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Short Communication

Effect of zinc on lysozyme-like activity of the seastar *Marthasterias glacialis* (Echinodermata, Asteroidea) mucus

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ABSTRACT

Lysozyme represents the best characterized enzyme involved in the self-defense from bacteria. In this study we analysed the effects of zinc on the lysozyme-like activity of the seastar *Marthasterias glacialis* mucus. This activity, detected by measuring the cleared lysis area of dried *Micrococcus lysodeikticus* cell walls on Petri dishes, was significantly reduced in presence of zinc. The results are discussed in the light of elucidating the possible relationship between environmental contaminants and increased disease susceptibility in seastars due to the decrease of antibacterial protection. The benefits of using the test of lysozyme activity to monitoring environmental pollution are highlighted.

1. Introduction

Environmental pollution by metals, especially cadmium, mercury, lead, copper and zinc, has become one of the most important problems in marine coastal areas as a consequence of anthropogenic inputs, via industrial run-off, river outflows, and domestic sewage (UNEP, 1996). Zinc, is an essential trace element potentially harmful at elevated levels. The concentrations of this metal, in pristine water systems, range from 2×10^{-6} to 1×10^{-4} mg/L, but in contaminated areas concentrations up to 1.5×10^{-2} mg/L can be found (Van den Berg et al., 1987). Zinc is used as fertiliser, furthermore its release in the Mediterranean Sea, including Italian coasts, is due to industrial pollution from the mines, refineries and other industrial activities (Çelo et al., 1999).

In marine invertebrates heavy metals affect survival, growth, reproduction, metabolism and immunity (Matozzo et al., 2001; Mydlarz et al., 2006). A number of studies on the impact of heavy metals on the immune system have been carried out in molluscs, crustaceans and oligochaetes (Mydlarz et al., 2006) whilst, little information is known about echinoderms. Canicattì and Grasso (1988) observed that high zinc ion concentrations produce an inhibitory effect on the hemolytic activity of *Holothuria polii*.

Lysozyme, identified in a wide range of organisms, represents the best characterized enzyme involved in self-defense from bacteria (Jolles and Jolles, 1984). This enzyme cleaves the β1–4 bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid of bacterial cell walls. A lysozyme-like activity was evidenced in the eggs and mucus of the seastar *Marthasterias glacialis* (Canicattì and D'Ancona, 1990; Stabili and Pagliara, 1994). Mucus typically forms a slippery coating and contains antimicrobial agents (Suzuki et al., 2003) that prevents bacteria and debris from accumulating on the body surface. The basal levels of lysozyme in the mucus protect the organism from bacteria living in the same environment. Consequently, a reduction of mucus antibacterial activity, due to high concentrations of contaminants, could make the seastars more susceptible to bacterial infection. A variety of studies have demonstrated that in invertebrates environmental perturbations, affecting immunological competence, can increase susceptibility to infectious disease (Galloway and Depledge, 2001; Rivera et al., 2003). In this framework we analysed the effects of a high zinc concentration on the lysozyme-like activity in the mucus of the seastar *M. glacialis*. We evaluated the effects of zinc on lysozyme-like activity by comparing control and stressed seastars. In addition, we measured the capacity of recovery of *M. glacialis* lysozyme-like activity after the zinc removal. As already observed by Goven et al. (1994) for the earthworm *Lumbricus terrestris* and Marcano et al. (1997) for the polychaete *Eurythoe complanata,* the evaluation of lysozyme-like activity reduction in seastars may give an early indication of disease susceptibility and, ultimately, survival.

2. Materials and methods

After the reproduction season (March) 80 adult specimens of *M. glacialis* (Echinodermata, Asteroidea) were collected, by using SCUBA equipment, in a coastal area of the Northern Ionian Sea (S. Cate-

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rina, Lecce–Italy), where Zn concentration was 1×10^{-6} mg/L (Buccolieri et al., 2004). In the laboratory, each animal was placed on a large Petri dish, left in this position for 15 min and then the undiluted mucus was collected (T_0) by a sterile plastic pipette. The mucus was immediately centrifuged at 400*g* for 15 min at 4 °C and the supernatant was stored at −80 °C and successively employed for lysozyme activity assay. The mucus protein concentration was determined using the protein–dye binding kit Biorad (Bradford, 1976). Individual mucus was diluted to obtain an equal protein concentration for the assays (0.29 ± 0.01 mg/mL).

After the mucus collection the seastars were divided into two sets of 40 individuals each. Individuals of each set were separated into five groups (8 individuals each one). The first set (control) was placed in five aquaria filled with filtered seawater (0.2 μm) and the second set (treatment) in five aquaria with filtered seawater and $ZnCl_2$ (final concentration of 5 mg/L). Aquaria were maintained at 20 °C and 37‰ salinity. After 48 h (T_{48}) individuals were removed from the aquaria and the mucus was collected. Control specimens were then replaced in fresh filtered seawater. Instead, zinc treated animals were washed gently in seawater (15 min) to remove residual of zinc and placed into fresh filtered seawater (post-zinc treatment). After 48 h of this treatment (T_{96}) all the seastars were removed from aquaria and mucus was collected again. Along all the experimental period the survival rates of both control and treated seastars were evaluated.

Lysozyme-like activity was assayed at T_0, T_{48} and T_{96} by using the agar diffusion lysozyme test as described by van Bijsterveld (1974). Briefly 700 μL of 5 mg/mL of dried *Micrococcus lysodeikticus* cells (Sigma) were diluted in 7 mL of 0.05 M phosphate buffer (PB) agarose, pH 5.2 then spread on a Petri dish. Five replicate wells (6.3 mm diameters) per individual organism were sunk and filled with 30 μL of mucus. The diameter of the cleared zone of the five replicates was recorded after overnight incubation at 37 °C, averaged for each individual, and compared with those of reference samples represented by hen-egg-white lysozyme (Merck), carefully measured as concentration and volume. Zinc concentrations ranging from 1 to 10 mg/L were employed as negative control.

Analysis of variance (ANOVA) was used to test for differences in the lysozyme-like activity between control and treatment seastars. Prior to analysis the assumption variance homogeneity was tested by Levene test. Given the unbalanced design and the skewed nature of the data, *p*-values were obtained by a permutational procedure. Therefore, tests for the significance of all terms involved in the full ANOVA model were made by using a distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) based on Euclidean distances on untransformed data. Each term in the analysis was tested using the highest number of possible random permutations of the appropriate units (Anderson and ter Braak, 2003). The analyses were performed using the add-on package PERMANOVA + (Anderson et al., 2008) in the PRIMER v6 computer program (Clarke and Gorley, 2006). Analysis of covariance (ANCOVA) among diameter of lysis and seastars size was also performed.

3. Results

Lysozyme-like activity of *M. glacialis* mucus, evaluated at the different times, and expressed as mean values of lysis diameter, is shown in Fig. 1. Before zinc treatment (T_0), the mean diameter of lysis was 6.5 ± 0.3 mm corresponding to 0.39 mg/mL of hen-egg-white lysozyme. After 48 h (T_{48}), in control conditions the mean diameter of lysis was 6.47 ± 0.34 mm and in treated organisms was 1.9 ± 0.4 mm (corresponding to 0.11 mg/mL of hen-egg-white lysozyme). The difference in lysozyme-like activity between controls and treatments was significant ($P < 0.05$). After 48 h of zinc removal (T_{96}), we evidenced the absence of significant differences in lysozyme–like activity between control and post-zinc seastars (mean diameter 6.48 ± 0.33 mm and 6.23 ± 0.35 mm respectively). Thus the treated specimens showed the capacity of recovery from the chemical stress. In Table 1 we summarized data on the body sizes as well as the mucus lysozyme activity of both control and post-zinc treated animals.

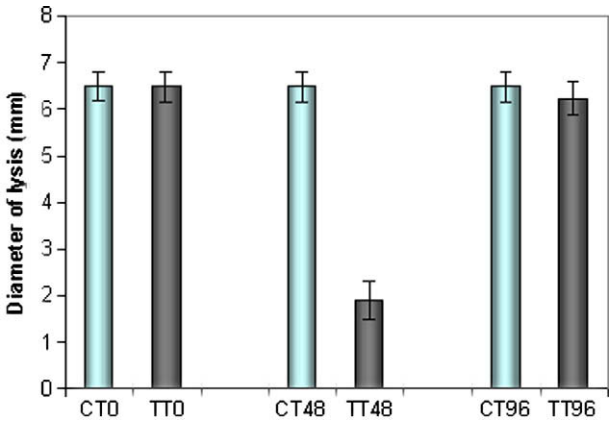

Fig. 1. *Marthasterias glacialis* mucus lysozyme-like activity in control and zinc treated seastars at T_0, T_{48} and in control and post-zinc treated seastars at T_{96}. CT_{48} = control at T_{48}; TT_{48} = treated seastars at T_{48}; CT_{96} = control at T_{96}; TT_{96} = treated seastars at T_{96}. Each column represents the mean value ±SD ($n = 40$ for CT_0, TT_0, CT_{48}, TT_{48} CT_{96}; $n = 24$ for TT_{96}).

After 48 h of zinc treatment, the mortality rate was approximately 40% with no differences among the seastars with different body sizes (Table 1). The control groups *of M. glacialis* instead showed no mortality.

ANCOVA showed that diameter of lysis and seastars size significantly covariate both at T_0 (MS_{cov} 7.1439; MS_{denom} 0.01756, $P < 0.01$) and T_{96} (MS_{cov} 6.1036; MS_{denom} 0.018544; $P < 0.01$). ANOVA highlighted the significance of the interaction Treatment × Time (Tr × Ti) suggesting that differences between control *vs* zinc treatment varied with times. Post-hoc comparisons of treatment within each of the three levels of the factor time identified these differences as occurring in T_{48}, where the mean diameter of lysis in control was by far higher than that observed in zinc treatment (Fig. 1). No significant differences between treatments were observed in T_{96} and, as expected, in T_0. ANOVA also indicated the lack of a tankness effect that is differences between treatments in time did not vary among tanks, as indicated by the lack of significance of the Ta (Tr) × Ti (Table 2).

4. Discussion

Available literature on zinc impact on marine invertebrates suggests that concentrations ranging from 0.097 to 11.3 mg/L, are highly toxic (Martin et al., 1989). In this study we used a high zinc ion concentration taking into account of both its high concentration in some marine invertebrates eaten by seastar (Viviani, 1992) and the accumulation of this heavy metal by metallothionein-like proteins in seastars (den Besten et al., 1990). Our results show that:

— lysozyme-like activity of *M. glacialis* mucus was inhibited in short-term by the presence of high zinc ion concentrations.

Zinc might act directly on the enzyme or indirectly through the organism itself. Anyway the suppression of lysozyme-like activity, by increasing the susceptibility of seastars to bacteria, may portend a decrease in immunocompetence and pathological manifesta-