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Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associate molecular patterns

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Abstract

This study investigates the effects of two pathogen-associated molecular patterns (PAMPs), LPS and zymosan, on the Sydney rock oyster (*Saccostrea glomerata*) immune system. Phenoloxidase and phagocytic activities, total and differential haemocyte frequencies, as well as peroxide and superoxide concentrations were measured after the injection of lipopolysaccharide and zymosan. All of the immunological parameters were induced by both PAMPs. Phenoloxidase (monophenolase and diphenolase) and phagocytic activities, as well as the frequencies of phenoloxidase-positive haemocytes, hyalinocytes and granulocytes in the haemolymph, increased within 24 h of PAMP injection. Values for all of these parameters peaked within 48 h of challenge and began to decrease to levels that were indistinguishable from those of controls within 96 h. The only exception to this pattern was diphenolase activity, which remained elevated for at least 96 h. Control saline injections that lacked PAMPs also induced responses in most of the parameters measured. However, reactions to saline injections were of far lower magnitude compared to those induced by PAMPs. All of the data suggest that the phenoloxidase and phagocytic systems of oysters are inducible components of the Sydney rock oyster immune system, and that induction is primarily due to increased frequencies of specialised haemocytes in the haemolymph.

Keywords: Sydney rock oyster (*Saccostrea glomerata*); Zymosan; LPS; Phenoloxidase; Phagocytosis

1. Introduction

Invertebrates lack immunoglobulin antibodies and their associated adaptive immune responses. Instead, they have highly effective innate immune reactions that defend them against a broad spectrum of infectious microorganisms [1]. These innate systems consist of both cellular and humoral components, and do not show an obvious capacity to discriminate between antigens [2].

Abbreviations: LPS, lipopolysaccharide; FSW, sterile filtered sea water; L-DOPA, L-3,4-dihydroxyphenylalanine; 4HA, hydroquinine monomethyl ether; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MAC, marine anti-coagulant; OD, optical density; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species; PRR, pattern recognition receptor.

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The cellular immune responses of invertebrates comprise phagocytosis, encapsulation and nodule formation [1,3,4]. Phagocytosis has been studied in many invertebrates, including molluscs like the snail, *Lymnaea stagnalis*, and the clam, *Mya arenaria* [5,6]. Circulating haemocytes are the key phagocytes of molluscs. According to morphological and immunological criteria, molluscan phagocytes have been classified into two major types, granulocytes and hyalinocytes [7], with granulocytes usually being the most actively phagocytic [8].

In many invertebrates, phagocytosis is accompanied by the induction of intracellular killing mechanisms, primarily involving reactive oxygen species (ROS), such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2). ROS and their induction by phagocytosis have been studied in a number of molluscs, including bivalves [9,10].

The humoral components of invertebrate immune systems include a variety of cytotoxic molecules, lectins and enzymatic cascades, such as the complement and phenoloxidase systems [11,12]. The phenoloxidase cascade seems to be a particularly important effector mechanism in many invertebrates [13,14]. Phenoloxidase usually exists as an inactive intracellular proenzyme, prophenoloxidase, that can be activated by the presence of fungal β-1,3-glucans, bacterial peptidoglycans and lipopolysaccharides (LPS) [15–20]. Conversion of prophenoloxidase into active phenoloxidase is accomplished, usually after exoctyosis, by a cascade of serine proteinases [17]. This process is triggered by pattern recognition receptors (PRRs) that act as surveillance molecules for microbial antigens [21].

Active phenoloxidase is a multifunctional enzyme with distinct monophenoloxidase and diphenoloxidase activities. Monophenoloxidase activity catalyses the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by α-hydroxylation, whilst subsequent diphenoloxidase reactions oxidise α-diphenols to dopaquinone. Dopaquinone undergoes a series of non-enzymatic reactions to form the pigment, melanin [22,23]. Melanisation has a number of uses among invertebrates. It contributes to the sclerotisation of cuticle in crustaceans and is also involved in wound healing, nodule formation, encapsulation and possibly phagocytosis [17,24–27]. Several intermediate metabolites of the phenoloxidase cascade are also antimicrobial [17]. Although the phenoloxidase cascade has been extensively studied in arthropods, there is relatively very little information about this system in molluscs [28–30].

In this study, changes in the phenoloxidase system, phagocytosis and its associated superoxide and hydrogen peroxide production, total haemolymph protein content and circulating haemocyte frequencies in Sydney rock oysters (*Saccostrea glomerata*) responding to injection of PAMPs (LPS and zymosan) were investigated. The data indicate that all of these immunological parameters are significantly enhanced by antigenic challenge.

2. Materials and methods

2.1. Oysters

Sydney Rock oysters (30–40 mm long from hinge to end of shell) were purchased from the Sydney Fish Markets (Sydney, Australia). They were kept in aerated aquaria containing seawater (20 l) at room temperature (25 °C). Seawater for the aquaria was collected from the Hawkesbury River, NSW, Australia.

2.2. PAMPs injection

The yeast cell wall antigen, zymosan A (Sigma Aldrich, Castle Hill, NSW) and LPS from *Escherisha coli* (055:B5, Sigma Aldrich) were suspended in sterile filtered seawater (FSW; 0.22 μm filtration; Millipore, North Ryde, NSW). Oysters were injected with 300 μl of zymosan or LPS with concentrations sufficient to yield doses in haemolymph ranging from 20 μg ml^{-1} to 400 μg ml^{-1}. These doses were based on the assumption that oysters, on average, contained 3 ml haemolymph. In all experiments, groups of control oysters were injected with 300 μl sterile FSW without PAMPs. Injections were made through the shell hinge using a 22-gauge needle fitted to a 1 ml syringe. Trial injections of 200 μl India ink showed that most of the injected material was localised in the adductor muscle.

2.3. Haemolymph collection

At various times after injection, oysters were removed from aquaria and allowed to air dry for 5 min. Haemolymph to be used for cytology or phagocytosis experiments was withdrawn using a 22-gauge needle fitted to a 5 ml syringe. A notch was made in the shell hinge and haemolymph was withdrawn from the foot sinus through the notch. Oysters were then shucked and the remaining haemolymph was extracted from the shell cavity. The semi-sterile haemolymph