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White Shrimp *Litopenaeus vannamei* that have received *Petalonia binghamiae* Extract Activate Immunity, Increase Immune Response and Resistance against *Vibrio alginolyticus*

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Abstract

This study was to examine immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus* when shrimp received *Petalonia binghamiae* extract in vitro and in vivo. Shrimp hemocytes incubated with 1 mg/ml extract showed increased Phenoloxidase (PO) activity and respiratory burst (RB, release of superoxide anion). The total hemocyte count (THC), Phenoloxidase (PO) activity, respiratory Burst (RB) of shrimp received the extract at 6 and 10 μg/g were significantly higher than those of shrimp received saline and the control shrimp after 48, 96 and 144 h. Superoxide Dismutase (SOD) activity of shrimp received the extract at 6 and 10 μg/g were significantly higher than those of shrimp received saline and the control shrimp after 48 h. Phagocytic activity and clearance efficiency to *Vibrio alginolyticus* in the shrimp that received the extract at 6 and 10 μg/g were significantly higher than those of shrimp received saline and the control shrimp after 24, 48, 96 and 144 h. In another experiment, shrimp which had received the extract at 2, 6, and 10 μg/g after one day were challenged with *V. alginolyticus* at 1.4×10^6 colony-forming units (cfu)/shrimp, and then placed in seawater. The survival rate of shrimp that received the extract at 6 and 10 μg/g was significantly higher than that of control shrimp after 12-144 h. It was concluded that *P. binghamiae* extract could cause activation of shrimp immunity, and concluded that shrimp received the extract at 6~10 μg/g increased immune response of shrimp as well as resistance to *V. alginolyticus* infection.

Keywords: *Litopenaeus vannamei*; *Vibrio alginolyticus*; *Petalonia binghamiae* extract; activation of immunity; Immune parameter; Phagocytic activity; Clearance efficiency

Introduction

White shrimp *Litopenaeus vannamei* is the primary crustacean species currently being cultured mainly in the Pacific rim countries, and its farmed production has reached 2.88 million tone in year 2011 [1]. However, shrimp farming has encountered disease outbreaks caused mainly by viral disease and vibriosis since last two decades under intensive culture activities that resulted in deteriorated pond environments [2,3]. The bacterium *Vibrio alginolyticus,* isolated from diseased *L. vannamei* with whitish musculature and inactivity is considered to be a secondary opportunistic pathogen and has caused mortality of shrimp under environment stress like ammonia and sulfide [4-6]. Therefore, prevention of incidence of disease epidemics and applications of immunostimulants and probiotics have become feasible ways to prevent pathogen infections [7,8].

Shrimp like other invertebrate rely on innate immune response in defending against hostile microbes [9]. The innate immune response is based on the hemocytes: hyaline cells, semi-granular cells and granular cells [10]. Circulating hemocytes are involved in pattern recognition of foreign particles that trigger degranulation, and initiate the prophenoloxidase (proPO) activating system, a well known component of the defense system [11]. Phenoloxidase (PO), an enzyme in the proPO cascase, is activated by minute amounts of cell-wall moiety like lipopolysaccharide (LPS), peptidoglycan (PG), and β-1,3-glucan (βG) from Gram-negative bacteria, Gram-positive bacteria and fungi, leading to formation of melanin [12].

Hemocytes are also involved in inflammatory-type reactions including phagocytosis, production of several Reactive Oxygen Species (ROS), and release of antimicrobial peptides [13]. Starting this process, the membrane-bound enzyme complex, NADPH oxidase, assembles after binding of a foreign particle to the cell, and reduces molecular oxygen to superoxide anion (O_2^-) [14]. The release of superoxide anion, known as the respiratory burst plays an important role in anti-bacterial activity [15]. An enzyme, Superoxide Dismutase (SOD) is considered to scavenge extra-amount of superoxide anion to less reactive hydrogen peroxide, and provide self-protection against oxidative stress.

Several bacterial polysaccharides like βG, lentinan, PG, schizophyllan, scleroglucan, and several brown seaweed polysaccharides like alginate, carrageenan, fucoidan and laminarin have been reported to increase the immune response of fish and shrimp [8,16-18]. Hot-water extracts of brown seaweed *Undaria pinnatifida* and *Sargassum autumnale* have been reported to increase the resistance of teleost against bacterial pathogen [19]. White shrimp *L. vannamei* that received the extract of *Sargassum cristaefolium*, *S. fusiforme, S. duplicatum, S. polycystum* and *S. hemiphyllum* var. *chinese* showed resistance against *V. alginolyticus*, *V. harveyi,* and WSSV challenges [20-25]. However, none is known about the mechanism and immune response of shrimp that received other species of brown seaweed.

The purpose of the present study was to examine immune response of white shrimp *L. vannamei* and its resistance against *V. alginolyticus* when shrimp were received the extract of *Petalonia binghamiae*. Several

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immune parameters were examined including Total Hemocyte Count (THC), PO activity, RB, SOD activity, phagocytic activity and bacterial clearance efficiency, as well as resistance to *Vibrio alginolyticus* infection when shrimp received the *P. binghamiae* extract.

Materials and Methods

Culture of *V. alginolyticus*

A pathogenic strain of *V. alginolyticus* isolated from diseased white shrimp *L. vannamei*, which displayed symptoms of anorexia, inactivity, poor growth and necrotic musculature, was used for the study [4]. It was cultured on tryptic soy agar (TSA supplemented with 2.0% NaCl, Difco) for 24 h at 25°C before being transferred to 10 ml tryptic soy broth (TSB supplemented with 2.0% NaCl, Difco), where it remained for 24 h at 25°C as stock culture for tests. The broth cultures were centrifuged at 7155×g for 15 min at 4°C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution to make 7×10^7, and 9×10^7 colony-forming unit (cfu)/ml as bacterial suspensions for the test of challenge, and tests of phagocytosis and clearance, respectively [26].

Preparation of *P. binghamiae* hot-water extract

P. binghamiae was collected from the North east coast of Taiwan. The algal fronds were washed with water and dried naturally at room temperature. They were ground, and the hot-water extract was prepared based on a method describe before [22]. The yield of extract obtained from the powder was 23.4% based on dry algal weight. It contains 3.5% of sulfate and 86.3% of sugar that contains galactose (35.10%), glucose (34.65%), fucose (14.36%), mannose (10.69%), xylose (3.47%), and arabinose (1.73%), as analyzed by GC-MS after hydrolysis, reduction and acetylation [27,28].

Experimental design

The extract was prepared with a shrimp marine saline to make concentrations of 1, 3, and 5 mg/ml, respectively [29]. About one thousand shrimp from the University Marine Station were shipped to the laboratory. Shrimp were placed in fiberglass tanks (5 m^3) containing aerated seawater, and acclimated for 2 weeks. During the acclimation period, shrimp were fed twice daily with a formulated shrimp diet (Tairou Feed Company, Tainan, Taiwan). Only shrimp in the intermoult stage were used for the study. The moult stage was determined by examination of uropoda in which partial retraction of the epidermis could be distinguished [30]. Four studies were conducted. (1) For the study on the activation of PO activity and generation of superoxide anion *in vitro*, 8 shrimp were used. (2) For the study examining the resistance of shrimp to *V. alginolyticus*, test and control groups comprised 10 shrimp each in triplicate. (3) For the examination of immune parameters, test and control groups comprised 8 shrimp each. (4) For the studies of phagocytic activity and clearance efficiency to *V. alginolyticus*, test and control groups also comprised 8 shrimp each. The weight of shrimp ranged 9.6-12.3 g, averaging 10.8 ± 0.8 g (mean ± SD) with no significant size differences among treatments.

Activation of proPO and generation of superoxide anion *in vitro*

Trypsin is known to convert inactive proPO to active PO. Four shrimp were used for the PO activity assay, and another four shrimp were used for generation of superoxide anion assay, known as respiratory burst (RB). There were one stimulant (extract) with one positive control (trypsin) for PO activity assay, and one positive control (zymosan) for generation of superoxide anion (respiratory burst, RB) assay. Trypsin and zymosan were obtained from Sigma Co. (St Louis, MO, USA). Trypsin, zymosan, and the extract was prepared separately at concentration of 1 mg/ml in cacodylate buffer (10 mM sodium cacodylate, 450 mM sodium chloride, 10 mM calcium chloride, and 260 mM magnesium chloride, pH 7.0) or MCHBSS (modified complete Hank's balanced salt solution) for PO activity assay or generation of superoxide anion assay, respectively. Therefore, there were 3 treatments (1 stimulant solution, 1 positive control, and 1 background control) for both assays. Hemolymph (300 µl) was individually withdrawn from the ventral sinus of each shrimp, mixed with 2700 µl of an anticoagulant solution, and placed in six tubes for the experiment.

Total PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as previously described [23,31]. Briefly, 500 µl of diluted hemolymph from each tube was centrifuged at 800 × g at 4°C for 20 min. The supernatant was discarded, and the pellet was rinsed, re-suspended gently in 500 µl cacodylate-citrate buffer, and then centrifuged again. The supernatant was discarded, and the pellet was re-suspended in 100 µl cacodylate buffer. The aliquot was placed equally into two tubes. One tube was for measuring PO activity, and the other tube was for measuring background PO activity. The optical density at 490 nm of the shrimp's PO activity was measured for the formation of dopachrome using a spectrophotometer (model U-2000, Hitachi, Tokuo, Japan).

RB of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of the superoxide anions produced, as described previously [15,23]. One hundred microlitres of diluted hemolymph was sampled, and placed in triplicate on microplates (96-wells) previously coated with 100 µl of a poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 800×g for 20 min and 4°C. Supernatant was removed, and 100 µl zymosan or extract (in MCHBSS) was added and allowed to react for 30 min at room temperature. The optical density at 630 nm for the shrimp's RB was examined for the formation of formazan.using a microplate reader (model VERSAmax, Moledular Devices, Sunnyvale, CA, USA).

Effect of *P. binghamiae* extract on the resistance of *L. vannamei* to *V. alginolyticus*

There were 3 concentrations of extract (2, 6, and 10 µg/g). Shrimp received injected individually with 20 µl of *P. binghamiae* extract at 1, 3, and 5 mg/ml to reach doses of 2, 6, and 10 µg/g on the first day. The shrimp that were injected with saline (20 µl) served as the saline group. The shrimp without receiving saline or extract served as the control group. The challenge test was conducted on the second day by the injection of 20 µl bacterial suspension (7×10^7 cfu/ml) resulting in 1.4×10^6 cfu/shrimp into the ventral sinus of the cephalothorax. The shrimp that received no extract, and then received *V. alginolyticus* at 1.4×10^6 cfu/shrimp served as the challenged control. The shrimp that received no extract, and then received saline (20 µl) served as the unchallenged control. Experimental and control shrimp (10 shrimp per aquarium) were kept in 40 l aquarium containing 20 l of seawater (35‰). There were a total of 6 treatments. Each treatment was conducted in triplicate with 10 shrimp each. Therefore, 180 shrimp (6×3×10) were used for the study. Water was renewed daily. Survival of shrimp was examined every 6-24 h and the experiment lasted 144 h.

Immune parameters of shrimp received *P. binghamiae* extract

There were 3 concentrations of extract (2, 6, and 10 µg/g) and 5 exposure times (0, 24, 48, 96, and 144 h). Shrimp were injected in the ventral sinus of the cephalothorax with *P. binghamiae* extract solution of 1, 3, and 5 mg/ml to reach 2, 6, and 10 µg/g. For each concentration and exposure time, ten shrimp were used for the studies. In addition,

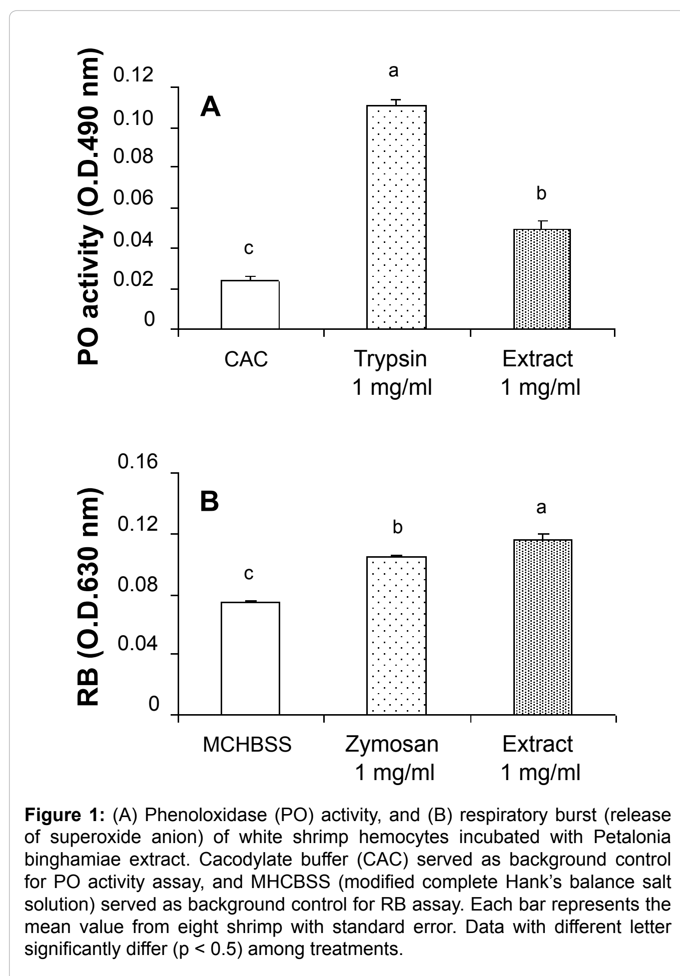

Figure 1: (A) Phenoloxidase (PO) activity, and (B) respiratory burst (release of superoxide anion) of white shrimp hemocytes incubated with Petalonia binghamiae extract. Cacodylate buffer (CAC) served as background control for PO activity assay, and MHCBSS (modified complete Hank's balance salt solution) served as background control for RB assay. Each bar represents the mean value from eight shrimp with standard error. Data with different letter significantly differ (p < 0.5) among treatments.

10 shrimp injected with saline (20 µl), and 10 shrimp with no injection served as the saline group and control group, respectively. Therefore, there were 25 treatments and 250 shrimp (5 x 5 x 10) in total were used for the study.

Measurements of immune parameters

For each treatment, eight shrimp were sampled. Hemolymph (200 µl) was individually withdrawn from the ventral sinus of each shrimp using a 1 ml sterile syringe with 25-gauge needle), and diluted with 1800 µl of an anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 M, EDTA 10 mM, pH 7.55, osmolality adjusted with 0.115 M glucose to 718 mOsm/kg). The hemolymph-anticoagulant mixture (diluted hemolymph) was placed in three tubes. Each tube contained 500, 1000, and 500 µl of diluted hemolymph, and was used to measure 1) hemocyte count and RB, 2) PO activity, and 3) SOD activity, respectively. Total hemocyte count (THC) was examined following the previously described method [26]. The PO activity was examined spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) based on previously described methods [31,32]. The RB (measurement of superoxide anion) was quantified using the reduction of NBT (nitroblue tetrazolium) to formazan according to the previously published methods [15,32]. The SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod kit (Randox, Crumlin, UK) following the method previously described [26,33].

Phagocytic activity and clearance efficiency of *L. vannamei* to *V. alginolyticus*

White shrimp that received saline and *P. binghamiae* extract were the same as that described in the immune parameter assay. There were five treatments (control, saline, 2, 6, and 10 µg/g) with five exposure times (0, 24, 48, 96 and 144 h). Eight shrimp for each treatment and time were used for the studies. Therefore, 200 shrimp (5 x 5 x 8) were used for the study. For the phagocytic activity and clearance efficiency tests, 20 µl of bacterial suspension (9×10^{7} cfu/ml) resulting in 1.8×10^{6} cfu/shrimp were injected into the ventral sinus. The shrimp that received no extract, and then received saline (20 µl) served as the saline group, and the shrimp that received no extract or saline served as control group. After injection, the shrimp were kept in a separate tank containing 40 l of water at 26 ± 1°C. Then, 200 µl of hemolymph was collected from the ventral sinus and mixed with 200 µl of sterile anticoagulant. Examinations of phagocytic activity and clearance efficiency were conducted following the previously described methods [18,34,35]. Phagocytic activity was expressed as PA (%) = [(phagocytic hemocytes)/(total hemocytes)] x 100. The number of bacterial colonies in the control shrimp served as the control group and the number of colonies in shrimp that received saline as well as shrimp that received *P. binghamiae* extract at 2, 6, and 10 µg/g served as the test group. The clearance efficiency was calculated as 100- [(cfu in test group)/(cfu in control group)]×100.

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA). If significant differences were indicated at the $p<0.05$ level, then Duncan's multiple-comparison test was conducted to examine significant difference among treatments using SAS computer software (SAS Institute Inc., Cary, NC, USA). Before analysis, percent data (resistance test) were normalized using an arcsine transformation. For statistically significant differences, it was required that $p<0.05$.

Results

The activation of proPO and generation of superoxide anion *in vitro*

The PO activity of shrimp hemocytes that incubated with trypsin (positive control group) was significantly higher than that of shrimp hemocytes incubated with extract. The PO activity of shrimp hemocytes incubated with the extract showed significant increase as compared to the background control (CAC) (Figure 1A). Shrimp hemocytes incubated with zymosan and the extract showed higher RB (generation of superoxide anion), as compared to the background control (MCHBSS) (Figure 1B).

Resistance against *V. alginolyticus* in shrimp receiving *P. binghamiae* extract

All the unchallenged control shrimp survived. In contrast, mortalities had occurred after 6 h in the challenged shrimp that received saline and extract. Survival rate of shrimp received the extract at 6 and 10 µg/g was significantly higher than that of shrimp received the extract at 2 µg/g, received saline as well as the control shrimp after 12-48 h. Survival rate of shrimp received the extract at 6 and 10 µg/g was significantly higher than that of shrimp received saline as well as the control shrimp after 12-144 h (Figure 2).

Immune parameters of shrimp that received the *P. binghamiae* extract

THC of shrimp received the extract at 2 µg/g was significantly higher

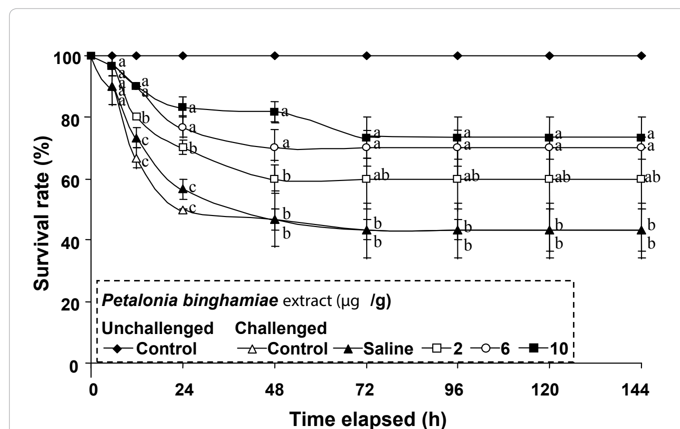

Figure 2: Survival rate of white shrimp *Litopenaeus vannamei* received *Petalonia binghamiae* extract at 2, 6, and 10 μg/g for one day, shrimp received saline, and the control shrimp, and then challenged with *Vibrio alginolyticus* at a dose of 1.4×10^6 cfu/shrimp. Each data represents mean of three replicates (10 shrimp in each replicate) with the standard error. Data (mean ± SE) in the same time with different letters significantly differ (p<0.0) among treatments.

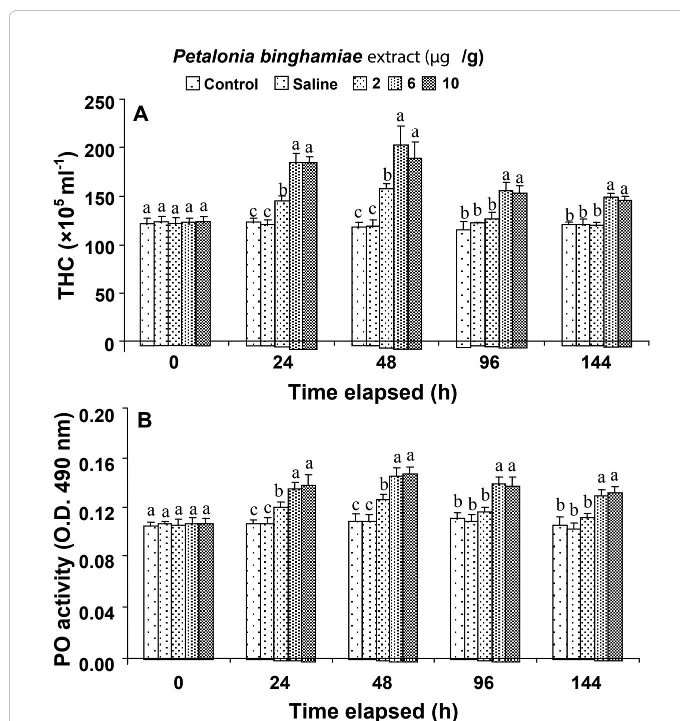

Figure 3: Mean (± SE) THC (A) and phenoloxidase activity (B) of white shrimp *Litopenaeus vannamei* received *Petalonia binghamiae* extract at 2, 6, and 10 μg/g, shrimp received saline, and the control shrimp. Each bar represents mean value from eight shrimp with standard error. Data at the same exposure time with different letters are significantly different (p<0.05) among treatments.

than that of shrimp received saline as well as the control shrimp after 24 and 48 h. THC of shrimp received the extract at 6 and 10 μg/g was significantly higher than that of shrimp received the extract at 2 μg/g, shrimp received saline and the control shrimp after 24, 48, 96, and 144 h (Figure 3A). PO activity of shrimp received the extract at 2 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 24 and 48 h. PO activity of shrimp received the extract at 6 and 10 μg/g was significantly higher than that of shrimp received the extract at 2 μg/g, shrimp received saline and the control shrimp after 24, 48, 96 and 144 h (Figure 3A). RB of shrimp received the extract at 2 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 96 h. RB of shrimp received the extract at 6 and 10 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 48, 96, and 144 h (Figure 4A). SOD activity of shrimp received the extract at 6 and 10 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 48 h. No significant difference in SOD activity was observed for the shrimp among six treatments after 24, 96 and 144 h (Figure 4B).

Phagocytic activity and clearance efficiency of *L. vannamei* to *V. alginolyticus*

Phagocytic activity of shrimp received the extract at 6 and 10 μg/g was significantly higher than that of shrimp received the extract at 2 μg/g, shrimp received saline and the control shrimp after 24, 48, 96 and 144 h. Phagocytic activity of shrimp received the extract at 2 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 24 and 48 h. Phagocytic activity was 12%, 13%, 20%, 26%, and 29% for the control shrimp, shrimp received saline and the shrimp that received the extract at 2, 6, and 10 μg/g, respectively after 48 h (Figure 5A). Clearance efficiency of shrimp received the extract at 2, 6 and 10 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 24, 48, 96 and 144 h. Clearance efficiency of shrimp received the extract at 6 μg/g was significantly higher than that of shrimp received saline and the control shrimp after 24 h and 48 h. Clearance efficiency increased significantly to 41%, 48%, and 54% for the shrimp that received the extract at 2, 6, and 10 μg/g after 48 h as compared to the control shrimp (Figure 5B).

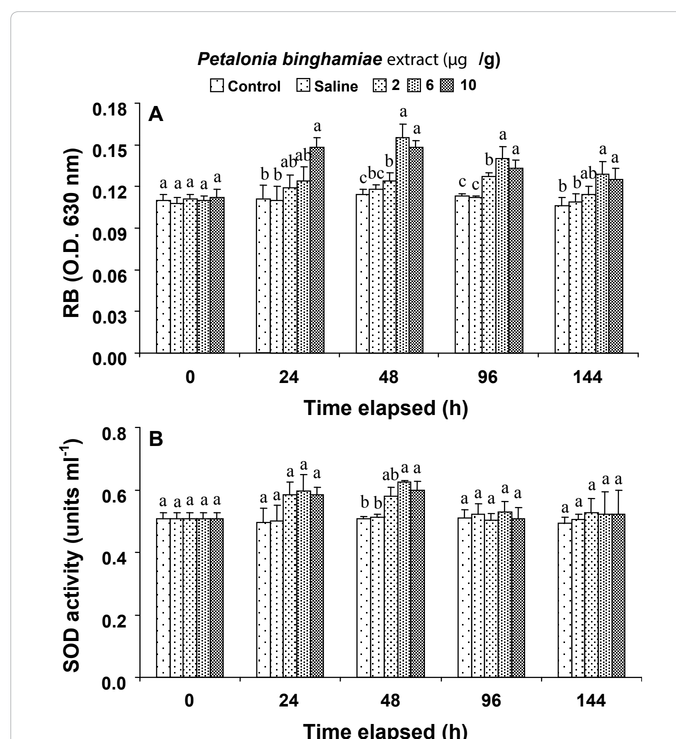

Figure 4: Mean (±SE) respiratory burst (A) and superoxide dismutase (SOD) activity (B) of white shrimp *Litopenaeus vannamei* received *Petalonia binghamiae* extract at 2, 6, and 10 μg/g, shrimp received saline, and the control shrimp. See Figure 3 for statistical information.

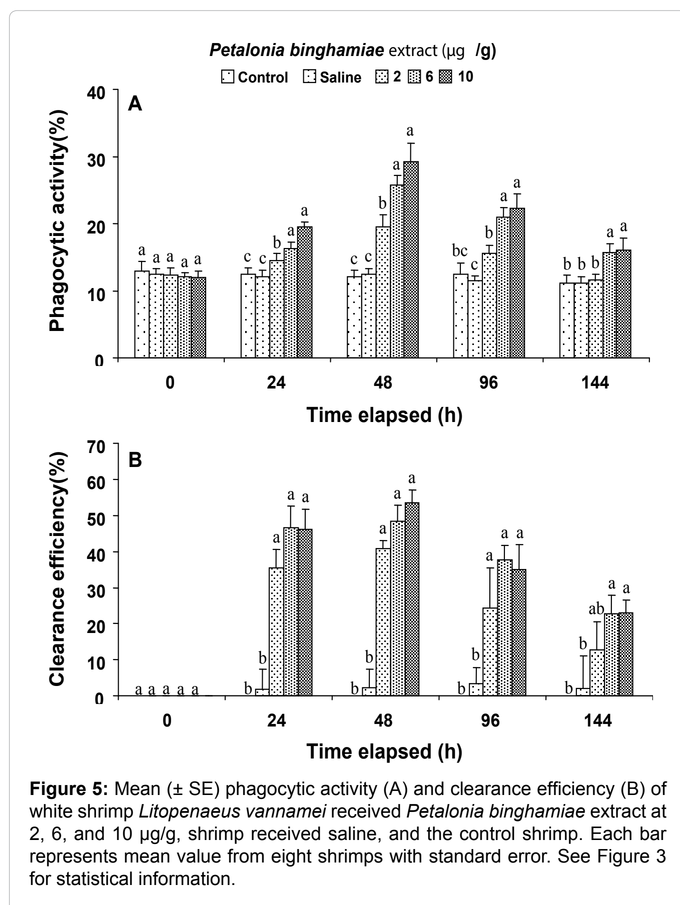

Figure 5: Mean (± SE) phagocytic activity (A) and clearance efficiency (B) of white shrimp *Litopenaeus vannamei* received *Petalonia binghamiae* extract at 2, 6, and 10 µg/g, shrimp received saline, and the control shrimp. Each bar represents mean value from eight shrimps with standard error. See Figure 3 for statistical information.

Discussion

Several *in vitro* studies indicated that the PO activity of shrimp hemocytes incubated with βG, laminarin, LPS, and PG increased in penaeid shrimps [31,36-38]. An i*n vitro* study also indicated that shrimp hemocytes incubated with *S. hemiphyllum* extract could increase PO activity and superoxide anion [23]. The present study indicated that shrimp hemocytes incubated with *P. binghamiae* extract could increase PO activity and release of superoxide anion indicating activation of innate immunity. An *in vitro* study also indicated that the PO activity and RB of shrimp hemocytes incubated with alginate, βG, curdlan, and laminarin significantly increased [18,39]. The PO activity and RB of shrimp hemocytes incubated with fucoidan increased together with degranuation of hemocytes and cell size change indicated an activation of immunity [18]. It is suggested that *P. binghamiae* extract could cause degranulation, change in cell size of shrimp hemocytes and leads to increases in PO activity and RB.

Brown seaweed which contains biologically active polysaccharide shows antibacterial activity by the activation of macrophages and promotion of wound healing [40,41]. White shrimp *L. vannamei* received *S. duplicatum* extract, and received *S. hemiphyllum* var. *chinese* extract showed resistance against *V. alginolyticus* challenge [22,23]. Fleshy shrimp *Fenneropenaeus chinensis* fed a diet containing *S. fusiforme* extract showed resistance against *V. harveyi* challenge [21]. Tiger shrimp *P. monodon* that fed diets containing *S. glaucescens* extract, and white shrimp *L. vannamei* that fed diets containing *S. cristaefolium* extract showed resistance against *V. alginolyticus* challenge [24,25]. Similarly, shrimp received *P. binghamiae* extract showed resistance against *V. alginolyticus* challenge in the present study. Furthermore, white shrimp *L. vannamei* that fed a diet containing fucoidan extracted from *Fuscus* showed resistance against *V. alginolyticus* challenge [18]. Tiger shrimp *P. monodon* that fed diets containing fucoidan extracted from *S. polycystum* showed resistance against white spot Syndrome virus (WSSV) challenge [20]. White shrimp *L.vannamei* that received *S. hemiphyllum* var. *chinese* extract increased resistance against WSSV challenge [23]. Fucoidan is widely present among all the brown seaweed including species of *Cladosiphon, Fuscus*, *Petalonia* and *Sargassum* [42]. Therefore, fucoidan, *Sargassum* extract, and *Petalonia* extract all showed positive effects on the resistance of shrimp against pathogen infections.

Several scientists reported the immune parameters of shrimp that received brown seaweed extract *in vivo*. For instance, white shrimp *L. vannamei* received *S. duplicatum* extract, *S.* var *chinese* extract increased the THC, PO activity and RB [22,23]. Similarly, shrimp received *P. binghamiae* extract at 6 and 10 µg/g increased THC, PO activity, and RB in the present study. White shrimp *L. vannamei* that received fucoidan showed increased hemocyte count together with increase in the mitotic index of hematopoietic tissue (HPT) [18]. The fact that the hemocyte count increased together with increases of PO activity and RB of shrimp that received the extract also indicate a proliferation of HPT.

Longevity effect has become a concern for shrimp receiving βG, PG and other immunostimulants [43]. The PO activity and RB of shrimp that received *Sargassum* extract via injection increased after 2-3 days, but slightly decreased after 4-6 days [22,23]. The PO activity and RB of shrimp that received *Sargassum* extract via immersion increased after 3 h, but slightly decreased after 5 h [23]. The PO activity and RB of shrimp that received carrageenan via immersion increased after 3 h, but slightly decreased after 5 h [44]. The PO activity and RB of shrimp that fed diets containing *Gelidium* extract and *Gracilaria* extract increased after 14 days, but slightly decreased after 28-35 days [32,45]. The RB of shrimp that fed a diet containing βG increased after 12-24 days, but decreased after 30-40 days [46]. Similarly, increase and decrease of immune parameters were observed in shrimp that received *P. binghamiae* extract in the present study. Therefore, the immune parameters of shrimp that once received immuno stimulants increased to a plateau after a certain time, but were likely to decline afterward to maintain homeostasis.

Despite the increase and decrease of immune parameters occurred in shrimp that received immunostimulant, shrimp still maintain its immunity to defend against pathogen. The phagocytic activity and clearance efficiency to *V. alginolyticis* in white shrimp that received the extract at 6 µg/g still maintained higher at 96 h post injection, despite the decline of RB [22]. The present study indicated that the phagocytic activity and clearance efficiency to *V. alginolyticus* in white shrimp that received *P. binghamiae* extract at 6 and 10 µg/g increased at 24 h, reached to a plateau at 48 h, but declined at 96 and 144 h post injection. However, phagocytic activity and clearance efficiency of shrimp received 6 and 10 µg/g extract still maintained higher than those of control shrimp at 96 and 144 h post injection. Therefore, despite the decrease of immune parameters occurred in shrimp that received immunostimulant, shrimp still maintain its immunity to defend against pathogen.

In conclusion, shrimp hemocytes incubated in *P. binghamiae* extract provoked activation of immunity. Shrimp received *P. binghamiae* extract experienced enhanced immunity by increasing HC, GC, THC, PO activity, RB, phagocytic activity and clearance efficiency and resistance against *V. alginolyticus*, *P. binghamiae* extract could be used in shrimp farming to increase immunity of shrimp and its resistance against *Vibrio* pathogen.

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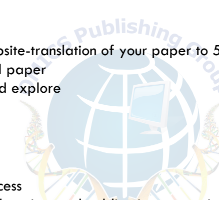

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