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Comparative Biochemistry and Physiology, Part A 146 (2007) 54 – 62
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Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen

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Received 27 May 2006; received in revised form 27 August 2006; accepted 1 September 2006
Available online 16 September 2006

Abstract

The enzymatic defense mechanisms of *Gorgonia ventalina* to the fungal pathogen *Aspergillus sydowii* may play important roles in colony resistance to infection. In this study, we examined the role of the superfamily of peroxidase enzymes in the coral response to a naturally occurring pathogen. We examined the inducibility of peroxidases by experimentally exposing corals to *A. sydowii* and found that peroxidase activity was induced after an 8 day incubation period. In contrast, naturally infected corals collected from the reef had lower peroxidase activity when compared to healthy corals. Infected sea fans from the field also had less measurable protein in their tissues and increased purple sclerites near infection sites and it is likely that these infections are months old. Using native-PAGE activity gels, we detected 5 peroxidase isozymes in healthy corals, indicating that multiple isoforms of peroxidase with a plurality of possible functions are present in this coral. The role of the peroxidase enzymes in disease resistance was examined by testing anti-fungal activity of commercially available and partially purified sea fan peroxidases. In both cases there was significant, dose-dependent anti-fungal activity. While peroxidases are ubiquitous enzymes involved in many cellular pathways, we also hypothesize that *G. ventalina* utilizes these enzymes as an integral component in disease resistance pathways. As such, they may also contribute to the initiation of physiochemical defenses such as melanization and lipid soluble anti-fungal metabolites.

Keywords: Coral disease; Peroxidase; *Aspergillus sydowii*; Induced resistance; Anti-fungal activity; Innate immunity

1. Introduction

In invertebrates, pathogens can trigger the production of defense-related proteins and induce host immune responses. However, exploration of immunity in invertebrates has been dominated by mechanistic studies of model organisms, with little attention to natural populations (Little et al., 2005). Thus, identifying what is known about key components of the innate immune system in different invertebrate phyla, especially the most basal of invertebrates, remains a critical priority (Mydlarz et al., 2006). In basal invertebrates, including the Porifera and Cnidaria, allorecognition and identification of self/non-self have been the main immune responses characterized (Bigger and Hildemann, 1982), while the effector response pathways in signaling and inducing immune responses during pathogen invasion remain understudied. Anti-oxidant enzymes (Hawkridge et al., 2000), radicals of oxygen (Mydlarz and Jacobs, 2006) and nitrogen (Morall et al., 2000) as well as cellular responses such as the involvement of amoebocytes in wound healing (Olano and Bigger, 2000; Meszaros and Bigger, 1999) have been identified in Cnidaria. In this study we examine the relationship between the multifunctional oxidoreductase proteins known as the peroxidases, and coral response to disease.

The super-family of peroxidase enzymes (H_2O_2 donor and consumer) contains many isoforms which partake in a variety of metabolic functions. In terrestrial plants and marine algae, peroxidases are involved in resistance to pathogens (He et al., 2001; Nagy et al., 2004; Yedidia et al., 1999), wound healing (Ross et al., 2005; Bernards et al., 1999), heavy metal stress (Pinto et al., 2003), production of reactive oxygen species (Blee et al., 2001; Bolwell and Wojtaszek, 1997; Bolwell et al., 1998) and scavenging and detoxification of reactive oxygen species (Pinto

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doi:10.1016/j.cbpa.2006.09.005

et al., 2003; Asada and Takahashi, 1987). In animals, peroxidase enzymes are involved in phagocytosis and immune cell function (Torreilles et al., 1997; Rodriguez et al., 2003; Soares-da-Silva et al., 2002), cell adhesion (Holmblad and Soderhall, 1999), anti-oxidant function (Gamble et al., 1995; Galloway and Depledge, 2001; Hawkridge et al., 2000) and the oxidative polymerization of hydroquinones to melanin (D'Ischa et al., 1991). In many benthic marine invertebrate systems such as mussels and clams (Dyrynda et al., 1998; Cossu et al., 1997; Cossu et al., 2000), peroxidase is measured as part of a suite of immunological measurements. In these studies, changes in peroxidase levels can signify immunomodulation due to contaminants and other environmental stressors. Due to the overarching importance of the peroxidase proteins in the defenses of many different organisms, we examined their role in the sea fan–aspergillosis pathosystem.

In recent years, populations of the common sea fan *Gorgonia ventalina* have suffered high mortality in an epizootic of the fungal pathogen *Aspergillus sydowii* (Kim and Harvell, 2004; Nagelkerken et al., 1997; Alker et al., 2001; Harvell et al., 1999). Immune responses of *G. ventalina* detected against pathogen infection include production of anti-fungal lipid metabolites (Kim et al., 2000a,b) and melanin deposition as a physical barrier to prevent fungal expansion (Petes et al., 2003; Mullen et al., 2004; Alker et al., 2004). These are likely secondary responses that are preceded by more rapid enzymatic immune responses which have not been examined to date.

Several studies have linked coral peroxidase activity with anti-oxidant potential (Hawkridge et al., 2000; Olano and Bigger, 2000) and oxidation of fatty acid hydroperoxides (Koljak et al., 1997). However, their role in the coral defense responses against pathogens has not yet been investigated. In this study, we examined the function and inducibility of peroxidases in the defense responses of the gorgonian coral *G. ventalina* to infection by the fungus *A. sydowii*. We hypothesize that peroxidase activity is an inducible response to infection that occurs early in the infection process. We also hypothesize that peroxidases may serve a direct role in the anti-fungal defense mechanism and therefore confer anti-fungal activity against *A. sydowii*.

2. Materials and methods

2.1. Materials

Sea fan pieces were collected using SCUBA from the Looe Key Reef research site and from Tennessee Reef in the Florida Keys, USA. Specimens were collected from depths of 5–10 m. For peroxidase activity analysis, all corals were immediately flash frozen in liquid nitrogen and stored at −80 °C. For histological analysis, corals were immediately placed in zinc buffered formalin sea water for at least 24 h and then decalcified in an aqueous solution of calcium citrate and formic acid. Histological samples were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H and E) at the Cornell Veterinary School Histology Laboratory.

2.2. Peroxidase detection

Frozen coral pieces were weighed and homogenized in liquid nitrogen using a mortar and pestle in 0.2 M phosphate buffer, pH 7.8 with 5 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis MO). Extracts were centrifuged at 405 × *g* to remove large tissue pieces and again at 14,000 × *g* to remove small cellular debris. The protein concentration of the crude protein extracts was determined using Bio-Rad RC DC Protein Assay Kit (Hercules, CA) with bovine serum albumin as a standard. To measure peroxidase activity, 5 μL of the crude protein was diluted with 50 μL of 0.01 M phosphate buffer, pH 6.0 and added to 50 μL of 25 mM guaiacol (Sigma-Aldrich) in 0.01 M phosphate buffer pH 6.0 in a 96-well plate (guaiacol final concentration of 10.8 mM). The reaction was initiated with the addition of 10 μL of 20 mM hydrogen peroxide (Sigma-Aldrich) (1.7 mM final concentration) prepared in 0.01 M phosphate buffer, pH 6.0 and optical density was measured over time at 470 nm using a Thermo Electron Corporation Multiskan Spectrum microplate reader with ScanIt software (Waltham, MA, USA). Peroxidase activity was represented as the change in absorbance at 470 nm/min and normalized among samples to mg protein.

To detect peroxidase activity by gel electrophoresis, protein extracts were run on 10% polyacrylamide gels under native conditions and detected using 25 mM *o*-dianisidine (Sigma-Aldrich) and 5 mM hydrogen peroxide in sodium acetate buffer (pH 5.2). Protein concentrations were normalized among samples to 0.5 mg/mL of protein. Colored bands on the gel refer to peroxidase proteins which have oxidized *o*-dianisidine to a brown product.

2.3. Experimental infection of G. ventalina

Pieces of healthy sea fans ($n=13$, 9 cm × 5 cm) were collected and brought back to Mote Tropical Research Laboratory (Summerland Key, FL, USA). The pieces were sectioned into clonal replicates approximately 4 cm × 3 cm in size, 1 piece was immediately frozen to represent the initial levels of peroxidase activity, and the remaining pieces were kept in closed tanks in a flowing sea water table for a 2 day acclimatization period. Fungus was applied to the surface of the sea fan using a gauze patch. Controls received the same gauze patch without the fungus. Controls and fungal exposed sea fans were harvested and processed 8 days post-inoculation. Histological samples were obtained and the remaining tissue was frozen in liquid nitrogen. Protein extracts of the corals were prepared and analyzed as described above. Histological preparations of corals exposed to *Aspergillus* showed no sign of an active infection and there were no visible signs of disease even after 8 days of exposure.

2.4. Collection and analysis of naturally infected G. ventalina specimens

Sea fans showing signs of fungal infection such as lesion formation and purple tissue were collected. Sea fan tissue within the