Immunomodulatory effects of seagrass *Halophila ovalis* polysaccharide mixed feed in adult black tiger shrimp *Penaeus monodon* and its protective efficacy against white spot syndrome virus infection

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Received: July 2016 Accepted: October 2016

Abstract

As white spot syndrome virus (WSSV) can be highly pathogenic in penaeid shrimp, various feed supplements have been tested to help to protect farmed shrimp against WSSV disease. Here a polysaccharide extract from *Halophila ovalis* (HO) seagrass was added to feeds at concentrations of 0.25, 0.5, and 1.0 g/kg to assess its ability to protect Black Tiger shrimp (*Penaeus monodon*) against WSSV challenge. Following feeding on these diets for 25 days, *P. monodon* were challenged by muscle injection and monitored for 21 days. On Day 0 and on Days 7 and 21 post-injection (pi), total haemocyte counts (THC), total protein concentrations, prophenoloxidase activity and respiratory burst activity were compared using haemolymph collected from 10 shrimp. All shrimp fed the basal diet died by Day 7 pi but survival times were extended among shrimp fed diets containing HO polysaccharide (HOP), and significantly at concentrations of 0.5 or 1 gkg^{-1}. Concomitantly with improved survival, all haemolymph immune parameters examined were enhanced significantly ($p<0.05$) among shrimp fed diets containing higher amounts of HOP. WSSV infection loads determined by real-time PCR were also lowered. The data suggest that if shrimp growth performance is not affected, inclusion of 0.5-1 gkg^{-1} HOP in commercial feeds might increase resilience of pond stocks of *P. monodon* against WSSV disease and when disease occurs, provide farmers with a longer management window to minimize economic losses.

Keywords: *Halophila ovalis*, Immune response, Polysaccharide, RT-PCR, WSSV

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Introduction

White spot syndrome virus (WSSV) has become established in most parts of the world as the most serious pathogen of farmed shrimp (Sanchez-Paz, 2010; Simrouni *et al.,* 2014). While there is growing data to suggest that shrimp and other invertebrates possess an immune system with limited capacity for memory, defense against pathogens appears to be mediated primarily by non-specific innate responses (Sarathi *et al.,* 2007).

Traditional medicinal plant extracts and various other immune-stimulants fed to shrimp have proved useful in providing limited protection against WSSV challenge (Balasubramanian *et al.,* 2007; Sanchez-Paz 2010). For example, Kuruma (*Penaeus japonicas*) and Black Tiger shrimp (*P. monodon*) shrimp fed on diets including peptidoglycan or β–1,3-glucan have been found to more tolerant of WSSV disease (Chang *et al.,* 2003; Wang *et al.,* 2008) and studies examining dietary polysaccharides such as glucan (Chang *et al.,* 2003), sodium alginate (Cheng *et al.,* 2004), lipopolysaccharide (Felix, 2005), peptidoglycan (Purivirojkul *et al.,* 2006), and fucoidan (Immanuel *et al.,* 2012) have shown to enhance factors believed important to shrimp immune function. Potent immuno-stimulatory effects enhancing protection against WSSV have also been obtained with feeds including crude extracts of various medicinal plants (Balasubramanian *et al.,* 2007; Rameshthangam and Ramasamy, 2007; Immanuel *et al.,* 2012).

Haemocytes play a crucial role in the innate immune system of crustaceans. Their numbers can increase in response to infection and environmental stress. They become degranulated when activated and have a key function in generating active phenoloxidase (PO) from prophenoloxidase (proPO) to catalyze the stepwise oxidation of phenols to quinines and produce melanin. PO activity is thus a useful marker of immune stimulation. NADPH-oxidase driven superoxide anion generation produces toxic metabolites that are capable of destroying invasive pathogens. This cellular defense reaction is immunologically vital. nitroblue tetrazolium (NBT) reduction assay appears to be a simple and reliable method for rapid quantification of intracellular superoxide anion production.

While feeds containing polysaccharides extracted from seaweed have been found to reduce WSSV disease impacts in shrimp (Immanuel *et al.,* 2010; Immanuel *et al.,* 2012), little is known about what immune parameters are stimulated by the polysaccharides. To investigate this, feeds including 3 concentrations of a crude polysaccharide extract of seagrass *Halophila ovalis* (HO) were fed to *P. monodon* before injection challenge with WSSV. Each supplemented diet was found to enhance haemolymph-mediated immune parameters including total haemocyte count (THC), protein concentration, prophenoloxidase activity (proPO) and respiratory burst

activity (NBT assay) as well as extend shrimp survival in a dose-dependent manner.

Materials and methods

Polysaccharide extraction

Halophila ovalis (HO) seagrass was collected at low tide from Chunnambar estuary, Pondicherry, India, in September 2015. Seagrass was rinsed in seawater followed by tap water and distilled water to remove epiphytes and other contaminants and then dried under shade. Portions (20 g) of dried whole plant were ground to fine powder, suspended in 400 mL 0.1 M sodium acetate pH 6.0 containing 2 g papain, 5 mM EDTA and 5 mM cysteine and incubated at 60°C for 24 h. The extract was then clarified by centrifugation at 6000×*g* for 10 min and the supernatant filtered through glass (G-3) filter paper. Sulfated polysaccharides in the supernatant were precipitated by mixing with 800 mL absolute ethanol for 24 h and collected by centrifugation at 2560 × *g* for 20 min at 4°C. Drying the pellet at 60°C for 12 h resulted in ~1.3 g (dry weight) of crude HO polysaccharide (HOP)/20 g extracted seagrass.

Feed preparation

Feeds were prepared to include 0.25, 0.5 and 1 g kg^{-1} HOP with concomitant decreases in the 0.2% cellulose component of the basal diet formation described previously (Yeh *et al.*, 2008). All dry ingredients were mixed thoroughly, and 2.5% gelatin solution containing active ingredients was added along with the oils and water and mixed in to produce a dough-like consistency. This dough was cold-extruded through a pelletizer into feed pellets of an appropriate size that were then dried at 40°C in an oven and stored at 4°C in airtight containers until used.

Shrimp

Healthy *P. monodon* (15±2 g) were collected from ponds at a farm in Marakanam, Tamilnadu, India, transferred to the laboratory and stocked into 3000 L capacity fiberglass tanks containing filtered and aerated sea water (32 ± 1 ppt salinity, >6 ppm dissolved oxygen, 28±1°C, pH 8.2±0.1). Shrimp were fed commercial feed pellets (CP Aquaculture, India, 41% crude protein, 6% fat, 2% fiber, 13% ash, 11% moisture) before the feeding experiment commenced.

Feeding experiment

Uniform-sized *P. monodon* were selected and transferred to 1000 L capacity tanks with flow through seawater and aeration as for the 1000 L tanks and fed *ad libitum* a rate of 10% body weight at 7:00, 15:00 and 23:00 h. Waste and uneaten food were removed before feeding and water flow through resulted in 25% water exchanged daily. Triplicate tanks each containing 25 shrimp (*n*=75 shrimp/diet) were feed the basal diet or basal diets formulated to contain 0.25, 0.5 or 1 g/kg HOP for 25 days.

WSSV inoculum

P. monodon infected with WSSV and displaying typical gross signs of white spot disease were collected from a farm near Marakanam, Tamilnadu, India. Soft head tissues (1 mg) including gills from each of 10 shrimp were homogenized in 2 mL PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at 1:10 (w/v) and centrifuged at 8000×*g* for 20 min at 4°C. The supernatant was filtered through 0.22 µm filter and stored at –20°C until used. The presence of WSSV in all was confirmed by nested PCR (Yoganandhan *et al.,* 2003; Immanuel *et al.,* 2010).

WSSV challenge

After feeding for 25 days, tail muscle at the second abdominal segment of each of the 75 *P. monodon* fed the different diets were injected with 10 µL of WSSV inoculum. Shrimp in another group of 75 spread across three tanks and fed the basal diet were injected with 10 µL WSSV inoculum (positive control) and 10 µl saline as a negative control. Survival was monitored at 6 h intervals, and any dead shrimp found from Day 6 post-injection (pi) onwards were removed. Survival data at each monitoring time were collected for each group and presented for each day up to Day 21 pi as described below.

Haemolymph collection

Haemolymph used to determine THC, prophenoloxidase activity (proPO), superoxide anion activity (NBT) was sampled from shrimp at the time (Day 0) of WSSV injection and on Days 7 and 21 pi. Haemolymph (0.2 mL) was withdrawn from the base of the second leg of ventral segments using 1mL syringe fitted with 26 gauge needles. Each syringe was pre-filled with 800 µL ice-cold Alsever's solution as anticoagulant (Kakoolaki *et al.,* 2011).

THC and total protein concentration

For determine THCs, 10 µL haemolymph in Alsever's solution was diluted immediately with 0.5% tryphan blue in 2.6% NaCl and haemocytes were then counted (total haemocytes/mm^3) using a Neubauer chamber hemocytometer and phase contrast microscope (Kakoolaki *et al.,* 2010a). Total protein concentrations were estimated spectrophotometrically (A_{660nm}) using the Lowry method and bovine serum albumin as a standard (Lowry *et al.,* 1951).

Prophenoloxidase activity

ProPO activity in haemolymph was determined spectrophotometrically using L-Dihydroxyphenylalanine (L-DOPA) as a substrate (Afsharnasab *et al.,* 2016) with slight modifications. Briefly, 200 µL of haemolymph was mixed with 200 µL of anticoagulant and centrifuged at 800*g* for 10 min at 4°C. The upper layer was used as plasma in this experiment. Twenty µL of plasma were strewed in cuvette as an unknown sample and 20 µL anticoagulant strewed in another cuvette used as a control. After 1 min, 880 µL L-DOPA was added to both cuvette and the amount of dopachrome produced were

determined by measuring A_{490nm}. Each 1 U of enzyme activity was defined as a 0.001/min increase in absorbance per mL haemolymph.

Respiratory burst activity

Respiratory burst of haemocytes was quantified using NBT to formazan reduction method (Chen *et al.*, 2014) with slight modifications. Briefly, 100 µL of diluted haemolymph in anticoagulant solution was dispensed in triplicate into microplate wells coated beforehand with 100 µL 0.2% poly-L-lysine solution to improve cell adhesion. Plates were centrifuged at 500×*g* for 20 min at 4°C, the supernatant removed and 100 µL zymosan (0.1% in Hank's Balanced Salt Solution) was added and allowed to react for 30 min at room temp, followed by addition of 100 µL 0.3% NBT solution, staining for 30 min at room temp and addition of 100 µL methanol. The solution was then discarded; the plates washed three times with 100 µL 70% methanol and air-dried. Formazan was dissolved in 120 µL 2 M KOH and 140 µL DMSO. Plate well absorbance was determined at A_{630nm}.

DNA extraction and PCR

After challenge with WSSV, gill tissue was collected from 10 shrimp from each treatment group and preserved in 70% ethanol. Before use, gill tissue was rehydrated in distilled water for 1 h. Total genomic DNA was extracted from ~10 mg gill tissue using a Shrimpex DNA extraction kit and quantified at A_{260nm} using a Nanodrop spectrophotometer (Thermo Scientific Inc., USA). To quantify WSSV DNA copy numbers, 50-200 ng total DNA was added to SYBR green RT-PCR master mix (Applied Biosystems) in a 25 µL reaction containing WSSV PCR primers provided by Mangalore Biotech, India. A WSSV fragment containing a 341 bp target amplicon for RT-PCR was ligated into the plasmid pGEM-T-Easy vector (Promega, Wisconsin, USA) and cloned into *E. coli* (DH5α). Automated DNA sequencer (Applied Biosystems Inc., USA) confirmed the target segment in the recombinant plasmid. The copy number of the target amplicon was estimated and 10-fold dilutions were made to use as standards for quantification. DNA samples were amplified in triplicate using the thermal cycling conditions 95°C for 5 min, 35 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 30 s using a StepOne™ 48-well real-time thermal cycler (Applied Biosystems). Cycle threshold (Ct) data obtained were analyzed by using StepOne software v2.1. A set of standard 10-fold dilutions (from 10^{10} to 10^2 WSSV DNA copies/µL) was run simultaneously with 3 no-template controls and duplicate DNA samples were used to generate a linear standard curve of Ct value vs DNA copy number from which WSSV DNA copies in each sample could be determined (Immanuel *et al*., 2012).

Statistical analysis

All data generated were expressed as a mean±standard deviation and compared

using the SPSS Version 7.5 statistics software (SPSS Inc.). Statistical differences between the experimental groups were analyzed using Students '*t*' test with a *post hoc* multiple comparison of Tukey's test at a significant level of $p<0.05$.

Results

Shrimp survival following WSSV challenge

Survival rate among triplicate groups of 25 *P. monodon* (n=75) challenged with WSSV after being fed on a basal diet or diets containing 0.25, 0.5 and 1 g HOP/kg was determined (Fig. 1).

Shrimp fed the basal diet began dying from Day 3 of challenge and none of the shrimp remained alive by Day 7 post-challenge. Shrimp fed the diet containing 0.25 g HOP/kg di*et al*so began dying from Day 3 but it took until Day 10 before none remained alive.

Shrimp fed the diet containing 0.5 g HOP/kg diet began to die from Day 6 with deaths accumulating progressively, and on Day 21 when the bioassay was terminated, 18% of the shrimp remained alive. Shrimp fed the diet containing 1 g HOP/kg diet began to die from Day 9 with deaths accumulating progressively but more slowly, and on Day 21, 41% of the shrimp remained alive. Deaths were commonly preceded by shrimp displaying a pinkish-red body discoloration, lethargy, loss of appetite and anorexia known to be causative of WSSV disease.

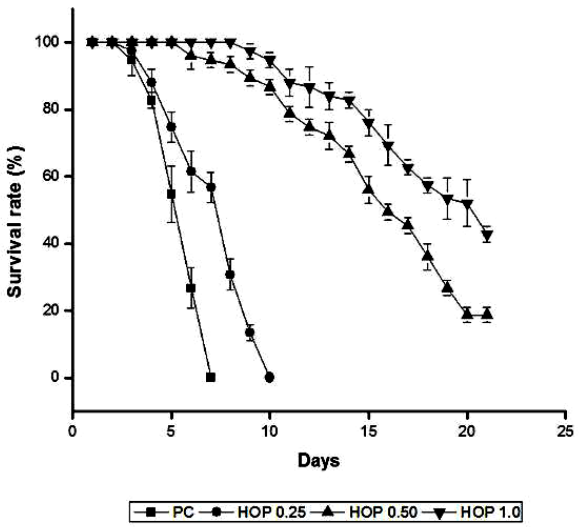

Figure 1: Survival rate of *Penaeus monodon* fed on diets containing *Halophila ovalis* polysaccharide extract during challenge experiment with WSSV in different day's interval. Values are mean±SD of three determinations. PC-positive control shrimp fed the basal diet and challenged with WSSV; HOP-WSSV challenged shrimp feed using fed containing *Halophila ovalis* polysaccharide at concentrations of 0.25, 0.5 or 1 g/kg.

THC

After feeding *P. monodon* on various diets for 25 days, there was little variation in mean haemocyte numbers counted in haemolymph of shrimp fed the basal diet (1.23×10^7 cells/mL) compared to shrimp fed the diet containing 0.25 g HOP/kg diet (Fig. 2). However, mean THCs in shrimp fed on diets containing 0.5 and 1 g HOP/kg diet showed dose-related significant ($p<0.05$) increases. On Day 7 pi of WSSV, THCs decreased markedly (0.25×10^7 cells/mL) in shrimp fed the basal diet, but only marginally in shrimp fed diets containing 0.25, 0.5 and 1 g HOP/kg diet (1.19×10^7, 1.27×10^7 and 1.40×10^7 cells/mL, respectively). On Day 21 pi of WSSV the THCs had recovered in shrimp fed the diet containing 0.5 g HOP/kg diet (1.44×10^7 cells/mL) and were elevated significantly in shrimp fed the diet containing 1 g HOP/kg diet (1.81×10^7 cells/mL).

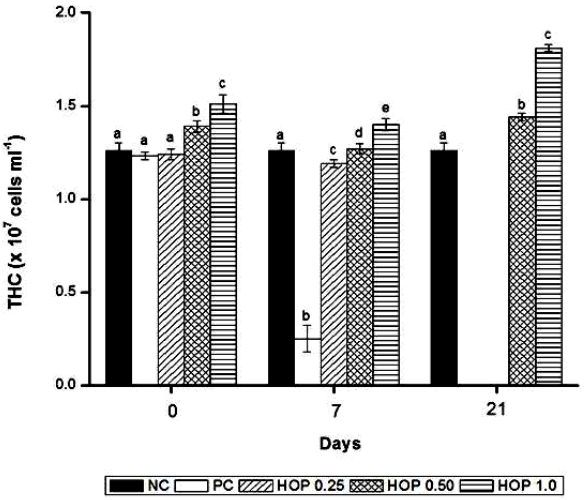

Figure 2: Total hemocyte count (THC) of shrimp *Penaeus monodon* fed on diets containing *Halophila ovalis* polysaccharide extract on different concentrations (0, 0.25, 0.5 or 1 g kg^{-1} HOP) during challenge experiment with WSSV in different day intervals. Values are mean±SD of three determinations; bars with different letters are statistically significant from each other (t-test; $p<0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC-Negative control, PC-Positive control, HOP-*Halophila ovalis* polysaccharide).

Haemocyte total protein

The mean total protein amounts present in haemolymph withdrawn from 10 shrimps selected at random from the groups fed the basal diet for use as either saline-injected or WSSV-injected controls ranged from 105-110 $mgmL^{-1}$ (Fig. 3). On Day 7 pi of WSSV, relative protein amounts increased significantly ($p<0.05$) in shrimp fed the basal diet and diets containing lower amounts on HOP in a dose-related manner (159 mg mL^{-1} with basal diet, 148 $mgmL^{-1}$ and 139 $mgmL^{-1}$ with the 0.25 and 0.5 g HOP/kg diets, respectively), but reduced slightly (127 mg mL^{-1}) in shrimp fed the 1 g HOP/kg diet.

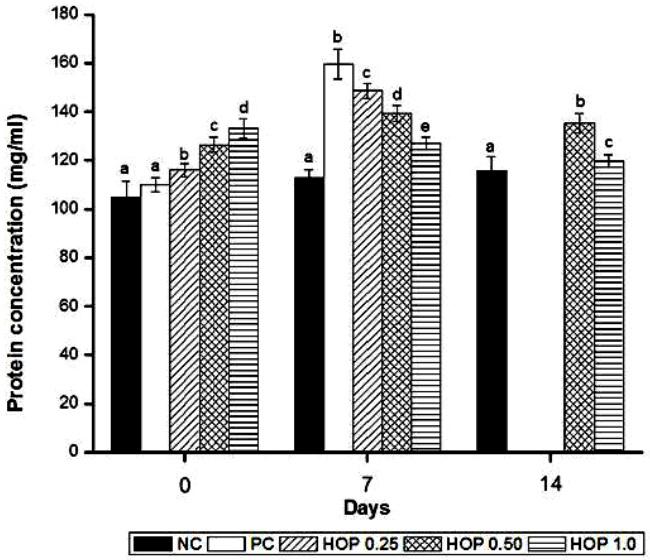

Figure 3: Total protein concentration in haemolymph of *Penaeus monodon* fed on a basal diet or diets containing 0, 0.25, 0.5 or 1 g/kg HOP during challenge experiment with WSSV in different day's interval. Values are mean±SD of three determinations; bars with different letters are statistically significant from each other (t-test; $p<0.05$ subsequent post hoc multiple comparison with Tukey's test) (NC-Negative control, PC-Positive control, HOP-*Halophila ovalis* polysaccharide).

On Day 21 pi of WSSV, relative protein amounts in shrimp remaining alive in 0.5 and 1 g HOP/kg diet groups reduced significantly ($p<0.05$) to 135 $mgmL^{-1}$ and 119 $mgmL^{-1}$, respectively.

Prophenoloxidase activity

Prophenoloxidase activity (proPO) in haemolymph showed dose-related significant ($p<0.05$) increases in groups fed diets containing 0.25, 0.5 and 1 g HOP/kg diet(0.162 to 0.198 OD_{490nm}) compared to shrimp fed the basal diet (0.147 OD_{490nm}) (Fig. 4). On Day 7 pi of WSSV, mean proPO activity levels decreased markedly and proportionally in shrimp fed the basal diet (0.026 OD_{490nm}) and 3 diets containing 0.25 to 1 g HOP/kg diet (0.052 to 0.077 OD_{490nm}). On Day 21 pi of WSSV, relative proPO activities in shrimp remaining alive in 0.5 and 1 g HOP/kg diet groups increased again to levels slightly below those detected before WSSV challenge (0.174 and 0.181 OD_{490nm} for the 0.5 and 1 gkg^{-1} HOP diets, respectively).

Respiratory burst activity (NBT assay)

Similar to proPO activities the respiratory burst activity in haemolymph showed dose-related significant ($p<0.05$) increases in the 3 shrimp groups fed diets containing 0.25 to 1 gkg^{-1} HOP (0.41 and 0.81 OD_{630nm}, respectively) compared to shrimp fed the basal diet (0.34 OD_{630nm}) (Fig. 5).

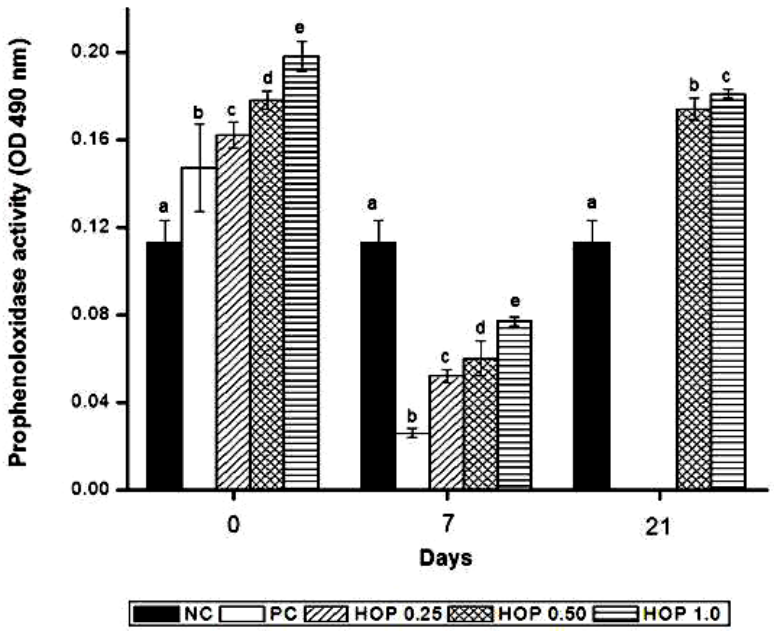

Figure 4: Prophenoloxidase (PO) activity in haemolymph of *Penaeus monodon* fed on a basal diet or diets containing 0, 0.25, 0.5 or 1 gkg^{-1} HOP during challenge experiment with WSSV in different day intervals. Values are mean±SD of three determinations; bars with different letters are statistically significant from each other (t-test; p<0.05 subsequent post hoc multiple comparison with Tukey's test) (NC-Negative control, PC-Positive control, HOP- *Halophila ovalis* polysaccharide).

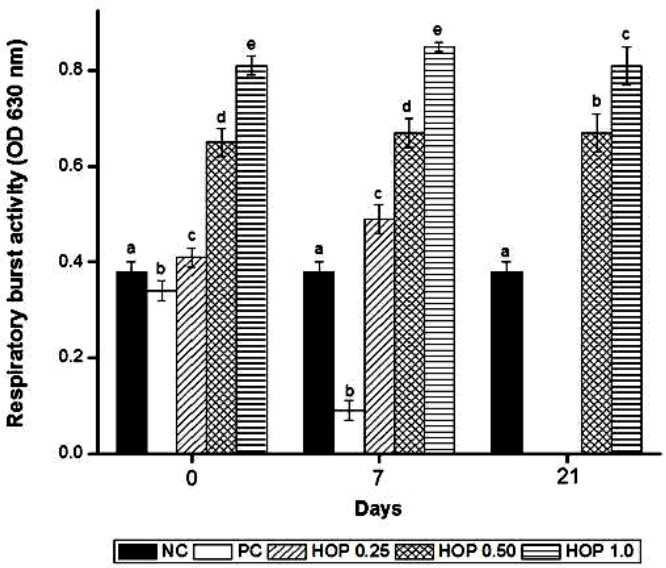

Figure 5: Respiratory burst activity (NBT assay) in haemolymph of *Penaeus monodon* fed on a basal diet or diets containing 0, 0.25, 0.5 or 1 gkg^{-1} HOP during challenge experiment with WSSV in different day intervals. Values are mean±SD of three determinations; bars with different letters are statistically significant from each other (t-test; p<0.05 subsequent post hoc multiple comparison with Tukey's test) (NC-Negative control, PC-Positive control, HOP- *Halophila ovalis* polysaccharide).

On Day 7 pi of WSSV, respiratory burst activity dropped significantly in shrimp fed the basal diet (0.09 OD_{630nm}) but increased slightly but significantly ($p<0.05$) in the 3 shrimp groups fed 0.25 to 1 gkg^{-1} HOP (0.49 to 0.85 OD_{630nm}). On Day 21 pi of WSSV, relative respiratory burst activities in shrimp remaining alive in 0.5 and 1 g/kg HOP diet groups dropped slightly to be similar to the levels detected before WSSV challenge (0.67 and 0.81 OD_{630nm} for the 0.5 and 1 g/kg HOP diets, respectively).

WSSV quantification by real-time PCR

The WSSV infection loads in challenged *P. monodon* fed the different diets were quantified by real-time PCR using a 10-fold dilution series of WSSV DNA of known copy number. Gill tissue (10 mg) collected from each of 10 shrimp selected from each diet group on Day 7 (0.25 gkg^{-1} HOP diets) and 21 pi (0.5 and 1 gkg^{-1} HOP diets) of WSSV was pooled for DNA extraction and amplified using a real-time PCR test. Among the groups of WSSV challenged shrimp, the group fed the basal diet generated a Ct = 11.78 (2.12 x 10^8 WSSV DNA copies/ng total DNA). WSSV DNA amounts in shrimp fed the diet containing 0.25 g/kg HOP were higher (Ct=7.94, 4.42×10^9 WSSV DNA copies/ng total DNA) but significantly reduced in shrimp fed either 0.5 gkg^{-1} HOP (Ct=24.92; 6.45×10^3 WSSV DNA copies/ng total DNA) or 1 gkg^{-1} HOP (Ct=31.94; 25.05 WSSV DNA copies/ng total DNA).

Discussion

Immunostimulants have been investigated intensively as a means of boosting the defense capabilities of farmed shrimp against pathogens (Huynh *et al*., 2011). The solvent extracts of seagrass *Halophila ovalis* have been reported to possess wide ranging biological activities (Hua *et al.*, 2006; Yuvaraj *et al.*, 2012). A crude polysaccharide extract of this seagrass species was incorporated into shrimp feed to investigate its ability to enhance defense responses in haemolymph and to protect *Penaeus monodon* against challenge by WSSV.

Among groups of *P. monodon* fed on either a basal diet or the same diet containing 0.25, 0.5, and 1 g HOP/kg diet, the rate at which mortalities accumulated over a 21 day period following injection of a standardized dose of WSSV was slowed markedly, particularly among shrimp fed on diets containing the 2 higher concentrations of HOP. Similar results were observed in WSSV challenge of *P. monodon* fed on diets supplemented with increasing concentrations of fucoidan (Immanuel *et al.*, 2012). However, this is the first report on polysaccharide from seagrass *H. ovalis* showing anti-viral properties.

In invertebrates, haemocytes have a primary pathogen defense role that includes clotting, non-self-recognition, phagocytosis, melanization, encapsulation, cytotoxicity and cell-to-cell communication (Johansson *et al.*, 2000). Among *P. monodon* fed on diets containing 0.25, 0.5 and 1 g HOP/kg diet for 25 days, haemocyte numbers

were elevated in shrimp fed at the 2 higher HOP concentrations, suggesting that they might better resist pathogen challenge. Following challenge with WSSV, this was found to be the case. THCs were higher than prior to challenge, particularly in the shrimp fed the 1 g HOP/kg diet. Feeding *P. monodon* on diets containing 1-3% polysaccharide gel (PG) extracted from *Durio zibethinus* has similarly been noted to elevate THCs and protect them against WSSV (Pholdaeng and Pongsamart, 2010). Feeding shrimp on diets containing HOP thus appears to accelerate haemocyte maturation and release from haematopoietic tissue.

Crustaceans have an open circulatory system in which the hemolymph carries out several physiological functions. The protein concentration in hemolymph of crustaceans is found to be high due to WSSV infection (Lo *et al*., 1997). In the *P. monodon* fed on diets containing 0.25, 0.5 or 1 g HOP/kg diet, the total protein concentration significantly increased in a dose dependent manner. However, on Day 7 after challenge with WSSV, haemolymph protein amounts increased considerably relative to HOP concentration, and decreased somewhat among shrimp fed the 1 g HOP/kg diet. Such findings are consistent with relative haemolymph protein levels seen following WSSV challenge of *P. monodon* feed on diets containing herbal supplements (Citarasu *et al.*, 2006). The nature of the proteins that become more elevated in heamolymph when more HOP was included in the feed and that potentially help suppress WSSV replication and mortality in *P. monodon* remains to be determined.

The proPO system was found to be essential for the shrimp defense against invading pathogenic microorganisms. Activation of the proPO system promotes the release of other factors mediating non-self-recognition, melanin formation, adhesion and cell-to-cell communication (Amparyup *et al.*, 2013). In the *P. monodon* examined here, PO activity levels became more elevated as feed concentrations of HOP increased, and relative levels remained comparable even when activities were reduced markedly on Day 7 post-challenge with WSSV. The proPO activity rebounded among those shrimp fed 0.5 and 1 g HOP/kg diets that remained alive on day 21 post-challenge. Similarly, dietary effect of β-1, 3-glucan and fucoidan showed higher PO activity of *P. monodon* challenged with WSSV than the control group at the end of challenge experiment (Chang *et al*., 2003; Immanuel *et al*., 2012), and supports a key role for elevated proPO in helping protect shrimp against virus-induced disease.

Reactive oxygen intermediates (ROIs) including superoxide (O_2^-) anions are formed during respiratory bursts of phagocytosis that are part of the shrimp defense mechanism against microbial infection. While the levels of O_2^- anions can thus provide an accurate measure of the relative effectiveness of a potential immunostimulant (Munoz *et al.*, 2000), excessive production can be extremely toxic (Halmblad and

Soderhall, 1999). In the present study, the respiratory burst activity of experimental groups of shrimp at the beginning (0 day) was significantly (p<0.05) higher than that of the control group after WSSV challenge test. On the 7th day of challenge experiment, the respiratory burst activity decreased in the control group, whereas in experimental group, it increased with increasing concentrations. Nevertheless, at the end of challenge experiment (21st day), the shrimp recovered normal respiratory burst activity in galactan sulfate supplemented diets fed shrimp. Marked elevation in haemolymph O_2^- anion levels of up to 15.7-fold have been noted following feeding of adult *P. monodon* on beta-1,3-1,6-glucan extracted from yeast cell walls (Song *et al.,* 1997) as well as juvenile *P. monodon* fed an extract of an Indian traditional medicinal plant (*Cynodon dactylon*) before challenge with WSSV (Balasubramanian *et al.,* 2008). Any feed supplements capable of substantially elevating the capacity of shrimp to generate circulating ROIs thus seem to have potential to afford elevated protection against disease caused by WSSV.

As found previously in shrimp fed diets containing immune-stimulants derived from various sources (Balasubramanian *et al.,* 2008; Jang *et al.,* 2009; Immanuel *et al.,* 2012), WSSV DNA amounts and replication levels in gill tissue of *P. monodon* fed diets containing 0.25 to 1 gkg^{-1} HOP reduced the mortality rate. The present study revealed that the polysaccharide extract derived from *H. ovalis* has potent immune-stimulant properties when fed to shrimp as a feed component at concentrations of 0.5 to 1 g/kg, and that feeding of juvenile *P. monodon* for 25 days on such diets was sufficient to markedly slow the rate at which mortalities accumulated following injection challenge by WSSV. It now needs to be determined whether the commercial use of such diets might provide increased resilience of pond stocks of *P. monodon* against WSSV disease and when disease occurs, provide farmers with better management options to minimize economic losses.

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