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Endogenous molecules released by haemocytes receiving *Sargassum oligocystum* extract lead to downstream activation and synergize innate immunity in white shrimp *Litopenaeus vannamei*

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ABSTRACT

White shrimp *Litopenaeus vannamei* haemocytes receiving immunostimulating *Sargassum oligocystum* extract (SE) caused necrosis in haemocyte cells, which released endogenous EM-SE molecules. This study examined the immune response of white shrimp *L. vannamei* receiving SE and EM-SE *in vitro* and *in vivo*. Shrimp haemocytes receiving SE exhibited degranulation, changes in cell size and cell viability, necrosis and a release of EM-SE. Shrimp haemocytes receiving SE, EM-SE, and the SE + EM-SE mixture (SE + EM-SE) increased their phenoloxidase (PO) activity which was significantly higher in shrimp haemocytes receiving the SE + EM-SE mixture. Furthermore, shrimp haemocytes receiving EM-SE showed degranulation and changes in cell size and cell viability. Shrimp receiving SE, EM-SE, and SE + EM-SE all increased their immune parameters, phagocytic activity, clearance efficiency and resistance to *Vibrio alginolyticus,* being significantly higher in shrimp receiving SE + EM-SE. Meanwhile, the recombinant lipopolysaccharide- and β-1,3-glucan binding protein of *L. vannamei* (r*Lv*LGBP) was bound to SE, EM-SE, and SE + EM-SE. We conclude that in shrimp haemocytes receiving a non-self molecule, SE in dying cells released EM-SE which led to downstream activation and synergization of the immune response. This study demonstrated that the innate immunity of shrimp was elicited and enhanced by a mixture of endogenous molecules and exogenous substances (or immunostimulants).

1. Introduction

Like other crustaceans, shrimp rely on an innate immunity to defend against pathogen invasion and maintain their health [1]. Circulating haemocytes are the important components in protecting animals against pathogens through recognition, phagocytosis, melanization and cytotoxicity [2]. Three different classes of haemocytes, hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs) are identified based on cell size and degree of granularity [2,3]. The innate immune system is initiated via the recognition and binding of non-self molecules of microbial polysaccharides like β-1,3-glucan (βG), lipopolysaccharide (LPS), and peptidoglycan (PG), as well as seaweed polysaccharides like alginate, carrageenan, fucoidan, and laminarin [3,4]. These polysaccharides can be termed pathogen-associated molecular patterns (PAMPs) and are commonly used as immunostimulants to increase the immune response in fish and shellfish [4,5].

Once PAMPs have entered the haemocoel of a host, they are recognized by host proteins referred to as pattern recognition proteins (PRPs), or receptors (PRRs) [6–8]. In shrimp, several types of PRPs, like lipopolysaccharide- and β-1,3-glucan binding protein (LGBP), β-1,3-glucan binding protein (βGBP), and C-type lectin, have been identified [9,10]. Recombinant tiger shrimp *Penaeus monodon* LGBP (r*Pm*LGBP) and recombinant *Litopenaeus vannamei* LGBP (r*Lv*LGBP) are able to recognize and bind to LPS, βG, alginate, carerageenan, fucoidan, laminarin, red seaweed *Gracilaria tenuistipitata* extract, and brown seaweed *Sargassum duplicatum* extract, and subsequently activate innate immunity [11,12]. In shrimp, a number of extracts derived from brown seaweed *Sargassum* species like *S. fusiforme*, *S. duplicatum*, S. *cristaefolium,* and *S. hemiphyllum* have been reported to increase the immune response and resistance against *Vibrio alginolyticus* and white spot syndrome virus (WSSV) [13–15].

HCs are mainly involved in phagocytosis, an important cellular reaction in eliminating intruders [16]. SGCs and GCs can be triggered to cause degranulation (exocytosis) and release immune proteins such as prophenoloxidase (proPO), prophenoloxidase activating enzyme (ppA), peroxinectin, and antimicrobial peptides [3]. During the process of phagocytosis, superoxide anion (known as respiratory burst, RB) and other reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), singlet oxygen ($^1O_2$), and nitric oxide (NO) are generated and all play crucial roles in microbicidal activity [17,18].

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In crayfish haemocytes, degranulation and cell size change occur in response to microbial polysaccharides [19,20]. Degranulation is also induced in white shrimp *L. vannamei* haemocytes receiving fucoidan and spirulina [21,22]. Reductions in the percentage of large cells and increases in the percentage of small cells occur in white shrimp haemocytes receiving carrageenan, fucoidan, and LPS [21,23,24]. In addition, a decrease in the percentage of viable cells and an increase in the percentage of non-viable cells occur in white shrimp haemocytes receiving carrageenan [24]. Furthermore, cell necrosis and a release of endogenous molecules occur in white shrimp haemocytes receiving βG, LPS, and PG [25]. In mammals, intracellular molecules or endogenous molecules known as damage-associated molecular patterns (DAMPs) are released by dying cells due to exogenous pathogens, PAMPs or trauma, and convey a similar message upon stimulation [26,27]. However, nothing is known about the immune response of shrimp receiving endogenous molecules in the presence of exogenous substances.

We assumed that shrimp haemocytes that receive an immunostimulant or a PAMP like *Sargassum oligocystum* extract (SE) will suffer degranulation and changes in haemocyte size and viability as well as cell necrosis (cell death) and release endogenous molecules (called EM-SE herein), and assumed that shrimp haemocytes receiving EM-SE in the presence of SE exhibit downstream activation and synergize innate immunity. Therefore, we examined the recognition and binding of r*Lv*LGBP to SE, EM-SE, and the SE + EM-SE mixture (SE + EM-SE), and examined degranulation, cell size change, cell viability change and necrosis in shrimp haemocytes receiving SE and EM-SE. We examined phenoloxidase (PO) activity and RB in shrimp haemocytes receiving SE, EM-SE, and SE + EM-SE *in vitro*, and examined immune parameters, phagocytosis, and clearance efficiency under exposure to *V. alginolyticus* as well as resistance against *V. alginolyticus* in white shrimp *L. vannamei* receiving SE, EM-SE, and SE + EM-SE *in vivo*.

2. Materials and methods

2.1. Experimental animal

White shrimp L. *vannamei* were obtained from the Aquatic Animal Center, National Taiwan Ocean University, and shipped to our laboratory, placed in 8-tonne fiberglass tanks and acclimated to room temperature for two weeks prior to experiments. Shrimp were fed a formulated shrimp diet at 5% of their body weight daily during the acclimation period. The shrimp we used weighed 12.8–15.1 g and averaged 13.7 ± 1.2 g (mean ± SD).

2.2. Preparation of S. oligocystum extract (SE)

S. oligocystum was collected from the coast of Cagayan, Philippines. The preparation of *S. oligocystum* extract (SE) followed a previously described method [14]. A proximate analysis of SE showed that it contained 3.8% crude protein, 0.2% crude lipid, 60.2% ash and 35.8% carbohydrates (nitrogen-free extract and crude fiber). Carbohydrates were composed of 40% D-galactose, 55% D-fucose and 5% D-fructose based on HPLC after hydrolytic reduction and acetylayin [28].

2.3. Experimental design

Test solutions of SE were prepared by dissolving 0, 50, 100, 300, and 500 mg in 100 ml of shrimp salt solution (SSS, 30 mM trisodium citrate, 340 mM sodium chloride, pH 7.5, and 718 mOsm kg^{-1} osmolality) to become 0, 0.5, 1, 3, and 5 mg ml^{-1}. Eight experiments were conducted: (1) degranulation of shrimp haemocytes, (2) shrimp haemocyte cell size and cell viability, (3) cell necrosis and collection of the endogenous molecules, EM-C that was released from dying shrimp haemocytes receiving SSS, and endogenous molecules, EM-SE that was released from dying shrimp haemocytes receiving SE, (4) PO activity and RB in shrimp haemocytes receiving SE, EM-SE, and SE + EM-SE *in vitro,* (5) immune parameters of shrimp receiving SE, EM-SE, and SE + EM-SE, (6) phagocytytic activity, phagocytic index and clearance efficiency of *V. alginolyticus* in shrimp receiving SE, EM-SE, and SE + EM-SE, (7) the resistance of shrimp receiving SE, EM-SE, and SE + EM-SE against *V. alginolyticus* and (8) the binding of r*Lv*LGBP with SE, EM-SE, and SE + EM-SE.

2.4. Degranulation of shrimp haemocytes receiving SE

There were three SE test solutions (0, 1, and 3 mg ml^{-1}). Haemolymph (100 μl) was individually withdrawn from the ventral sinus of each shrimp and diluted with 900 μl of an anticoagulant solution (30 mM trisodium citrate, 340 mM sodium chloride and 10 mM EDTA, pH 7.5, and osmolality adjusted to 718 mOsm kg^{-1} with 115 mM glucose). One hundred microlitres (100 μl) of diluted haemolymph was placed on clean pyrogen-free coverslips, placed in 24-well multiple trays, and left to stand at 28 °C for 15 min to allow cells to settle and attach to the glass surface. A freshly prepared monolayer was overlaid with 200 μl of 0, 1, and 3 mg ml^{-1} SE and incubated at 28 °C for different periods of time (10–60 min). Haemocytes were fixed with 200 μl of 1% paraformaldehyde solution for 5 min and the coverslips were gently transferred onto a slide glass and examined under a phase-contrast microscope (Model BX51, Olympus, Tokyo, Japan) [19,21,22].

2.5. Cell size and cell viability in shrimp haemocytes receiving SE

There were five SE test solutions (0, 0.5, 1, 3, and 5 mg ml^{-1}). Cell size and cell viability assays were examined at different incubation times (0, 15, 30, 45, and 60 min) for each test solution. Cell size and the number of cells were expressed as dots and a quantified histogram was quantified and displayed on a log or linear scale of the size scatter height (SSC-H) and forward scatter height (FSC-H). Cell viability was expressed as a distribution histogram of PI fluorescence (585 nm yellow/orange fluorescence, PI-H). The details of haemolymph sampling and assays of cell size change and cell viability were conducted as previously described [21,23].

2.6. Cell necrosis of shrimp hemocytes receiving SE

There were three SE test solutions (0, 1, and 3 mg ml^{-1}). Cell necrosis examination was conducted similar to the cell size assay except for the final step. Briefly, we added 200 μl of SSS to make the cell suspension and then added 200 μl of 2% paraformaldehyde to fix the haemocytes. The mixture was centrifuged at 800 *g* for 10 min at 4 °C. Four hundred microlitres (400 μl) of SSS were added to suspend the precipitated cells, followed by the addition of 10 μl of RNase (1 mg ml^{-1}). This solution was incubated for 30 min at 37 °C. Twenty microlitres (20 μl) of PI solution (400 mg ml^{-1}) was added and the mixture was incubated for another 15 min at room temperature. Next, 50 μl of the mixture was sampled, placed on a glass slide and centrifuged on a cytospin machine (Thermo, Shandon, UK) at 1000 rpm for 3 min. Permanent slides were made and placed on a fluorescence microscope (Model BX 51, Olympus, Tokyo, Japan) for the observation of cell death (necrosis) [29].

2.7. Preparation of EM-SE

Five hundred microlitres (500 μl) of haemolymph were withdrawn from the shrimp, placed in a tube containing 500 μl of anticoagulant solution, and centrifuged at 800 *g* at 4 °C for 10 min. The supernatant was discarded and 200 μl SSS added to make a cell suspension. One hundred microlitres (100 μl) of SE solution (3 mg ml^{-1}) was added and the suspension incubated for 30 min at 4 °C. The mixed solution (300 μl) was centrifuged at 800 *g* at 4 °C for 10 min. The supernatant was discarded and the precipitated cells rinsed with 100 μl of marine saline