other xenobiotics, including industrial wastes.

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