NONSPECIFIC IMMUNE RESPONSE AND RESISTANCE OF *Litopenaeus vannamei* FED WITH NUCLEOTIDE, β-GLUCAN, AND PROTAGEN DIETS

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ABSTRACT

The objective of this research was to evaluate the nonspecific immune response and resistance of *Litopenaeus vannamei* fed with nucleotide, β-glucan, and protagen diets. Shrimp juveniles with an average weight of 5.39±0.56 g were reared in glass aquaria at a density of 15 shrimps/aquarium. Shrimps were fed three times a day for four weeks at a feeding rate of 3%/bw/day. Treatment diets consisted of A: basal diet (without immunostimulant), B: β-glucan, C: protagen, and D: nucleotide, each with three replicates. At the end of feeding period, the shrimps were intramuscularly injected with *Vibrio harveyi* 0.1 x 10^6 cfu.shrimp^{-1}. Total haemocyte count (THC) of shrimp fed with nucleotide-diet was significantly different compared to that of control shrimp (p=0.01), but not different compared to shrimp fed with protagen-diet. PO activity also increased significantly in shrimp fed with nucleotide-diet (p=0.02). β-glucan diet could also increase THC and PO activity, but compared to the control, the increase was not significantly different. Overall, PO activity of shrimp fed with nucleotide, β-glucan, and protagen diets was high (>0.35). Oral administration of nucleotide, β-glucan, and protagen for four consecutive weeks significantly increased resistance of shrimp to disease (<0.01) where the highest resistance rate was observed on shrimp fed with nucleotide-diet. Growth of shrimp fed with nucleotide-diet was significantly different compared to that of control shrimp (p<0.01), as well as to β-glucan, and protagen-treated shrimp. As a conclusion, supplementation of nucleotide into shrimp pellet enhanced nonspecific immune response and growth performance better than β-glucan, and protagen.

KEYWORDS: ***Litopenaeus vannamei,*** **nucleotide, total haemocyte count, PO activity, resistance, growth**

INTRODUCTION

Developing shrimp culture, especially species of *Litopenaeus vannamei* and *Penaeus monodon*, has been the main program of the Indonesian government. However, since the last two decades, many farmers and industries had suffered significant economic losses due to viral disease. WSSV destroyed the industry since 1992/1993, and starting 2006, new disease namely infectious myonecrosis virus (IMNV) has been found to infect many shrimp aquaculture in Indonesia. These two viral diseases are still unsolved.

A number of strategies that had been applied in diseases control included the use of probiotic bacteria, SPR or SPF shrimp, and

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biosecurity system. Many reports had shown that even though these methods can significantly increase production but disease still continues to occur because the susceptibility of shrimp to pathogen may differ according to life stages and the present of genetic mutation of pathogen in the environment. The use of immunostimulant is an alternative approach for disease control in shrimp aquaculture.

Immunostimulant is a substance that induces nonspecific immune response against infection of various pathogens simultaneously. This substance can be used as prophylactic treatment for unexpected disease or as suppressive treatment for latent and sub lethal pathogen (Nikl *et al*., 1993). Unlike vaccine, immunostimulant increases resistance of cultured shrimp against infectious pathogen simultaneously through stimulating the nonspecific immune response (Gannam & Schrok, 2001).

Immunostimulant can be grouped into bacteria and bacterial product, yeast, carbohydrate complex, nutrition factor, animal and plant extracts, and synthetic drugs (Sakai, 1999; Sealey & Gatlin III, 2001; Cook *et al*., 2003). Researches in fish and crustacean mostly used β-glucan because it occurs naturally, and no residue in fish and environment. The most common use of glucan products is *Saccaharomyces cerevisiae* (baker's yeast) and preparation of fungi *Schizophyllum commune* and *Selerotium glucanicum* (Sakai, 1999). Lopez *et al*. (2003) reported that administration of 2 g of β-glucan per kg diet could induce immune response of *L. vannamei*. Chang *et al*. (2003a) recommended the use of 2 g β-glucan per kg diet for 24 days for shrimp *P. monodon*, while Itami *et al*. (1998) recommended 2 g β-glucan per kg diet for *P. japonicus*.

This research used nucleotide as an immunostimulant. Nucleotides are semi-essential nutrient that have essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signaling as well as serving as components of coenzymes, allosteric effectors and cellular agonist (Li & Galtin III, 2006). Application of nucleotide for disease control in aquaculture has obtained more attention since 2001. Publications concerning the application of nucleotide in fish showed that nucleotide could enhance immune response and resistance of fish against various pathogens simultaneously, increase growth and tolerance to stress. On the other hand, report on the use of nucleotide in shrimp was still unavailable or very limited. In our previous research, it was found that oral administration of nucleotide at a level of 400 mg.kg^{-1} pellet significantly enhanced nonspecific immune response, resistance and growth of *Litopenaeus vannamei* (Manoppo *et al*., 2009). A comparative study with other immunostimulants is necessary to be conducted before this finding is applied in shrimp aquaculture. The objective of this research was to evaluate the nonspecific immune response, resistance and growth performance of *L. vannamei* fed with nucleotide β-glucan, and protagen diets.

MATERIALS AND METHOD

Shrimp

Shrimp juvenile (*Litopenaeus vannamei*) was collected from cultivation area in Bakauheni, South Lampung. Shrimps were placed into styrofoam boxes equipped with aerator and then transported to Fish Health Laboratory at the Bogor Institute of Agriculture, Bogor.

Immunostimulant

Immunostimulants used in this research consisted of pure nucleotide (Sigma-Aldrich), β-glucan, and Protagen (Diasham Resource PTE, Singapore). Nucleotide consisted of uridine-5'-monophosphate disodium salt, cytidine-5'-monophosphate disodium salt, guanosine-5'-monophosphate disodium salt, adenosine-5'-monophosphate sodium salt, and inosine-5'-monophosphate disodium salt.

Diet Preparation

Nucleotide, β-Glucan, and protagen were first diluted in small amount of distilled water, mixed thoroughly into basal diet, and dried at room temperature. The mixture was then coated with albumin (egg white), and dried at room temperature. Pellet was then put into plastic bags and stored in refrigerator until used.

Experimental Design

The research was carried out using Randomized Complete Design with four treatments, each with three replicates. The treatments included:

A. Basal diet (without immunostimulant)
B. β-glucan 2 g.kg^{-1} pellet
C. Protagen 2 g.kg^{-1} pellet
D. Nucleotide 0.4 g.kg^{-1} pellet

Research Procedure and Data Collection

Shrimp juveniles were reared for two weeks in 2 of 1,000 L circular fiberglass tank for adaptation process. During acclimatization, the shrimps were fed with basal diet three times a day at 09.00, 13.00, and 17.00, with feeding rate of 3%/bw/day. Juveniles were then distributed into 12 glass aquaria (60 cm x 30 cm x 30 cm) each equipped with aerator and water recirculation. Each aquarium contained 50 L of water with 15 juveniles. Juveniles were fed with treatment diets for four weeks at a feeding rate of 3%/bw/d and applied three times a day at 09.00, 13.00, and 17.00.

Sample of haemolymph for measuring immune parameters was gathered at the end of the feeding period. Haemolymph was collected according to procedure suggested by Liu & Chen (2004). Shortly after that, about 1 mL of haemolymph was withdrawn from ventral sinus at the base of first abdomen using 1 mL syringe previously inserted with 0.1 mL anticoagulant. 0.8 mL of anticoagulant was then added to the mixture to make the ratio between haemolymph and anticoagulant 1:9.

Immune Parameter

Immune parameters measured included total haemocyte count (THC) and phenoloxidase (PO) activity. THC was counted using light microscope at 40x magnification. PO activity was measured based on dopachrome formation produced by L-DOPA. The measurement was done according to the procedure of Liu & Chen (2004). First, 1 mL of haemolymph-anticoagulant mixture was centrifuged at 700 g for 20 minutes at 4°C. Supernatant was then removed and pellet was suspended into cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7) and centrifuged again. Pellet was suspended into 200 µL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7).

Aliquot of 100 µL was incubated with 50 µL trypsin (1 mg.mL^{-1} cacodylate buffer) as activator for 10 minutes at 25°C-26°C. Then, 50 µL L-DOPA (3 mg.mL^{-1} cacodylate buffer) was added, after 5 minutes, added 800 µL cacodylate buffer. Optical density (OD) 490 nm was measured using Spectrophotometer.

Resistance

Four weeks after the feeding, the shrimps were injected intramuscularly with 0.1 mL of *Vibrio harveyi* 1 x 10^6 cfu.mL^{-1} at the dorsal of third abdomen. During the challenge test, the shrimps were fed with basal diet three times a day at 3%/bw/d. Mortality was observed every day for 14 days. Disease resistance was determined based on survival rate of shrimp after challenge test.

$$SR\ (\%) = \frac{Nt}{No} \times 100\ \%$$

where:
SR = Survival rate
Nt = Number of live shrimp at time *t*
No = Number of live shrimp at the beginning of experiment

Growth rate

Weight gain of shrimp was measured every 2 weeks namely at day 14th and 28th. Weight gain was calculated based on the formula of:

$$G = Wt - Wo$$

where:
G = Weight gain
Wt = Final weight of shrimp (g)
Wo = Initial weight (g)

Data analysis

Data were presented as mean±Sd. The effect of immunostimulants on THC, PO activity, resistance, and growth of shrimp was evaluated through analysis of variance (ANOVA). Duncan Test was conducted to evaluate if there were different effects between treatments using SPSS 17 for windows.

RESULTS AND DISCUSION

Total Haemocyte Count

Supplementation of nucleotide, β-glucan, and protagen in shrimp pellet enhanced total haemocyte count (THC) of shrimp. One way ANOVA demonstrated that THC of shrimp fed with nucleotide diet was significantly differ-

ent from that of control shrimp (p=0.01, Table 1), but not different compared to shrimp fed with protagen diet. After four weeks of feeding, THC increased up to 87% higher than in control shrimp. Shrimp fed β-glucan also showed an increase in THC, but the increase was not different compared to that of control shrimp.

Similar result was observed in the previous research in which THC of shrimp fed with nucleotide-diet at 400 mg.kg^{-1} pellet for four weeks increased up to 76% which was higher than the control (Manoppo *et al*., 2009). Nucleotide is a semi essential nutrient required for growth and cell replication (Barnes, 2006). Supplementation of nucleotide in shrimp diet may optimize proliferation of cells including immune cells (Sajeevan *et al*., 2006). Protagen is a protein supplement (yeast extract) for fish and shrimp feed. It is known that besides protein, yeast is rich of immunostimulatory compounds such as glucan, nucleotide, and lipopolysaccharide, thus enhanced nonspecific immune response. β-glucan had been known to induce THC of *L. vannamei* (Lopez *et al*., 2003), *M. rosenbergii* (Sahoo *et al*., 2008). β-glucan supplemented to shrimp pellet will bind to molecule receptors present at the surface of phagocyte cells (Raa, 2000). The cells then become more active for phagocytosis of pathogen or foreign particles and at the same time, they produce signal molecule (cytokine) that stimulates the production of new haemocyte.

Phenoloxidase Activity

Oral administration of nucleotide, protagen, and β-glucan increased PO activity. Analysis of variance showed that PO activity of shrimp fed with nucleotide-diet for four weeks was different from that of control shrimp (p=0.02, Table 2). PO activity observed on protagen-fed shrimp was also different compared to control.

The process of nucleotide increases PO activity is as still unclear, but Li & Galtin III (2006) assumed that nucleotide added to the diet will participate in cell signaling pathway as well as be used as nutrient for biosynthetic processes. It was also observed that β-glucan increased PO activity even though the different was not significant. Several reports had shown that β-glucan could enhance PO activity of *P. monodon* (Chang *et al*., 2003a), *L. vannamei* (Lopez *et al*., 2003), and *Macrobrachium rosenbergii* (Sahoo *et al*., 2008). β-glucan enhanced PO activity after binding to β-glucan binding protein (Li *et al*.,

Table 1. THC (x 10^7 cell/mL) of *L. vannamei* fed nucleotide, β-glucan, and protagen diets for four weeks

Treatments	THC
Basal diet	1.119 ± 0.270^{a}
β-glucan	1.422 ± 0.175^{ab}
Protagen	1.955 ± 0.289^{bc}
Nucleotide	2.090 ± 0.438^{c}

Mean value with different superscripts was significantly different (p=0.01)

Table 2. PO activity of *L. vannamei* fed nucleotide, β-glucan, and protagen diets for four weeks

Treatments	PO activity
Basal diet	0.304 ± 0.028^{a}
β-glucan	0.376 ± 0.052^{ab}
Protagen	0.579 ± 0.149^{bc}
Nucleotide	0.633 ± 0.163^{c}

Mean value with different superscripts was significantly different (p=0.02)

2008; Vargas-Albores & Yepiz-Plascencia, 2000). Once it binds, inactive proenzyme PO (proPO) is activated to be PO enzyme necessary for melanization. Furthermore, Lopez *et al*. (2003) reported that β-glucan added to the diet will induce cell activating factors in haemocyte, thus increase PO activity and phagocytosis. In normal condition, shrimp having high THC (Table 1) displayed high PO activity too because haemocyte functions in producing and releasing proPO into haemolymph (Vargas-Albores & Yepiz-Plascencia, 2000). Overall, PO activity induced by nucleotide, protagen, and β-glucan was high (>0.35) (Gullian *et al*., 2004).

Resistance

Disease resistance was determined based on survival rate of shrimp after challenged with *Vibrio harheyi* 1 x 10^6 cfu.shrimp^{-1} (Figure 1). Mortality occurred one day after challenge, and continued until 4 day-post challenge. Afterward, no mortality was observed in all treatments.

Application of immunostimulant nucleotide, protagen, and β-glucan positively affected the resistance of shrimp to pathogens. One way Anova showed that 28 days post-challenge, resistance of shrimp fed with these three immunostimulants was significantly different from that of control shrimp (p=0.003). Nucleotide-treated shrimp had the highest resistance rate followed by β-glucan and then protagen (Table 3).

There was no report concerning the effect of nucleotide supplementation on shrimp resistance to disease. In fish, Li *et al*. (2004)

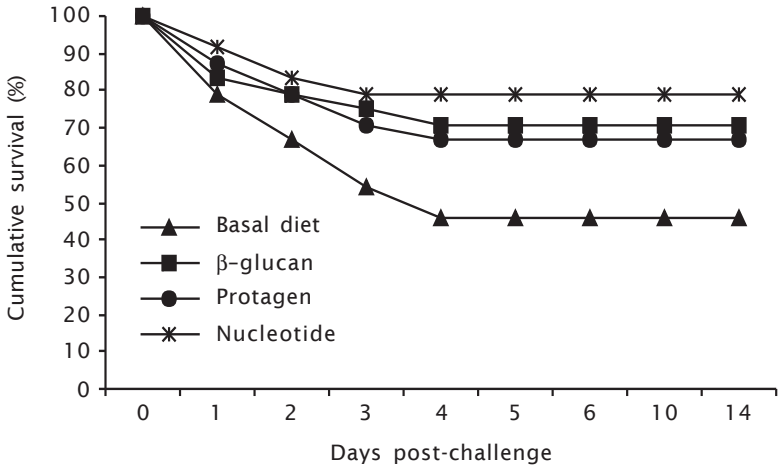

Figure 1. Cumulative survival of *L. vannamei* fed nucleotide, β-glucan, and protagen diets and challenged with *Vibrio harveyi* 1 x 10^6 cfu.shrimp^{-1}

Table 3. Resistance of *L. vannamei* fed nucleotide, β-glucan, and protagen diets for four weeks and challenged with *Vibrio harveyi* 1 x 10^6 cfu.shrimp^{-1}

Treatments	Resistance (%)
Basal diet	45.83±7.22^a
β-glucan	70.83±7.22^b
Protagen	66.67±7.22^b
Nucleotide	79.17±7.22^b

Mean value with different superscripts was significantly different (p=0.03)

reported that oxidative radical production of blood neutrophyl of hybrid striped bass increased after fed with nucleotide-diet for 6–7 weeks and infected with *Streptococcus iniae,* and survival of fish (80%) was higher than that of fish fed free nucleotide-diet (60%). Burrels *et al.* (2001) also reported that mortality of rainbow trout fed with nucleotide-diet (optimum) for 2 weeks and challenged with ISAV (infectious salmon anaemia virus) was 35.7% while mortality of fish fed basal diet was 48%.

β-glucan had been proved to increase resistance of shrimps including *Penaeus monodon* (Chang *et al*., 2003a; 2003b; Song *et al*., 2003; Sung *et al*., 2001), *Metapenaeus japonicus* (Itami *et al*., 1998), and *L. vannamei* (Burgents *et al*., 2004). β-glucan induced response immune by increasing phagocytosis activity of phagocyte cells (Yin *et al*., 2006).

Growth

Oral administration of nucleotide, protagen, and β-glucan for 2 weeks did not induce growth of shrimp. But after feeding for 4 consecutive weeks, growth rate of shrimp fed with nucleotide-died was significantly higher than control ($p<0.01$, Table 4) as well as higher than protagen and β-glucan.

Weight gain of shrimp fed with nucleotide-diet was 4,73 g or 65,38% higher than that of shrimp fed with basal diet (Figure 2). Similar result was observed in the previous research where weight gain of shrimp fed with nucleotide-diet at 400 mg.kg^{-1} pellet for 4 weeks achieved 50.74% higher than control shrimp (Manoppo *et al*., 2009).

Nucleotide might enhance growth through increasing feed efficiency and food intake.

Table 4. Growth performance of *L. vannamei* fed nucleotide, β-glucan, and protagen diets for four weeks

Treatments	Initial weight (g)	Final weight (g)		Weight gain (g)	
		14th day	28th day	14th day	28th day
Basal diet	5.39±0.56	7.37±0.36^{a}	8.25±0.71^{a}	1.98±0.36^{a}	2.86±0.71^{a}
β-glucan	5.39±0.56	7.21±0.53^{a}	9.13±0.45^{b}	1.82±0.53^{a}	3.74±0.45^{b}
Protagen	5.39±0.56	7.77±0.64^{a}	9.24±±0.79^{b}	2.38±0.64^{a}	3.84±0.79^{b}
Nucleotide	5.39±0.56	7.71±0.81^{a}	10.12±0.57^{c}	2.32±0.81^{a}	4.73±0.57^{c}

Mean value with different superscripts was significantly different (p=0.00)

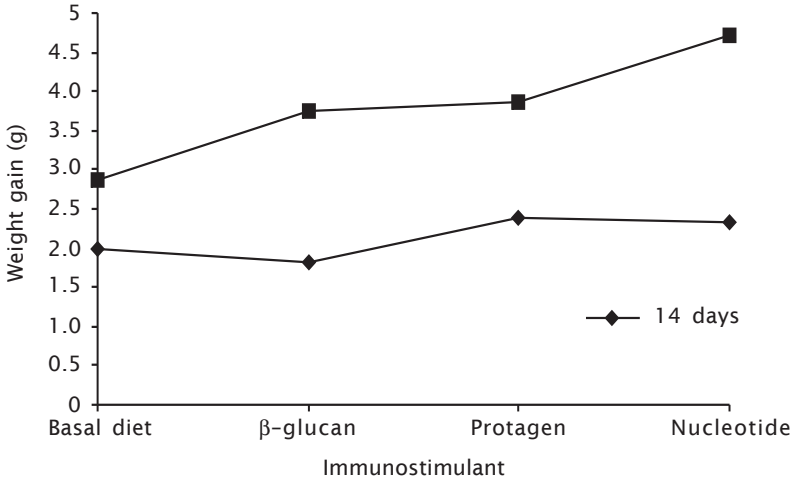

Figure 2. Weight gain of *L. vannamei* fed with different immunostimulans for 14 and 28 days

Adenosine and inosine are good chemo-attractants widely used for fish and crustacean. Therefore, application of nucleotide will increase feed intake and reduce leaching of food into water (Li *et al*., 2007). β–Glucan added to the diet might enhance growth but how this substance works is still unclear (Lopez *et al*., 2003).

CONCLUSION

Application of nucleotide in shrimp culture offered more benefits than β–Glucan and protagen. Besides enhancing nonspecific immune response, it induced growth of shrimp. Further research on leaching of nucleotide into the water and its effect on shrimp need to be conducted.

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