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The Effect of Peptidoglycan on Immune Response in Black Tiger Shrimp (*Penaeus monodon* Fabricius)

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ABSTRACT

The effect of peptidoglycan on immune response in Black Tiger Shrimp (*Penaeus monodon* Fabricius) was conducted under laboratory conditions. *P. monodon* (13±2 g) were fed for 7 days with diets containing graded levels of PG (0, 0.06, 0.12 and 0.18 g/kg feed). The immune responses were measured by phenoloxidase, superoxide anion, bactericidal activity, clearance ability and resistance against *Vibrio harveyi*. The results showed that shrimp which fed on diets containing PG 0.18 g/kg had these immune parameters at significantly higher ($P<0.05$) than PG 0.06, 0.12 g/kg and control.

The study for optimum time of using PG 0.18 g/kg was determined with *P. monodon* (13±2 g) for 10 weeks. In weeks 1-4, shrimps were fed with the diet containing PG at 0.18 g/kg at 0, 3, 5 and 7 days/week. In weeks 5-8, shrimps were fed with normal feed and in weeks 9-10, shrimps were fed with same concentration and frequency as weeks 1-4. The results showed that optimum time for use of PG 0.18 g/kg was 5 days/week. This application could increase total hemocytes, phenoloxidase, superoxide anion and bactericidal activity which were significantly different ($P<0.05$) from 3 days/week and control but not significantly different ($P>0.05$) from 7 days/week. In conclusion, oral administration of PG at 0.18 g/kg diet for 1 month (5 days/week), normal feed for 1 month and PG 0.18 g/kg diet for another month (5 days/week) could effectively enhance the immune system of *P. monodon*.

Key words: immune responses, black tiger shrimp, *Penaeus monodon*, peptidoglycan

INTRODUCTION

Black tiger shrimp (*Penaeus monodon*) is an important export product of Thailand. In 2004, export of this shrimp reached the value of $US 373.93 million and was expected to be slightly higher or just about the same in 2005 (Ministry of Commerce Thailand, 2005). One of the important problems in black tiger shrimp aquaculture is the luminous disease caused by *Vibrio harveyi* (Baticados *et al*., 1990). A common method to control the disease is the use of antibiotics. However, using of antibiotics in some cases cause residues in shrimps which are harmful for consumption and might cause the loss of confidence in Thai shrimps in commercial scale. Moreover, use of antibiotics for a period of time might cause resistances to antibiotics in the bacteria due to their adaptabilities for new resistance genes (Moriarty, 1998).

Many scientists try to solve the problem of the diseases by enhancing non-specific immune response which is a main defense mechanism in shrimp. Using of immunostimulants is another

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approach to increase immunity to defend shrimp against diseases. Peptidoglycan (PG), derived from the cell wall of gram positive bacteria, was reported to be used as an immunostimulant in aquaculture. It was found to increase survival rate and enhance protection against pathogens in shrimp (Soderhall and Cerenius, 1992; Boonyaratpalin and Boonyaratpalin, 1995; Sung *et al*., 1996; Itami *et al*., 1998; Vargas-Albores *et al*., 1998)

Although peptidoglycan can enhance immunity of shrimp, shrimp farmers need to use it wisely. If shrimp farmers use excess amount of PG supplement in feed, it may waste a lot of money. This research was conducted to determine the optimum concentration and time for effective use of PG which could be recommended for shrimp aquaculture.

MATERIALS AND METHODS

Experimental animals

The test shrimps were obtained from a farm in Chachoengsao Province, Thailand. Shrimps with approximate weight of 11-15 g per animal were used. They were acclimatized in a $45 \times 60 \times 45$ cm^3 aerated aquarium system filled with 25 ppt chlorinated sea water and changing of water was made everyday. Twenty shrimps per replication were used.

Experiment 1 Determination of optimum concentration

Immune parameters

Four treatments consisted of commercial shrimp pelleted feed mixed with PG at 0, 0.06, 0.12 and 0.18 g/kg feed, top-dressing with squid oil, were used in the experiment. Shrimps were fed five times daily at 3% body weight per day. The immune parameters, consisted of phenoloxidase, superoxide anion, bactericidal activity and clearance ability were measured after 7 days of the feeding trial.

Experiment 2 Determination of optimum time of application

The results from experiment 1 were evaluated to choose only one suitable concentration for this experiment. Four treatments consisted of control, feed with PG applied 3, 5 and 7 days/week were used in the experiment. Shrimp were fed five times daily at 3% body weight per day. Shrimps were continuously fed with PG for 4 weeks, following by normal feed for 4 weeks and PG for 2 weeks, respectively. Immune parameters consisted of phenoloxidase, superoxide anion and bactericidal activity were measured every week for 10 weeks.

All results were evaluated to establish the standard protocol for the application of peptidoglycan for *Penaeus monodon* culture including the concentration and the time of application.

Immune parameters

Preparation of hemolymph samples

Blood sample of 0.5 ml from each sample was withdrawn from the base of 3th walking leg of the shrimp by a syrynge containing 1.5 ml anticoagulant (K-199 + 5% L-cysteine).

1) Phenoloxidase activity assay

The method was modified from Supamattaya *et al.* (2000).

After the blood was withdrawn, the hemocytes were washed three times with shrimp saline (1,000 rpm 4 °C 10 min). Hemocyte lysate (HLS) was prepared from hemocytes in a cacodylate buffer pH 7.4 by using the sonicator at 30 amplitute for 5 second and the suspension was then centrifuged at 10,000 rpm., 4°C for 20 min. The supernatant was collected as HLS. Then 200 µl of 0.1% trypsin in cacodylate buffer was mixed to the 200 ml HLS followed by 200 µl of L-dihydroxyphenylalanine (L-DOPA) at 4 mg/ml as the substrate. Enzyme activity was measured as the absorbance of dopachrome at 490 nm.

wavelength. Measurement of protein content in HLS was made by using the method of Lowry *et al*. (1951). The phenoloxidase activity was calculated as the increasing of optimum density (OD) per minute per mg of protein as:

1 unit of phenoloxidase = Δ OD_{490} / min/ mg protein

2) Superoxide anion (O_2^-)

The method was modified from Supamattaya *et al*. (2000)

The O_2^- was detected by reduction of redox dye, nitroblue tetrazolium (NBT). By this method, hemocytes were washed 3 times in K-199 solution. Living cells were separated by using trypan blue solution and adjusted to 1×10^7 cell/ ml suspension. 200 µl of the cell suspension of each sample was dropped into well of a 96 microwell sterile plate. The plate was left for 45 min at room temperature for incubation period. Unattached cells were washed out by K-199 solution. 100 µl of the reaction mixture (0.5 mg zymozan in 0.5 ml serum + 20 mg NBT in 1 ml DMSO + K-199) was added to each well and the reaction mixture was incubated at 25 °C for 60 min. NBT was reduced by O_2^- during incubation period into a water insoluable blue formozan (Supamattaya *et al*., 2000). The reaction was inhibited by putting 70% methanol into the samples for 3 min. and the samples were then allowed to air dry. 120 µl of 2M NaOH and 140 µl of dimethyl sulfoxide (DMSO) were added to each well in order to dissolve the formozan. The concentration of the prussian-blue-colored solution was measured at 620 nm. KOH/DMSO was used as a blank control. The amount of O_2^- was indicated by the increasing in absorbance at 620 nm of 0.001 from control.

3) Clearance ability

The method of study was modified from Martin *et al.* (1993). The pathogenic bacteria, *V. harveyi*, was subcultured in Tryptic soy agar (TSA) with 1.5% NaCl and incubated at 35°C for 24 hrs. A single colony of *V. harveyi* was suspended in 1.5% NaCl sterile water. The solution with the OD of 0.1-0.15 measured by absorbance value at 640 nm wavelength was used to count for the number of bacteria after cultured on TCBS agar by spread plate technique. Then 0.1 ml of the bacterial suspension with the counted number was injected to each tested shrimp while the control was injected with saline water. Three hours after injections, 0.5 ml of the blood from each shrimp was withdrawn to determine the number of bacteria by spread plate technique and statistically compared.

4) Bactericidal activity

Serum was separated from blood of each shrimp sample and diluted by 2.6% NaCl at 1:2, 1:4, 1:8, 1:16 and 1:32. Then 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control were used in the study. *V. harveyi* suspension of 0.5 ml (prepared from the method as in 3) was put into each serum dilution and the control. The treatments were incubated at room temperature for 3 hr before enumerating the number of bacteria by a spread plate technique. Recording of the results were made from the dilution that could decrease 50% *V. harveyi* compared to the control.

Statistical analysis

Means were statistically compared by Analysis of Variance and Duncan's New Multiple Range Test at $p = 0.05$.

RESULTS

Experiment 1 Determination of optimum concentration

Immune parameters

1) Phenoloxidase

After 7 days of feeding, shrimp fed with PG 0.18 g/kg had phenoloxidase 358.26 ± 42.42 unit/min/mg. protein which were significantly

higher (P<0.05) than those fed with 0.06, 0.12 and control with the value of 281.90 ± 21.82, 300.03 ± 18.97 and 287.13 ± 48.89 unit/min/mg. protein respectively (Figure 1).

2) Superoxide anion

Shrimp fed with PG 0.18 g/kg had highest level of superoxide anion at 17.5 ± 6.758 unit which was significantly different from the control (P<0.05) with the value of 8.50 ± 2.65 unit but not significantly different (P>0.05) from those fed with 0.06 and 0.12 g/kg with the value of 9.75 ± 4.27 and 12.75± 5.38 unit respectively (Figure 2).

3) Bactericidal activity

Shrimp fed with PG 0.18 g/kg had bactericidal activity at the serum dilution of 1:16 while PG 0.06, 0.12 g/kg and control were 1:8, 1:8 and 1:4 respectively (Figure 3).

4) Clearance ability

Study on clearance ability after injected with *V. harveyi* in shrimp fed with PG 0, 0.06, 0.12 and 0.18 g/kg for 7 days revealed that number of *V. harveyi* in blood of all PG-treated shrimp were significantly lower than the control (P<0.05) (Figure 4). There were no significant differences among PG-treated shrimps (P>0.05).

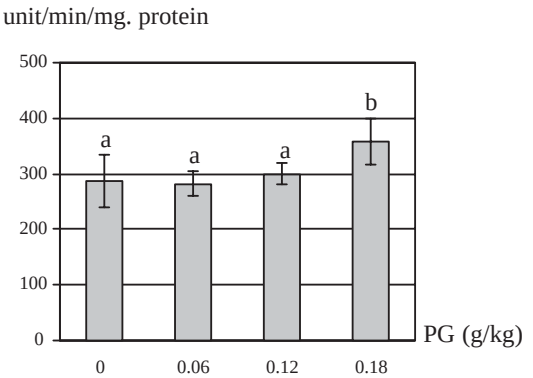

Figure 1 Phenoloxidase of *P. monodon* after 7 days of feeding with PG at 0, 0.06, 0.12 and 0.18 g/kg feed.

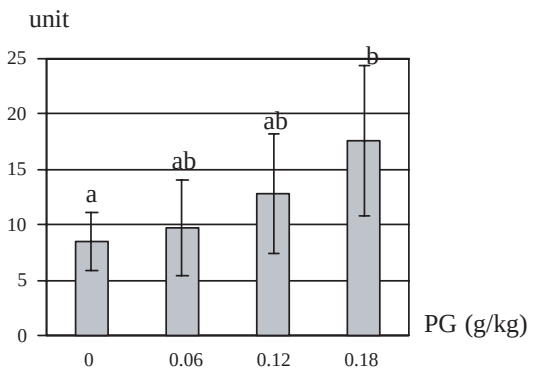

Figure 2 Superoxide anion of *P. monodon* after 7 days of feeding with PG at 0, 0.06, 0.12 and 0.18 g/kg feed.

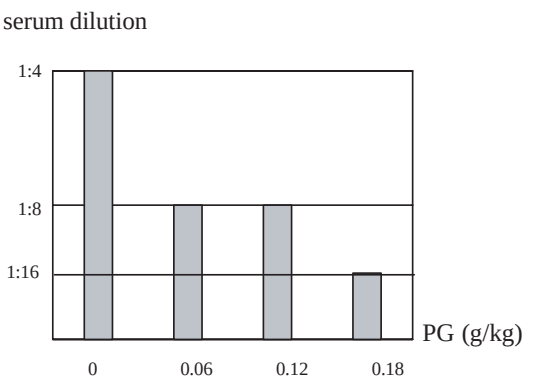

Figure 3 Bactericidal activity of *P. monodon* after 7 days of feeding with PG 0, 0.06, 0.12 and 0.18 g/kg feed.

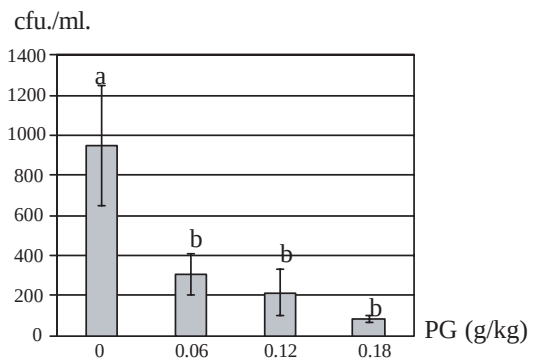

Figure 4 Number of *V. harveyi* in blood of *P. monodon* fed with PG at 0, 0.06, 0.12 and 0.18 g/kg after injected with *V. harveyi* for 3 hours (clearance ability).

The result from this experiment suggested that application of PG at 0.18 g/kg effectively enhanced the immunity of *P. monodon*. This concentration would be used in Experiment 2 to determine the optimum time of application.

Experiment 2 Determination of optimum time of application

Shrimps were fed with PG at 0.18 g/kg for 3, 5 and 7 days/week for one month, normal feed for one month and PG for other two weeks. Study on immune parameters revealed the same trend in which during PG feeding period for 4 weeks 5 and 7 days/week induced significant increase of immunity more than 3 days/week and control ($P<0.05$). After stop feeding of PG, these elevated levels persisted for 2-4 weeks depending on the parameters including phenoloxidase (Figure 5), superoxide anion (Figure 6) and bactericidal activity (Figure 7). And the immune

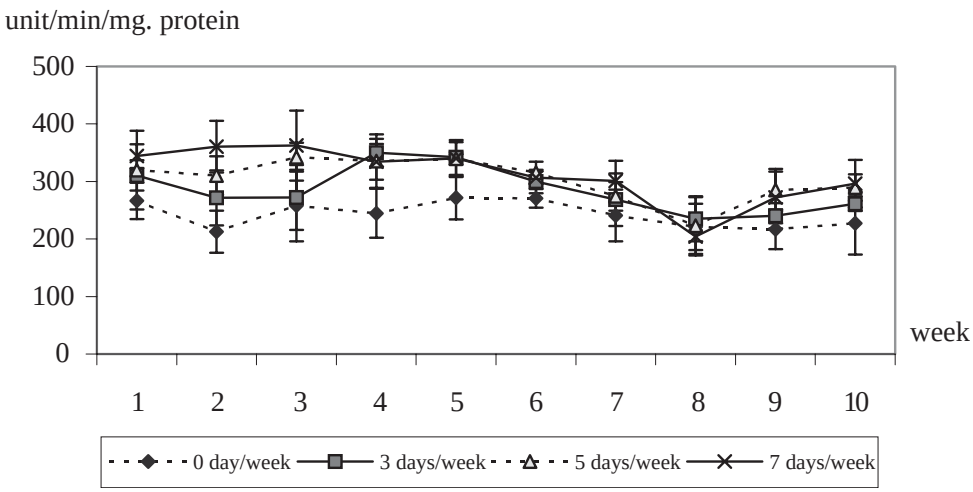

Figure 5 Phenoloxidase of *P. monodon* after 10 weeks of feeding with PG 0.18 g/kg. at week 1-4, normal feed at week 5-8 and PG 0.18 g/kg at week 9-10.

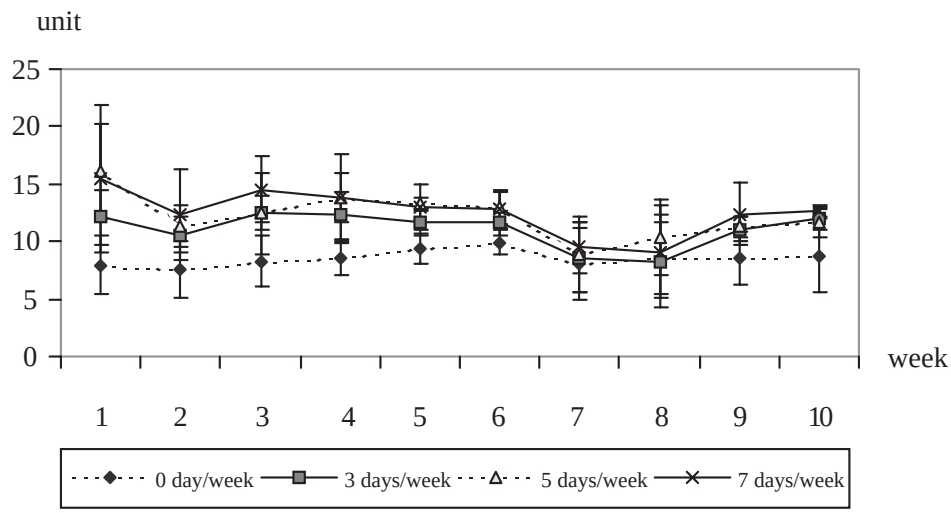

Figure 6 Superoxide anion of *P. monodon* after 10 weeks of feeding with PG 0.18 g/kg at week 1-4, normal feed at week 5-8 and PG 3 g/kg at week 9-10.

levels were significantly elevated again after two weeks of PG supplemented with the same protocol (5 and 7 days/week).

DISCUSSION

The results from this study indicated that PG 0.18 g/kg feed could effectively enhance immunity of *Penaeus monodon* consisting of phenoloxidase activity, superoxide anion, bactericidal activity and clearance ability. These values were significantly different ($P<0.05$) from 0.06, 0.12 g/kg and control. Boonyaratpalin *et al.* (1995) found that black tiger shrimp fed with PG supplement in the feed at 0.01% for 5 weeks showed better growth, survival and feed conversion ratio, phagocytic activity of hemocytes compared to those fed with normal diet.

Itami *et al.* (1998) reared kuruma shrimp (*Penaeus japonicus*) with PG supplemented feed at 0.2 mg/kg body weight/day for 7 consecutive days, alternated with 7 days without PG throughout a 95-day test period. The shrimps were sampled on day 65 and 95 and challenged by *Vibrio penaeicida* and white spot syndrome baculovirus (WSSV). Survival rate of PG-fed group was significantly higher than the control ($P<0.01$). Moreover, phagocytic activity of PG-fed shrimps was higher than the control ($P<0.05$). Itami (2002) found that kuruma shrimp fed with PG at a concentration of 0.2 mg/kg body weight/day with a 7-day intermittent schedule in which PG was fed for 4 days followed by 3 days of control diet (for a month) exhibited a higher survival rate (97.6%) than the control (19%) when challenged by WSSV.

All of the mentioned research and this study clearly indicated the effectiveness of peptidoglycan as immunostimulant for penaeid shrimp. But the optimum frequency for use peptidoglycan has not been established yet. Based on this study, peptidoglycan should be used at 0.18 g/kg feed for 5 days/week for one month. This will elevate the immune level for about 6-8 weeks. Therefore this application can be used during the first or second month of the crop, followed by normal feed for one month and the same protocol is repeatd for another month. The procedure will ensure the length of immunoenhancement effect that can protect the shrimp from disease throughout

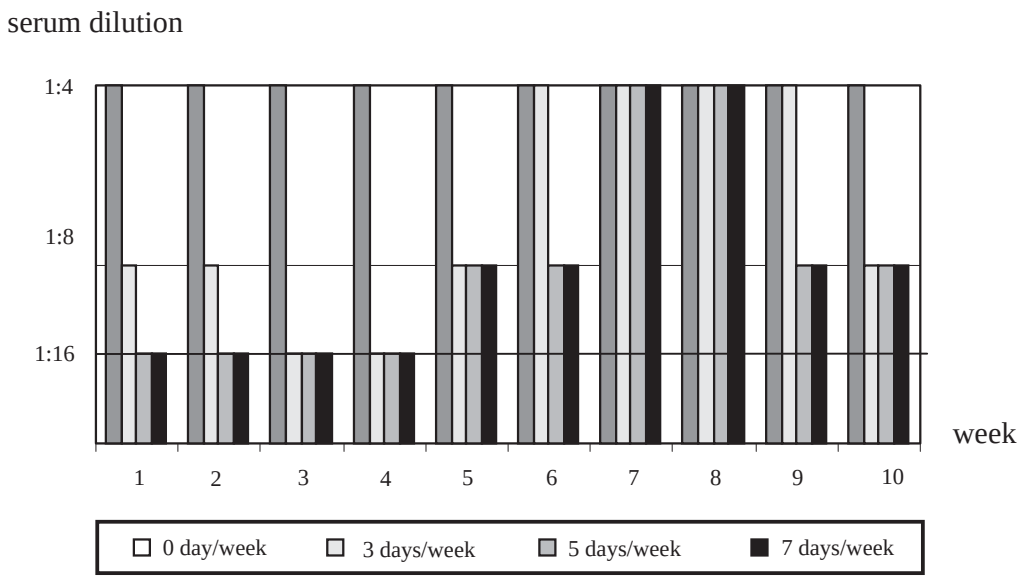

Figure 7 Bactericidal activity of *P. monodon* after 10 weeks of feeding with PG 0.18 g/kg at week 1-4, normal feed at week 5-8 and PG 0.18 g/kg at week 9-10.

the crop. The recommended protocol will slightly increase the cost of production.

CONCLUSION

Penaeus monodon fed with peptidoglycan supplemented in feed at 0.18 g/kg feed for 7 days developed enhanced immunity including phenoloxidase activity, superoxide anion, bactericidal activity and clearance ability.

Optimum time for peptidoglycan at 0.18 g/kg feed was 5 days/week for 1 month, which could elevate phenoloxidase activity, superoxide anion and bactericidal activity. These elevated immune level lasted for other 2-4 weeks after shrimps were fed with normal feed and the immunity can be enhanced again after the same scheme of peptidoglycan application.

ACKNOWLEDGMENTS

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