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Immunomodulatory and Growth Promoting Effects of Peptidoglycan Supplementation in Black Tiger Shrimp *Penaeus monodon* Fabricius 1798

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Abstract

A 30-day feeding trial was conducted to evaluate the influence of peptidoglycan supplementation on immune responses, growth, muscle fibre size and digestive protease activities of juvenile black tiger shrimp *Penaeus monodon* Fabricius 1798 (Penaeidae). Two treatments were run composed of a group supplemented with peptidoglycan at 0.18 $g \cdot kg^{-1}$ diet and a control maintained with basal diet without peptidoglycan. Results indicate elevated immune responses including total haemocyte count, phenoloxidase and respiratory burst activities in peptidoglycan-fed shrimp compared to the control. Significant enhancement of growth in terms of weight gain, protein and lipid retentions was eminent in peptidoglycan-supplemented shrimps compared to the control group. This growth improvement is associated with the increase in muscle fibre size and digestive protease activities that include trypsin and carboxypeptidase A. The present findings suggest that peptidoglycan supplementation in *P. monodon* can boost immunological responses and improve growth performance through the promotion of muscle growth and enhancement of digestive protease activities. Compounds with immune stimulating activities to augment disease resistance and with growth promoting activities are highly desirable in shrimp aquaculture. Peptidoglycan supplementation is suggested as a practical approach to improve disease resistance and enhance growth performance of *P. monodon* in culture.

Introduction

Economics and profitability are major factors defining the sustainability of industrial aquaculture. Since disease occurrence and poor growth performance of cultured animals are considered threats to attain sustainability in aquaculture, compounds able to promote disease resistance coupled with the bioactivity to enhance growth and nutrient utilisation efficiencies are highly sought for aquaculture use. Earlier practice in all types of animal rearing industries utilises low doses of antibiotics as feed additives, documented to elicit a significant growth promoting and disease suppression effects.

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However, collective concerns on the development of antimicrobial resistant strains led to a global consensus of policies on the controlled and limited use of antibiotics in aquaculture.

In shrimp aquaculture, application of immune stimulating compounds is currently gaining wide acceptance among aqua culturists as a practical prophylactic approach in managing disease occurrence. Studies revealed that these compounds could boost shrimp immune responses resulting to increased resistance against virulent pathogens and stress (Suphantharika et al. 2002; Burgents et al. 2004; Traifalgar et al. 2012). Earlier reports indicate that immunostimulation in shrimp results in significant enhancement of growth, improvement of feed efficiency and nutrient utilisation (Lopez et al. 2003; Felix et al. 2008; Traifalgar et al. 2009).

Peptidoglycan (PG), a bacterial polymer consisting of alternating strands of N-acetyl glucosamine and N-acetylmuramic acid (Schleifer and Kandler 1972) has been known as a potent immune stimulant that could activate and enhance shrimp immune responses (Boonyaratpalin et al. 1995). Like other immunostimulants, application of this compound on shrimp has been documented to elicit a positive influence on growth and nutrient utilisation (Itami et al. 1998). Purivirojkol et al. (2006) elucidated the optimum dose and frequency of application of PG to activate immune responses of juvenile *P. monodon* but aspects on how this compound improved growth performance and nutrient utilisation has not been fully elucidated to date. The present study evaluates the influence of PG as an immunostimulant on immune responses, growth performance, muscle fibre size and digestive protease activities of juvenile *P. monodon*.

Materials and Methods

Immunostimulant and experimental diets

Two experimental diets were prepared and used in the experiment. The control diet (without PG) was composed of 42.06% crude protein, 9.40% crude fat, 15.61% ash, 7.72% crude fibre (Table 1) and was formulated to satisfy the optimum nutrient requirement of *P. monodon* (Deshimaru et al. 1985). The treatment diet was composed of the control diet added with peptidoglycan (Chemoforma Ltd., Augst, Switzerland) at a dose of 0.18 $g \cdot kg^{-1}$diet. This dietary dose was based on the work of Purivirojkol et al. (2006) wherein it was observed to be their best treatment and was also previously optimised in our laboratory to elicit an optimum immune response in *P. monodon*.

Feeding trial

The study was conducted at the Institute of Aquaculture Multi-Species Hatchery, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miag-ao, Iloilo, Philippines. Pathogen-free experimental shrimps were procured from a commercial shrimp hatchery at Tigbauan, Iloilo, Philippines. Animals were transported to the laboratory and acclimated under laboratory conditions for a week. Commercial shrimp diet without immunostimulant was used to maintain the shrimp during acclimation.

Table 1. Composition of the basal diet.

Ingredients	g·100 g^{-1} diet
Peruvian Fishmeal	20
Shrimp Meal	27
Gelatin	5
Soybean Meal	20
Starch	15
Vitamin Mix	1.75
Mineral Mix	1.75
Cod Liver Oil	4
Soy Lecithin	0.5
Butylated Hydroxytoluene (BHT)	0.02
Cellulose	4.98
Peptidoglycan (PG)	0.0
Total	**100.00**

Following the acclimation, 120 shrimp juveniles (size: 0.40± 0.05 g) were collected from the acclimation tank and randomly distributed into 50 L tanks at a density of 20 individuals·tank^{-1} in a recirculation system. These constitute the two dietary treatment groups that include the 0.18 g PG·kg^{-1} diet (PEPTI) and a control (CTRL) in triplicate. Shrimps were feed three times a day (0800, 1200 and 1600 h) at a feeding rate of 10-7% of the average body weight. Periodic samplings were conducted every 10 days to determine the weight of the shrimps and adjust the feeding ration. The feeding experiment lasted until 30 days. Water parameters were maintained at the optimum condition throughout the experiment (salinity: 20 ppt, total ammonia nitrogen: 0-1 ppm, nitrite: 0.1-0.5 ppm, nitrite nitrogen: 0-0.15 ppm, dissolved oxygen: 7-8 ppm, temperature: 28.2-30.8 °C, pH: 8.4-9.0).

Upon termination of the feeding trial, shrimps were counted and weighed. Shrimp samples from each treatment were immediately processed for the immune assays and histology while specimens used for the carcass composition analyses and digestive enzyme assays were stored at -80 °C until analysed.

Immune assays

Haemolymph extraction

Haemolymph were extracted using a 1-mL syringe (26 gauge) from the base of pleopods at the first abdominal segment of *P. monodon* following the method of Hernandez-Lopez et al. (1996). Anticoagulant was prepared by adding 10 mM EDTA-Na_2 salt to shrimp salt solution (450 mM NaCl, 10 mM HEPES, pH 7.3, 850 mOsm·kg^{-1}). Collected haemolymphs were used in the various immune assays.

Total haemocyte count (THC)

An aliquot of 20 µL collected haemolymphs were stained with 1.2% rose bengal in 50% ethanol for 20 min and the haemocytes were counted under a compound microscope at 40x magnification using a Neubauer haemacytometer (Sritunyalucksana et al. 2005). Total haemocyte count was expressed as $THC \cdot mL^{-1}$ haemolymph.

Phenoloxidase (PO) activity assay

The amount of dopachrome formed from L-dihydroxyphenylalanine (L-DOPA) was measured to determine the PO activity of *P. monodon* following the protocols of Hernandez-Lopez et al. (1996). Cell–free haemocyte lysate was prepared by subjecting haemocytes to freeze-thaw cycle to induce cell lysis and degranulation. Lysed haemocytes were vortex mixed, centrifuged at 7,500 rpm at 4 °C for 15 min and supernatant was collected. An aliquot of 25 µL of the haemocyte lysate was collected and incubated with 0.1% trypsin solution for 30 min. Following the activation with trypsin, the reaction was started with the addition of 25 µL of 0.3% L-DOPA solution and the absorbance was read at 490 nm. Phenoloxidase activity was expressed as the increase in absorbance $min^{-1} \cdot 100\ \mu L^{-1}$ haemocyte lysate (Joseph and Philip 2007).

Respiratory burst (RB) activity assay

Haemocyte respiratory burst activity was measured following the method described by Muñoz et al. (2000). One hundred microlitres of haemocyte were allowed to settle at the bottom of microtitre well at room temperature for 2 h. The supernatant was then removed and replaced with 50 µL modified Hank's balanced salt solution. To the haemocyte solution, 50 µL of phorbol myristate acetate solution and 50 µL of 0.3% nitroblue tetrazolium solution were added and incubated in a dark place at room temperature for 30 min. Supernatant was removed and haemocytes were fixed and washed with methanol. To the fixed haemocytes, 120 µL 2 M potassium hydroxide and 140 µL of dimethyl sulfoxide were added to solubilise the formed formazan. The absorbance was measured at 620 nm and the enzyme activity was expressed as optical density $\cdot 100\ uL^{-1}$ haemolymph.

Growth performance

Percent weight gain was determined using the recorded weight upon the termination of the feeding trial and nutrient utilisations were computed based on the formula described by Hardy and Barrows (2002):

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Nutrient retention} = \left\{ \frac{\text{carcass nutrient content at the end of experiment} - \text{carcass nutrient content at the start of experiment}}{\text{nutrient intake during the experiment}} \right\} \times 100$$

Histology

Specimen processing for muscle histological analysis of *P. monodon* followed the protocol of Sung et al. (1994). Shrimp samples were collected, fixed in Davidson's solution, dehydrated with alcohol and embedded in paraffin. Specimens were sectioned (5 µm thick) and mounted on microscope slides. Tissue sections were stained with haematoxylin and eosin dye. Prepared tissue sections were viewed under the microscope and digital photomicrographs were taken under oil immersion. Mean size of muscle fibres was quantified by determining the area of muscle fibres within 1 μm^2 using the image analysis software Image J 1.42 (National Institute of Health, USA) as described by Tuller et al. (2012).

Digestive enzyme assays

Preparation of enzyme extract

Shrimp hepatopancreases were collected, weighed and homogenised in 50 mM citrate phosphate buffer, pH 7.0 at a proportion of 1:25 (weight :volume) using a teflon-glass tissue homogeniser. The homogenates were centrifuged at 4,000 rpm at 4 °C for 15 min and supernatants were collected and used in subsequent assays. Blank (absence of substrate or enzyme) and zero-time reaction were also performed. All enzyme assays were done in triplicates at 25 °C. One enzyme unit was defined as the amount of enzyme that catalysed the release of 1 mM of product·minute^{-1} under the assay condition. Enzyme activities were expressed as enzyme unit·mg^{-1} protein.

Protein determination

Protein content of the crude enzyme extracts were determined according to Bradford (1976) using bovine serum albumin as a standard.

Trypsin

One millimole of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride was used as substrate in this assay following the protocols of Kakade et al. (1969). The assay mixture consisted of 0.75 mL substrate solution, 1. 9 mL of 0.1 M Tris-HCl buffer, pH 8.0 and 0.1 mL enzyme extract which was added at timed intervals. After 5 min incubation, 0.25 mL of 30% acetic acid was added to stop the reaction and the absorbance was read at 405 nm. Trypsin activity was computed as the amount of released *p*-nitroaniline·min^{-1}·mg^{-1} protein.

Carboxypeptidase A

Hippuryl-L-phenylalanine was used as substrate to quantify carboxypeptidase A activity as described by Appel (1974). The reaction cocktail was composed of 1.2 mL 25 mM Tris-HCl buffer (pH 7.5) with 0.5 M NaCl, 1.4 mL substrate solution and 0.1 mL enzyme extract. Samples were incubated for 5 min and 0.3 mL of 30% acetic acid was added to stop the reaction.

The absorbance was read at 254 nm in a UV-VIS spectrophotometer and enzyme activity was determined by the amount of hippuric acid formed·min^{-1}·mg^{-1} protein of the extract.

Leucine aminopeptidase (LAP)

Assay was performed according to the methods of Serrano and Traifalgar (2012) wherein 1 mM L-leucine-p-nitroanilide was used as substrate. The reaction was initiated by adding 0.3 mL enzyme extract into the reaction tubes with 1.4 mL substrate solution and 1.0 mL of 60 mM Tris-HCl buffer, pH 8.5. The reaction was stopped by the addition of 0.3 mL 30% acetic acid after 5 min then the absorbance was read at 405 nm. LAP activity was quantified as the amount of released *p*-nitroaniline·min^{-1}·mg^{-1} protein of extract.

Statistical analysis

All data were tested for normality through Levene's test prior to the analysis using t-test in Sigma Plot 11.0 (Systat Software Inc., USA) at 0.05 significance level. As necessary, data were subjected to arcsine transformation prior to t-test analysis. Results were presented as mean ± standard error of the mean.

Results

Immunological indices

All immune indices (Table 2) were found to be enhanced in PEPTI as compared to the CTRL group. Total haemocyte count was significantly higher in PEPTI as compared to the CTRL. Phenoloxidase activity exhibited an almost four-fold increase in PEPTI in comparison to the CTRL. Similar to the other immune indices, a two-fold enhancement of RB activity is observable in PEPTI as compared to the CTRL group.

Table 2. Immune responses of *Penaeus monodon* fed with the CTRL and PEPTI.

Immune indices	Treatments	
	CTRL	**PEPTI**
THC (THC·mL^{-1}haemolymph)	$2.3 \times 10^5 \pm 5.6 \times 10^3$	$2.6 \times 10^5 \pm 3.0 \times 10^3$*
PO activity (Δ OD_{490}·min^{-1}·100μL^{-1}haemolymph)	0.005± 0.002	0.026± 0.0003*
RB activity (OD_{620}·100μL^{-1}haemolymph)	0.053± 0.001	0.079± 0.002*

Values are mean ±SEM.

Within parameters, means with asterisk (*) are significantly different ($p<0.05$).

Growth

PEPTI-fed shrimps exhibited significantly higher percent weight gain, protein retention and lipid retention than those fed with the CTRL diet (Table 3). There were no mortalities observed in both treatments during the growth trial.

Table 3. Growth performance of *Penaeus monodon* fed with the CTRL and PEPTI.

Growth Parameters	Treatments	
	CTRL	PEPTI
Weight Gain (%)	36. 21± 0.92	58. 39± 6.24*
Protein Retention (%)	8.68± 0.03	10.08± 0.56*
Lipid Retention (%)	4.00± 0.03	7.39± 0.66*

Values are mean ±SEM.
Within parameters, means with asterisk (*) are significantly different ($p<0.05$).

Muscle fibre size

Muscle fibre size (Fig. 1) of PEPTI-fed shrimps were found to be significantly bigger than that of shrimp in the CTRL group.

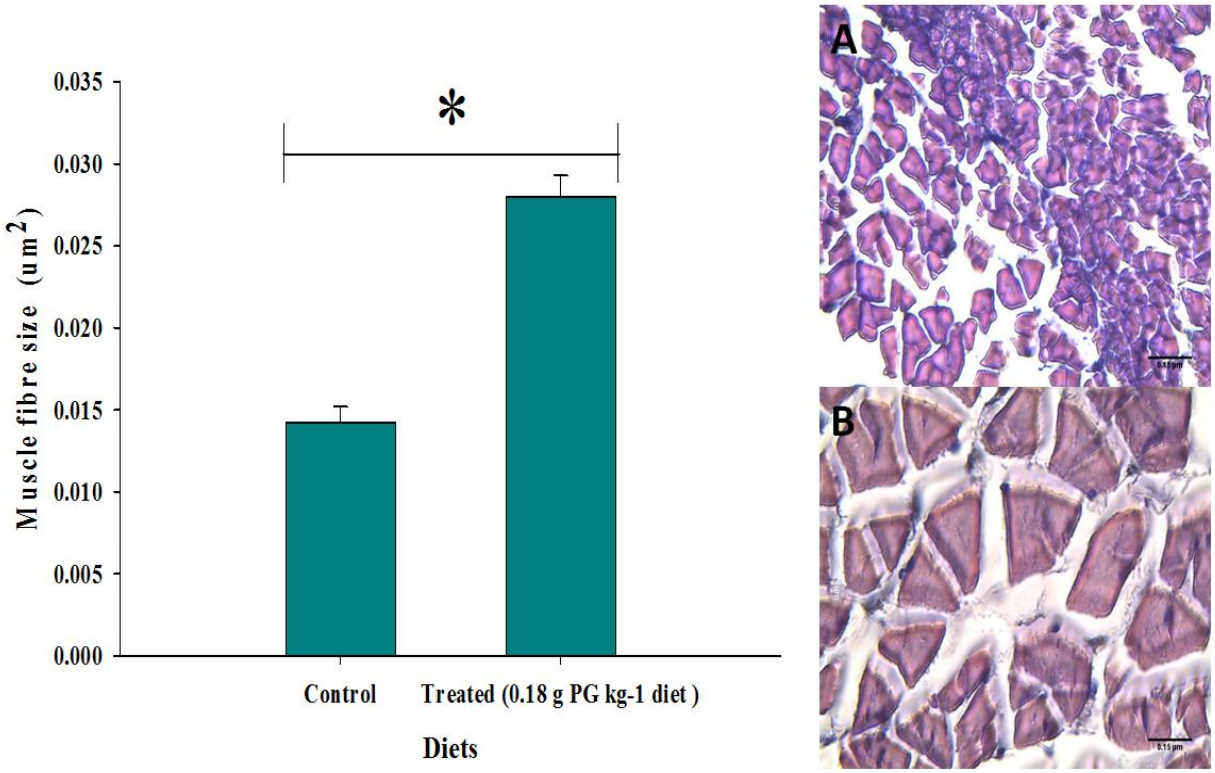

Fig. 1. Muscle fibre size of *Penaeus monodon* fed with the (A) CTRL and (B) PEPTI (mean ± SEM). Asterisk (*) indicates significant difference among treatments ($p<0.05$). Scale bar = 0.15 µm.

Digestive enzyme assays

Trypsin and carboxypeptidase A activities (Table 4) were evidently enhanced in PEPTI-fed shrimps than those given with the CTRL. However, LAP activities of *P. monodon* in both treatments were not significantly different from each other.

Table 4. Digestive enzyme activities of *Penaeus monodon* fed with the CTRL and PEPTI.

Digestive Enzymes (Units·mg^{-1} protein)	Treatments	
	CTRL	PEPTI
Trypsin	0.16± 0.005	0.21± 0.002*
Carboxypeptidase A	0.31± 0.100	0.64± 0.06*
LAP	0.05± 0.001	0.05± 0.0002

Values are mean ±SEM.
Within parameters, means with asterisk (*) are significantly different ($p<0.05$).

Discussion

Disease resistance and growth enhancement are aspects that are highly desired by aquaculturists. In recent years, increased attention has been focused on immune stimulating compounds that have been shown to elicit disease resistance coupled with significant growth enhancements in shrimp. Peptidoglycan is considered as one of the active immunostimulants capable of inducing disease resistance in both cultured crustacean and fish species (Sakai 1991; Raa et al. 1992). Improved disease resistance due to activation of immunological responses and significant growth improvements associated with the application of PG as a feed supplement in cultured aquatic animal species were earlier reported (Boonyaratpalin et al. 1995; Itami et al. 1998; Purivirojkul et al. 2006).

The mechanism of PG's immune enhancing activity has been a subject of several investigations in the past decade. However, like any other dietary immunostimulants the aspects on how these compounds influence the overall growth performance of aquatic animals are not fully evaluated until the present. Results of the present study indicate significant enhancement of immunological responses including THC, PO and RB activities in PEPTI as compared to the CTRL. This finding conforms with the results of Purivirojokol et al. (2006) on juvenile shrimp elucidating the activation of PO, superoxide anion, bactericidal activity, clearance ability and enhanced resistance against *Vibrio harveyi* in shrimp receiving PG supplemented diets. Furthermore, the results of the present study also agree with the earlier reports on the immune enhancing properties of PG in crustacean. Improvement in disease resistance in *Penaeus japonicus* Spence Bate 1888 (Itami et al. 1998), enhancement of immune responses in *P. monodon* (Boonyaratpalin et al. 1995) and activation of immune genes in *Penaeus vannamei* Boone 1931 (Song et al. 2013) are the reported effects of PG on cultured crustaceans. In these studies, significant improvement in survival following pathogen challenge were observed as a consequence of increased immune responses similar to that observed in the present study.

Moreover, findings of the present study indicate a significant enhancement of growth quantified as weight gain in PEPTI. Growth enhancement is commonly observed as a consequence of the application of dietary immunostimulant in cultured aquatic animals. Itami et al. (1998) documented similar effects in *P. japonicus* fed with PG supplemented diet.

Penaeus monodon maintained with diets supplemented with killed *Vibrio* cells were also documented to exhibit significant improvement in weight gain (Song and Sung 1990). In the present study, PEPTI exhibited a significant enhancement of muscle fibre size as compared to the CTRL. This suggests that positive growth as influenced by PG supplementation is attributed to muscle hypertrophy. While the link of immune stimulation and muscle growth is not fully understood in aquatic animals, some evidence suggests that immune activation could result to muscle hypertrophy. The muscle hypertrophy exhibited by PEPTI could be due to the activation of immune cytokines such as astakine. Astakine is a cytokine identified in shrimp known as a homologue of vertebrate prokinecitin that promotes proliferation of tissues including haematopoietic cells and inhibits cellular apoptosis (Lin and Soderhall 2011). This cytokine has been reported to be activated in shrimp by immune stimulating compounds, bacterial cells and viruses (Chang et al. 2013; Zhang et al. 2013). The enhanced muscle growth in PEPTI could be also due to the activation of other cellular growth promoting cytokine including the translationally controlled tumour proteins (TCTP) (Wu et al. 2013) and integrin (Zhang et al. 2012) known as cell proliferation regulator in shrimp. Both of these cellular proliferation factors have been reported to be activated during increased immune response and are transcribed during infection. Although these growth factors were not measured in the present study, it is tempting to speculate that these are involved in the enhanced muscle tissue growth in PEPTI in the present study.

In the present study, growth enhancement in PEPTI is supported with a significant improvement in body protein and lipid depositions measured as protein and lipid gains. These indicate that PG supplementation promoted accretion of protein and lipid in shrimp carcass. It has been speculated that growth promotion effects of immunostimulant in shrimp could have been attributed to activation of hepatopancreatic R cells that are involved in nutrient digestion (Alday-Sanz et al. 2002).

To our knowledge, findings of the present study on the enhancement of digestive trypsin and carboxypeptidase A enzymes as influenced by PEPTI supplementation is the first time to be documented in *P. monodon*. Shrimp hepatopancreas is an organ known to exhibit diverse functions. Its functions are not only limited to nutrient digestion and assimilation but are also involved in the synthesis and secretion of immune-related genes involved in the neutralisation of microbial invaders (Li et al. 2013). Immunostimulants including PG are compounds known to elicit their activity by the activation of enzymes classified as serine proteases (SPs) through protease-activated receptors (Shpacovitch et al. 2008). The high activity of digestive trypsin, an SP, observed in the present study could be due to its activation by PG. Serine protease homologues are not only participating in digestion but also involved in blood coagulation and immune defence responses (Ren et al. 2011).

In addition, PEPTI supplementation also enhances the activity of digestive carboxypeptidase A, a cysteine protease known to exhibit active roles in the immune processes and nutrient digestion and assimilation. In vertebrates, carboxypeptidases are cysteine proteases involved in lysosomal apoptosis pathway and T-cell migration (Manoury et al. 2011).

Ren et al. (2010) reported in *Fenneropenaeus chinensis* (Osbeck 1765) the presence of cysteine proteases that are activated in the presence of *Vibrio anguillarum* cells and white spot syndrome virus (WSSV). This indicates the activation of this enzyme with microbial antigens. The high activity of carboxypeptidase A in shrimp maintained with PEPTI conforms to these earlier findings.

Conclusion

The results of this study suggest that PG supplementation at 0.18 $g \cdot kg^{-1}$ diet in juvenile *P. monodon* enhances immunological responses and promotes growth that is associated with muscle hypertrophy, efficient nutrient retentions and enhancement of digestive trypsin and carboxyopeptidase A activities. Dietary PG supplementation could be a practical approach in the improvement of growth performance and enhancement of disease resistance in the culture of *P. monodon*.

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