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SHORT COMMUNICATION

First report of phenoloxidase and peroxidase activities in two intertidal sea anemone species of Argentina

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Abstract

The presence of immune responses within sea anemone species has received little attention, in comparison with coral species, so we decided to investigate the phenoloxidase and peroxidase activities in ectoderm, endoderm and tentacles of actiniarians *Aulactinia marplatensis* and *Bunodosoma zamponii,* the most common species in intertidal zone of Mar del Plata, Argentina. Enzyme activities were detected in all tissues evaluated with some differences among tissues and species. Phenoloxidase and peroxidase activities are associated with the mechanisms of innate immunity in invertebrates, and the high production of phenoloxidase observed in *B. zamponii* would provide a continual level of resistance to infection and this species to be less susceptible to stress and disease, compared to *A. marplatensis*. This study, represents the first step toward specific immune information about the mentioned sea anemone species of Argentina, and thus permits prediction of the potential effects of environmental factors on immune response.

Key Words: disease; immunity; peroxidase; phenoloxidase; sea anemone; stress; tissues

Introduction

Numerous studies have demonstrated that environmental factor variations such as temperature, salinity, oxygen, nutrients and contaminants can strongly affect immune parameters in invertebrates. In this context, immunomarkers have been proposed to be sensitive tools in eco-immunology studies to detect signs of impaired animal health (Matozzo *et al*., 2013). Palmer *et al*. (2010) concluded that immunological parameters, such as phenoloxidase activity, provide good indicator of coral immunity and underpin linkages between the susceptibility of corals to disease.

Immunity refers to the ability of an organism to resist infection with the nonspecific and immediate innate immune pathways providing the first line of internal defense. A key component of invertebrate innate immunity is the presence and activation of the melanin-synthesis pathway in response to invasion by foreign organisms or physical injury (Rinkevich, 2004). Melanin pathway activity, as indicated by levels of the activating enzyme phenoloxidase, has been documented in scleractinian corals, gorgonians and true soft corals from the Caribbean and Indo-Pacific (Palmer *et al.,* 2008, 2011; Mydlarz *et al*., 2009; Mydlarz and Palmer, 2011). Furthermore, melanin is a redox-active pigment and therefore has the potential not only to be cytotoxic and kill pathogens, but also to scavenge oxygen radicals that may be harmful to the host. Oxygen radical scavengers and enzymatic antioxidants are important during infection, as host responses frequently induce oxidative stress conditions (Palmer *et al*., 2011).

Several studies have linked cnidarian peroxidase activity with antioxidant potential (Hawkridge *et al.,* 2000; Olano and Bigger, 2000) and oxidation of fatty acid hydroperoxides (Koljak *et al*., 1997). Mydlarz and Harvell (2007) argued that enzymatically driven resistance measures, such as peroxidase activity, are important in the early responses of the sea fan *Gorgonia ventalina* to a fungal pathogen *Aspergillus sydowii*.

As immunity determines, the ability of an organism to resist and eliminate infection and to recover from injury, it can be used as a predictor of compromised health susceptibility. The immune defenses increase fitness by promoting survival, through disease resistance and the maintenance of tissue integrity. The study of marine invertebrate

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ecological immunity is advancing very quickly and critical signaling pathways and cytotoxic responses are being elucidated. The presence and relative activities of phenoloxidase and antioxidants, such as, peroxidase were investigated in several coral species, mainly scleractinian (Mydlarz and Harvell, 2006). The presence of immune responses within sea anemone species have received little or virtually no attention, but their biology suggests that disease-resisting defenses would be adaptive. Hutton and Smith (1996) investigated the antimicrobial defenses of *Actinia equina* and Hawkridge *et al.* (2000) determined the subcellular distribution of antioxidant enzymes in the temperate sea anemone *Anemonia viridis*. According to the scarce knowledge on this topic related with sea anemones we decided to investigate the phenoloxidase and peroxidase activities in ectoderm, endoderm and tentacles of actiniarians *Aulactinia marplatensis* and *Bunodosoma zamponii.* These species are the most common species in the rocky intertidal zone of Mar del Plata (38º 05' S-57º 32'W). They are found mainly attached to hard quartzitic substrate and the taxonomical status of both species was studied by Acuña *et al.* (2007) and Braga Gomes *et al.* (2012), respectively. Many aspects of their biology and ecology were also studied, like those related with reproduction (Zamponi and Excoffon, 1986; Excoffon and Zamponi, 1991, 1997), population ecology (Acuña and Zamponi, 1995a, 1996a, 1998), feeding (Acuña and Zamponi, 1995b; 1996b; Acuña, 1997; Acuña *et al*., 1999), as well as particular topics like analyses of the cnidae (Acuña and Zamponi, 1997) and mycosporine-like amino acid content (Arbeloa *et al*., 2010). However other aspects, like immunological, remain unknown. This study, represents the first record toward specific immune information about the mentioned sea anemone species of Argentina, and thus permits prediction of the potential effects of environmental factors on immune response.

Materials and Methods

Sample collection specimens of the sea anemones *Aulactinia marplatensis* and *Bunodosoma zamponii* were obtained from the intertidal zone of the rocky area with a quartzitic substrate in Punta Cantera, Mar del Plata (38° 05'S and 57° 38'W). The individuals, all around the same size (30 mm in basal diameter), were caught in December 2012 in the same area for both species during the low tide, but covered by water. The organisms, n = 10 for each species, were maintained at room temperature in an aquarium with decanted and aerated sea water and they were sampled one day after collection. For both anemone species, tissue of epidermis, endodermis (gastrodermis) and tentacles, were removed and homogenized with a 50 mM phosphate buffer at pH 7.8 on ice. Samples were then centrifuged for 5 min at 4,000 rpm and 4 °C, avoiding the mucus layer and the supernatant (protein extract) was carefully removed and stored at -20 °C. Soluble protein in protein extract was measured by the method described by Bradford (1976), using chicken egg white albumin as the standard.

Phenoloxidase and Peroxidase activities were determined according to Palmer *et al.* (2011). Phenoloxidase was assay using 50 µl of protein extract; 100 µl of phosphate buffer (50 mM pH 7.8) and 50 µl double distilled water pyrogen free were incubated for 20 min at room temperature, then 50 µl L-DOPA (3 mg ml^{-1}) (Aldrich, 333786) was added and after 10 min 350 µl of cacodylate buffer (200 mM pH7.4) was addedand absorbance at 490 nm was recorded (Shimadzu UV-2102 PC, UV-visible Scanning Spectrophotometer). Two control treatments were used, without L-DOPA or without protein extract. Peroxidase activity was determined using 60 µl of protein extract, 210 µl of phosphate buffer (10 mM pH 6) and 240 µl of pyrogallol (Sigma P0381) with 150 µl hydrogen peroxide 1.6 volumes to activate the assay, after 3 min the absorbance was recorded at 470 nm. Control treatment containing 60 µl of protein extract and 600 µl of phosphate buffer (10 mM pH 6) was done. Phenoloxidase and peroxidase activities were expressed as the change in absorbance per mg protein (Abs mg $protein^{-1}$). All assays were run by triplicate. Soluble protein content and enzymatic activity were analyzed with ANOVA after testing normality and homogeneity of variances. Significant differences were considered at $p \leq 0.05$. When significant differences were found, a Tukey-Kramer Multiple Comparison test was performed to locate these differences. Analysis were made using NCSS 8 Software.

Results and Discussion

The two sea anemone species used in this study, *A. marplatensis* and *B. zamponii,* demonstrated differing levels of constituent immunity, as indicated by the immune parameter activities. Soluble protein did not differ significantly among species and tissues, with levels between 15.8 and 20.2 mg ml^{-1} for *A. marplatensis* and 17.0 and 21.8 mg ml^{-1} for *B. zamponii*.

Phenoloxidase (PO) activity was observed in all tissues evaluated, having the ectoderm of *B. zamponii* the highest PO activity at 0.025 Abs 470 nm mg $protein^{-1}$. Mean PO activities of endoderm and tentacles for both species and ectoderm of *A. marplatensis* were significantly lower than *B. zamponii*'s ectoderm (Figs 1a, c).

Palmer *et al.* (2010) demonstrated that PO activity was present in different coral families, such as, Euphylidae, Acroporidae, Pocilloporidae, Alcyonacae, Merulinidae, Faviidae, Mussidae, Fungiidae, Poritidae and Oculinidae; and varied significantly among them.

Phenoloxidase is the activating enzyme of the melanin-synthesis pathway, a key component of invertebrate immunity and the melanin-synthesis which provides cytotoxic defense, a protective barrier and structural support (Palmer *et al*., 2011). For anthozoans, melanization was the first documented within a sea fan, as a barrier against a fungal infection (Petes *et al*., 2003; Mullen *et al*., 2004) described the amebocytes involved. In the same species, aggregations of amebocytes were documented around fungal infections, and their granular content was confirmed to be melanin

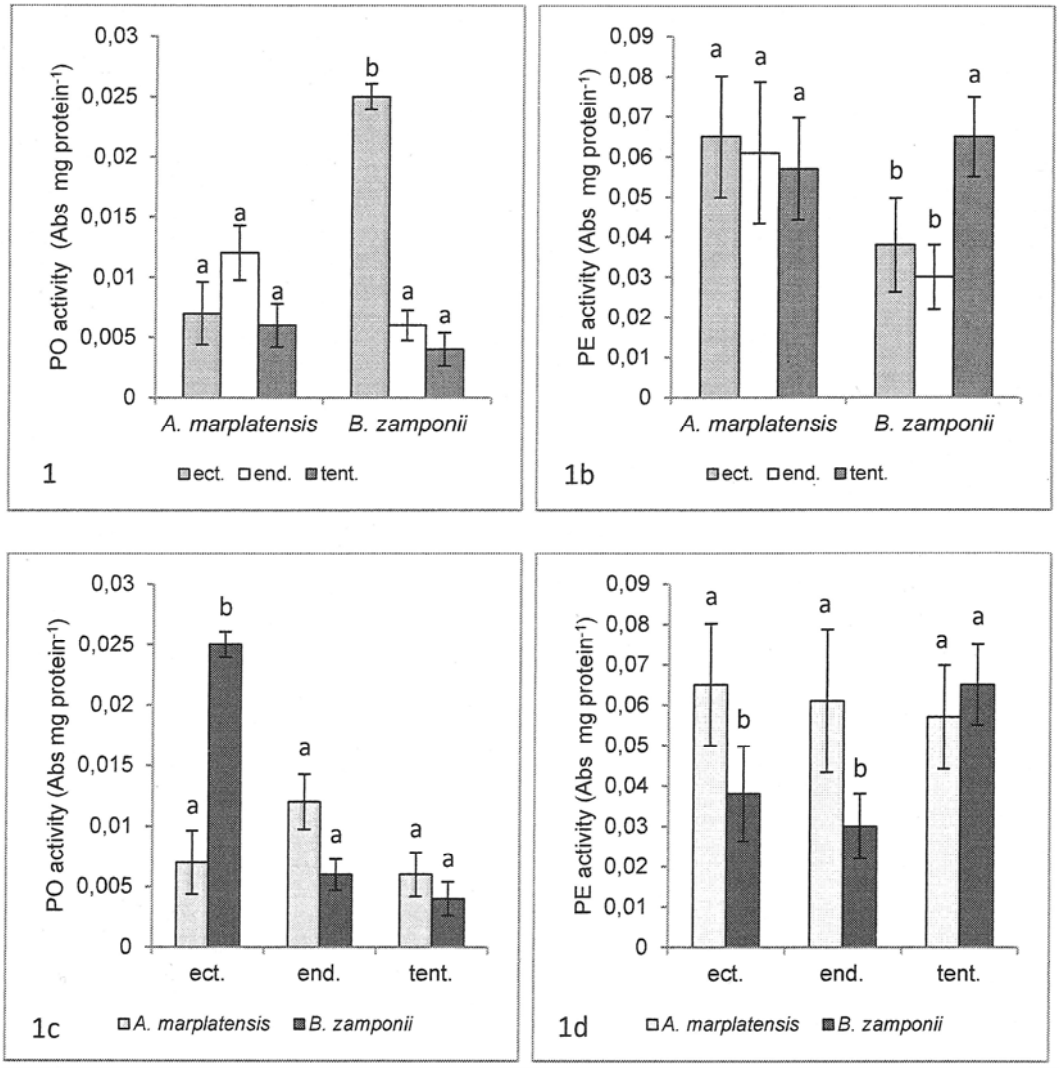

Fig. 1 (a, b, c, d). *Aulactinia marplatensis* versus *Bunodosoma zamponii* tissue comparisons for mean (± s.e.) phenoloxidase and peroxidase activities, ect = ectoderm, end = endoderm, tent = tentacles.

(Mydlarz *et al.*, 2008). Tucker et al. (2011) demonstrated that amebocytes were commonly encountered in the mesoglea, in the thick fibril free zone under the epidermis of anemone *Nematostella vectensis*, while melanin-containing granular cells were located predominantly in the epidermis in 15 scleractinian species by Palmer *et al*. (2010), this observation could be explain the highest PO activity registered in ectoderm of *B. zamponii* in the current study.

Invertebrates with low PO activity are more susceptible to disease and similarly, scleractinian corals are more susceptible to bleaching and disease, as recently documented in a wide range of coral families (Palmer *et al*., 2011). Differences in residual PO activity among coral families indicate physiological disparities that may have implications at an ecological scale in terms of disease resistance, with families having higher PO activity being more able to resist infection. This prediction is consistent with correlations found between PO activity and immunocompetence for numerous invertebrates (Palmer *et al*., 2010). According to previous information, we may be suggesting that *B. zamponii* is more able to resist infection, however additional research is necessary.

The super-family of peroxidase enzymes contains many isoforms which partake in a variety of metabolic functions. In animal, peroxidase enzymes (PE) are involved in disease resistance and stress responses. Changes in peroxidase levels can signify immunomodulation due to contaminants and other environmental stressors (Mydlarz and Harvell, 2007). These authors examined the inducibility of coral peroxidases by experimentally exposing corals to fungal pathogen and found that enzyme activity was induced after an incubation period and they also hypothesize that *Gorgonia ventalina* utilizes the peroxidases as an integral component in disease resistance pathways. Palmer *et al.* (2011)

evidenced peroxidase activity in bleached and healthy colonies of *Acropora millepora* and proposed that this enzyme is potentially important for mitigate the effect of oxidative stress.

Dikens and Shick (1984) established that peroxidase activity in the sea anemone *Anthopleura elegantissima* was highest in the tentacles and oral disc. A more detailed localisation of this enzyme was attemped by Hawkridge *et al.* (2000) who localised the antioxidant enzymes in granulated vesicles, accumulation bodies of endosymbiotic algae and all forms of cnida in the temperate sea anemone *Anemonia viridis* and tropical coral *Goniopora stokesi*, both species considered abundant in their respective habitats. In the current study, mean peroxidase activity was approximately equivalent (~0.06 Abs 470 nm mg protein^{-1}) for all tissues of *A. marplatensis* and tentacles of *B. zamponii*, and significantly higher than ectoderm and endoderm of *B. zamponii* (~0.03 Abs 470 nm mg protein^{-1}) (Figs 1b, d).

Phenoloxidase and peroxidase activities are commonly associated with the mechanisms of innate immunity in invertebrates, and were confirmed for the first time in the studied actiniarians. The high production of phenoloxidase observed in *B. zamponii* would provide a continual level of resistance to infection and this species to be less susceptible to stress and disease, compared to *A. marplatensis*. However the latter has a possible additional defense, the attached cover of gravel on its column that could constitute a physical barrier to pathogens. This cover is common on the column of intertidal sea anemones with verrucae, and can support a small but rich community of invertebrates and algae (Barcellini, 2011).

Thus, via collaborative efforts between ecologists, immunologists, cell biologists, and physiologists, we may expand the current understanding of innate immunity in naturally occurring ecologically important species. With greater understanding of the connections between environment and organismal immunity, we can make predictions about the effects of changing climate and environment on immunocompetence and disease outbreaks (Mydlarz *et al*., 2006).

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