CHAPTER III

RESULTS AND DISCUSSION

1. Determination of antimicrobial activity of PG

The *in vitro* antimicrobial activity tested of polysaccharide gel (PG) extracted from fruit-hulls of durian (*Durio zibethinus* L.) was performed against shrimp pathogenic bacterium, the result showed antibacterial activity of PG against *Vibrio harveyi* 1526. The luminescent bacterium *Vibrio harveyi* 1526, a gram negative bacterium, was used as test bacterium. The bacterium has been commonly found in various marine and caused luminous bacterial diseases in black tiger shrimp. The *Vibrio harveyi* 1526 has caused mass mortalities to black tiger shrimp in aquaculture industry of Thailand (Kasornchandra et al., 1995). The following tests were performed to evaluate antibacterial activity of durian polysaccharide gel against *Vibrio harveyi* 1526, a bacterial causing disease in back tiger shrimp.

1.1 Agar diffusion test

Antibacterial activity of polysaccharide gel against *Vibrio harveyi* 1526 was determined by agar diffusion method (Lorian, 1991; Brock et al., 1994). In the present study, the polysaccharide gel showed inhibitory activity against *Vibrio harveyi* 1526. The result of inhibition zone with sharp and clear margin was observed on plates of agar media added with PG at various concentrations using serial two-fold dilution to make PG concentration at 50.0, 25.0, 12.5, 6.3 and 3.2 mg/ml, respectively. The diameter of inhibition zones was found increasing with respect to increasing concentration of PG as indicated in Table 7. The result seemed similarly to those of Chotigeat et al. (2003). They has reported that the crude fucoidan, a sulfated polysaccharide extracted from brown seaweed *Sargassum polycystum*, at concentration 12 mg/ml inhibited the growth of *Vibrio harveyi* as determined by the agar plate diffusion method.

Table 7. Antimicrobial activity of PG on growth of bacteria *Vibrio harveyi* 1526 by agar diffusion method. Values are mean ± SD, Normal saline solution (NSS) = control, NZ = no inhibition zone.

Concentration of PG (mg/ml)	Diameter of inhibition zone, mm (mean ± SD)
50.0	20.43 ± 1.72
25.0	16.47 ± 1.32
12.5	12.15 ± 0.67
6.3	10.70 ± 0.56
3.2	8.88 ± 0.51
NSS	NZ

1.2 Broth microdilution test

1.2.1 Determination of MIC and MBC

According to the method of broth microdilution test, polysaccharide gel inhibited growth of *Vibrio harveyi* 1526 as demonstrated in table 16, the minimum inhibitory concentration (MIC) represented by the lowest concentration of PG that inhibited visible growth of bacteria was found at 6.3 mg/ml in MHB medium and the minimum bactericidal concentration (MBC) of PG represented by the minimal concentration in tube of non visible growth that showed no bacterial growth on the plates of agar media without PG after subculturing and incubating at 30 °C for 16 hours. The result was found the MBC volue of PG at 12.5 mg/ml concentration (Table 8). The crude fucoidan, a sulfated polysaccharide extracted from brown seaweed *Sargassum polycystum* has also found inhibit growth of *Vibrio harveyi* at the MIC value of 12.0 mg/ml (Chotigeat et al., 2003). The values of MIC and MBC indicated that bactericidal activity of PG against *Vibrio harveyi* 1526 was comparable to that of crude fucoidan extracted thus PG demonstrated completely kill tested bacteria in this study.

The mechanism of antibacterial activity against *Vibrio harveyi* 1526 of PG needed to be further studied. The polysaccharide gel (PG) has intrinsic viscosity and adhesive property due to electronegativity of polysaccharide rhamnogalacturonan in PG in which probable adhesively bound on the cells outer surface (Nantawanit, 2001; Hokputsa, 2004) and then produced interference of the cell normal function and alteration the membrane permeability (Tsai and Su, 1999). Including, the physical properties of PG has intrinsic acidic condition, pH 2.2-2.6 (Gerddit, 2002). PG is a polysaccharide composed of long chains acidic sugar, galacturonic acid, with branched of neutral sugars including rhamnose, fructose, glucose, galactose and arabinose, thus the mechanism of inhibition of *Vibrio harveyi* 1526 seemed to relate with property of eletronegavity of anionic carbonyl of acidic sugar, especially galacturonic acid.

Table 8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PG compared to gentamicin sulfate on growth inhibition against *Vibrio harveyi* 1526

Polysaccharide gel (PG)		Gentamicin sulfate	
MIC (mg/ml)	MBC (mg/ml)	MIC (μg/ml)	MBC (μg/ml)
6.3	12.5	4.0	8.0

The infectious disease *Vibrio harveyi* is found in a natural inhabitant of the seawater environment, it exists commonly in low numbers (Ruby and Nealson, 1978; Shilo and Yetinson, 1979; Lavilla-Pitogo, 1995). The growth of luminous bacterium has performed on the influence of optimal environmental factors such as high levels of organic matters, salinity range 10-60 ppt, water temperature range 25-32 °C, water pH 5-9 and dissolved oxygen range 0.5-7.8 mg/l. The condition of low salinity (5 ppt.), high water pH at 9.5 and low water pH at 3.0 inhibited the growth of *Vibrio harveyi* (Kiriantnikom et al., 2000). At pH 3 of seawater inhibited the growth of *Vibrio harveyi* which was indicated that luminous bacteria can not grow in seawater at low pH condition. The physical properties of PG were intrinsic acid condition due to acidic sugar especially galacturonic acid thus the mechanism of inhibition the *Vibrio harveyi* 1526 seems to relate with the property of acidic sugar in polysaccharide gel.

2. Polysaccharide gel additive diet

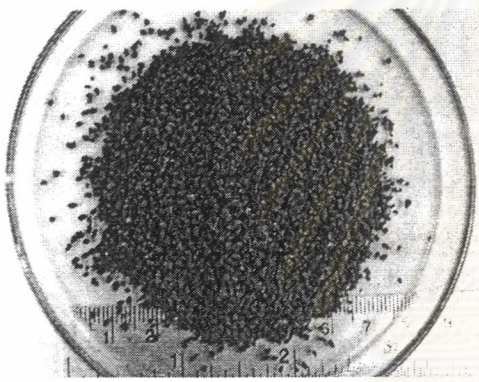
2.1 Diet contents

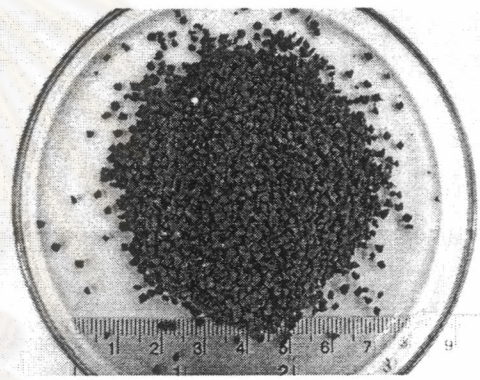
The feature of shrimp diets in various feed size was prepared from the durian polysaccharide gel as illustrated in Figure 21. Analysis of shrimp diets was determined after diet preparation. The nutritional contents are shown in Table 9. All ingredients in diets were not different except the amount of PG.

3. Growth performance

Durian polysaccharide gel (PG) additive diet showed potential effect on promotion of growth performance in black tiger shrimp *Penaeus monodon* juveniles which were clearly demonstrated in the present study. PG was a polysaccharide composed of long chain acidic sugar, galacturonic acid, with branch of neutral sugars including rhamnose, fructose, glucose, galactose and arabinose (Pongsamart, 1998; Hokputsa et al., 2004).

(a)





(b) (c)

Figure 21. The shrimp diets in various feed size were prepared from common diet supplemented with durian polysaccharide gel (PG). Shrimp in the rearing trial fed with the different feed size of shrimp diets due to feeding period, using the small size of shrimp diet in the early of trial rearing (a) and the large size of shrimp diet (b and c) in the bigger shrimp, respectively.

Table 9. Nutritional content of the shrimp diets in treated and control groups in the trial rearing. Control = diet without PG, 0.5% PG = diet added 0.5% PG, 1.0% PG = diet added 1.0% PG, 2.0% PG = diet added 2.0% PG

Ingredients	Compositions (g/100g)	Nutritional content of shrimp diet (g/100g)			
		Control	0.5% PG	1.0% PG	2.0% PG
Moisture		8.70	7.45	6.41	7.35
Protein	40.00	40.00	40.40	40.60	39.80
Fat	6.00	5.98	6.00	5.99	5.72
Fiber	1.50	1.38	1.43	1.53	1.33
Ash	9.00	8.74	8.92	9.18	9.33
Calcium	1.50	1.52	1.55	1.58	1.57
Phosphorus	1.50	1.52	1.60	1.67	1.80
Total carbohydrate	35.50	35.20	35.80	36.29	36.47
PG (g in 100 g diet)	0.5-2.0	0.00	0.50	1.00	2.00

Proximate results standard deviations were less than 1.0%. Moisture was less than 10.0%.

The durian polysaccharide gel (PG) added in diets of *Penaeus monodon* juvenile shrimps as a food additive significantly increased body weight ($P<0.05$) in black tiger shrimp *Penaeus monodon* juvenile, thus PG seemed to be valuable as a diet component of shrimps. Since, the black tiger shrimp *Penaeus monodon* juvenile culture has been the most economic important aquaculture developed in Thailand (Department of Internal Trade, 2003). The growth performance of shrimp after 8 weeks feeding period, Septemper to October, showed the increasing of body weight ($P<0.05$) in treatment groups fed with the 2.0% PG additive diet compared with the control group of shrimp fed with diet without PG (Figure 22). The total length was comparable in treated groups as well as in control group (Figure 23). The survival rate, feed conversion ratio (FCR) and biomass of *Penaeus monodon* juvenile shrimps in groups feeding with the PG additive diet (Figure 24, 25 and 26) were significantly better ($P<0.05$) than that of control group. The size and total length of *P. monodon* juvenile shrimp in the trial rearing are demonstrated in Figure 27. These results indicated that adding durian polysaccharide gel (PG) in a diet of *Penaeus monodon* juvenile shrimp as a food additive seemed to promote growth, feed conversion ratio (FCR), biomass and also survival of black tiger shrimps. In the present study, the durian polysaccharide gel (PG) additive diet showed promotion growth performance of black tiger shrimps (*Penaeus monodon*). Treatment groups of shrimps after fed with the PG additive diet showed higher in body weight than that of control group of shrimp fed with diet without PG. The total length was also increased in treated groups as well as control group as indicated in Figures 23 and 27 that total length of shrimps fed with 0, 0.5, 1.0 and 2.0% PG for two months were 93.6, 93.4, 94.4 and 97.3 mm, respectively. There have been reported that total length of *Penaeus monodon* reaching 330 mm or more is largest in body length (Lee and Wickins, 1992) and weight reching 25-30 g is the highest body weight within 3-4 months after PLs stocking in culture ponds (Rosenberry, 1997). Since *Penaeus monodon* shrimp can well digest the carbohydrate such as β-glucan and used it as an energy source (Wigglesworth and Griffith, 1994). These results suggested that PG additive diet had beneficial effect in providing extra carbohydrate for the *Penaeus monodon* shrimps which required as the energy source for their growth. The carbohydrate is the most economical dietary important energy source (cost/kcal).

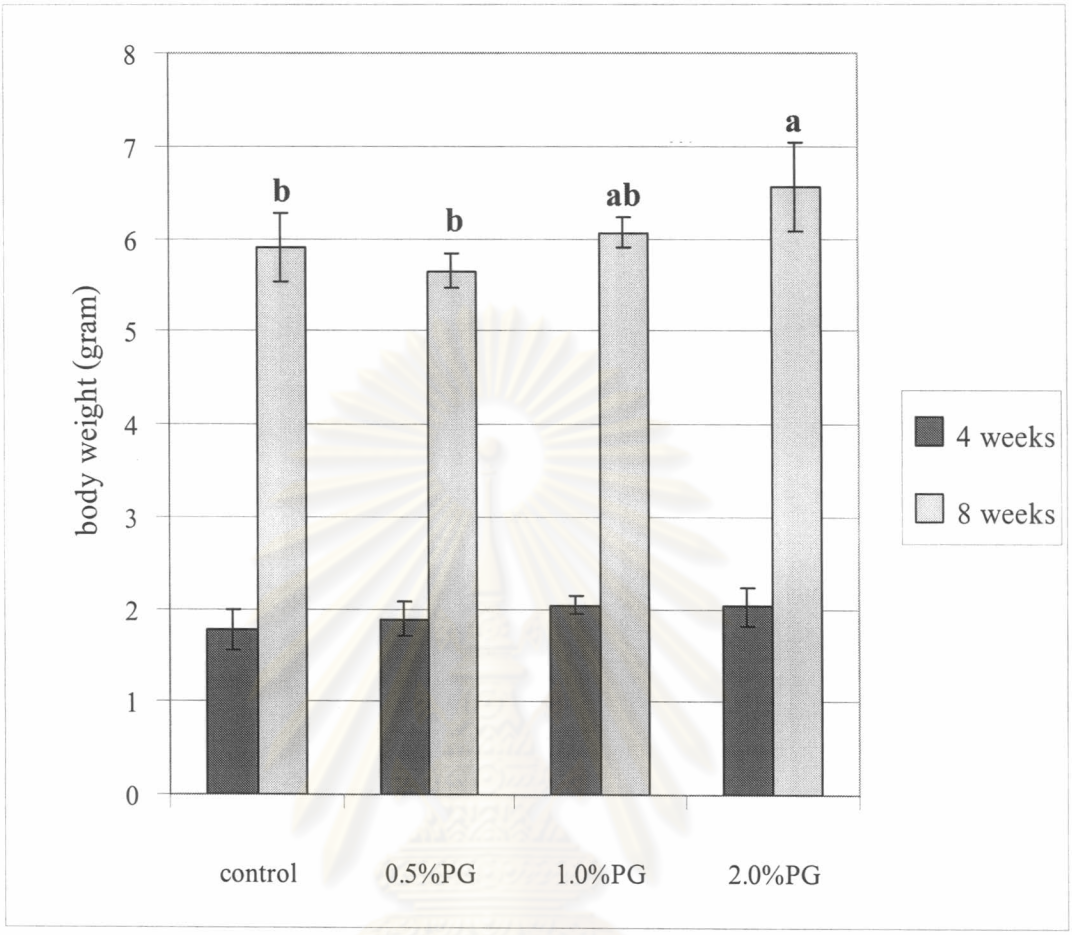


Figure 22. Body weight of *Penaeus monodon* juvenile in the trial rearing after 4 weeks and 8 weeks feeding with diets containing polysaccharide gel (PG) from durian fruit-hulls. Values are mean ± SD. control = 0% PG. a, b = significant difference between groups, ($P<0.05$).

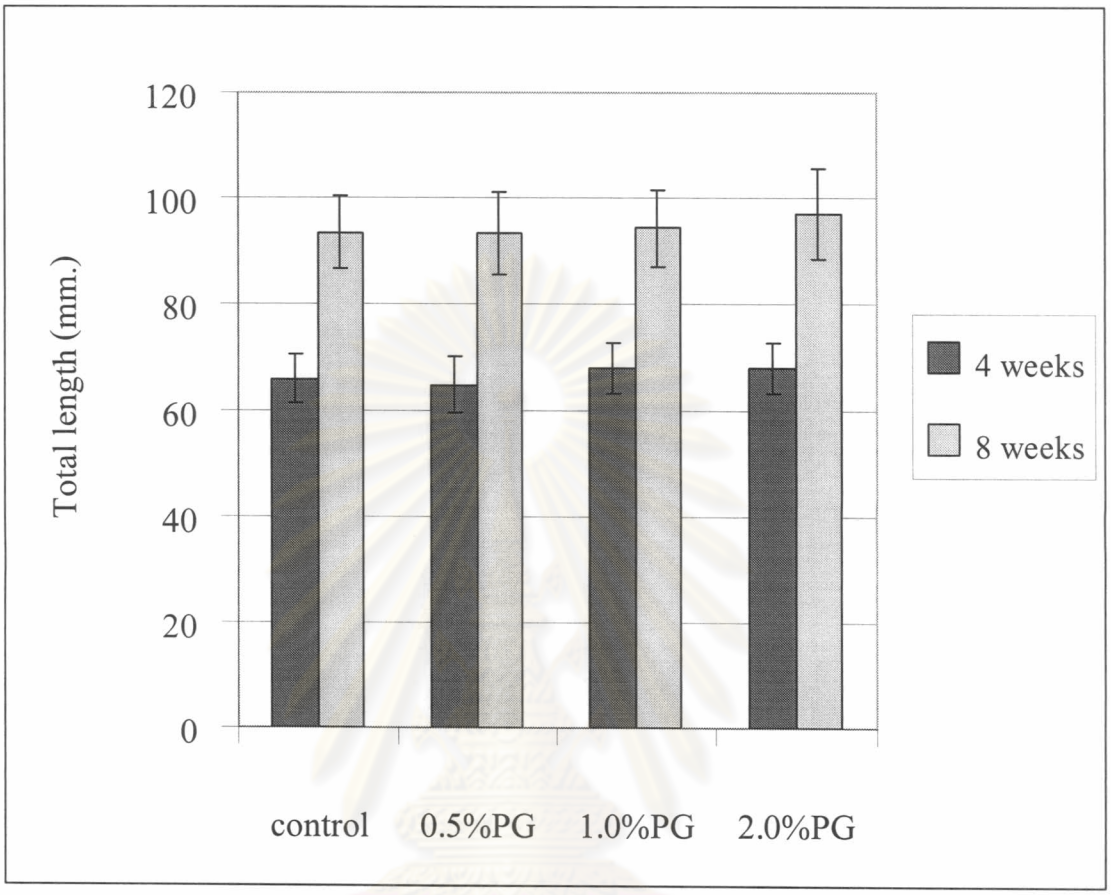


Figure 23. Total length of *Penaeus monodon* juvenile in the trial rearing after 4 weeks and 8 weeks feeding diets containing polysaccharide gel (PG) from durian fruit-hulls. Values are mean ± SD. control = 0% PG.

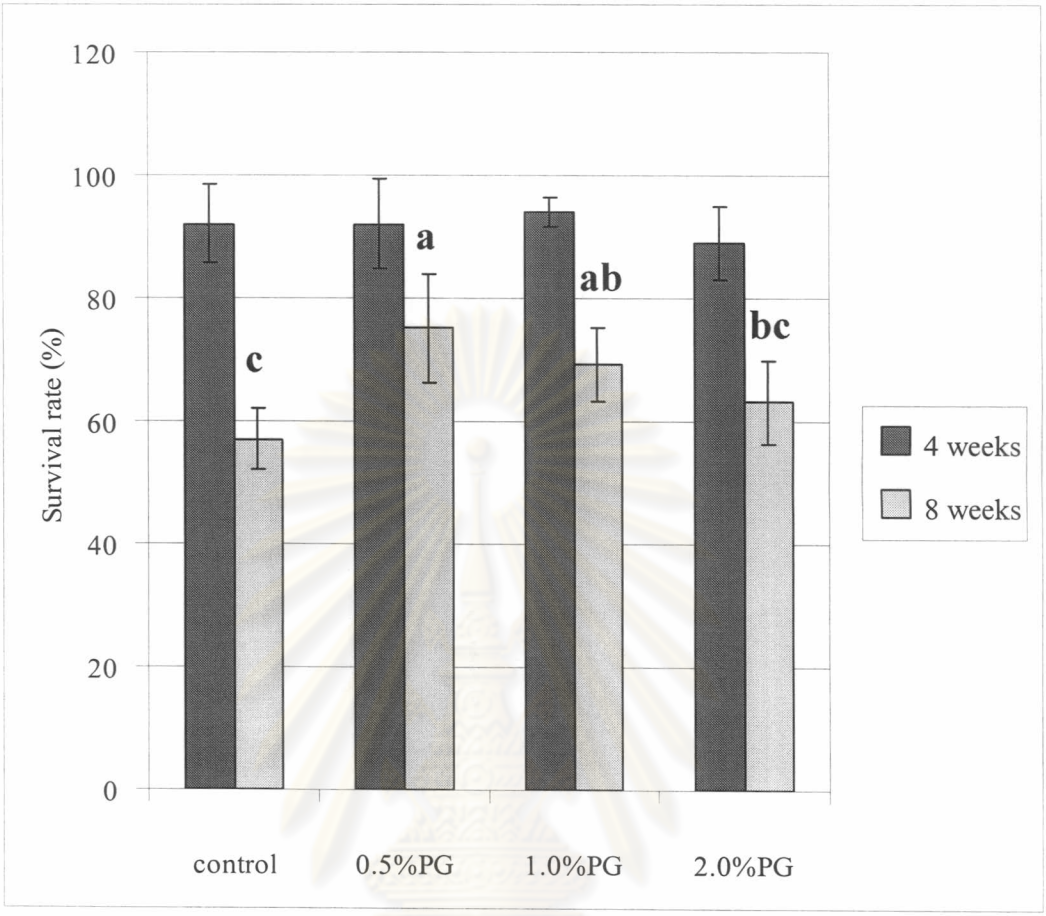


Figure 24. Survival rate of *Penaeus monodon* juvenile in the trial rearing after 4 weeks and 8 weeks feeding with diets containing polysaccharide gel (PG) from durian fruit-hulls. Values are mean ± SD. control = 0%. a, b, c = significant difference between groups, ($P<0.05$).

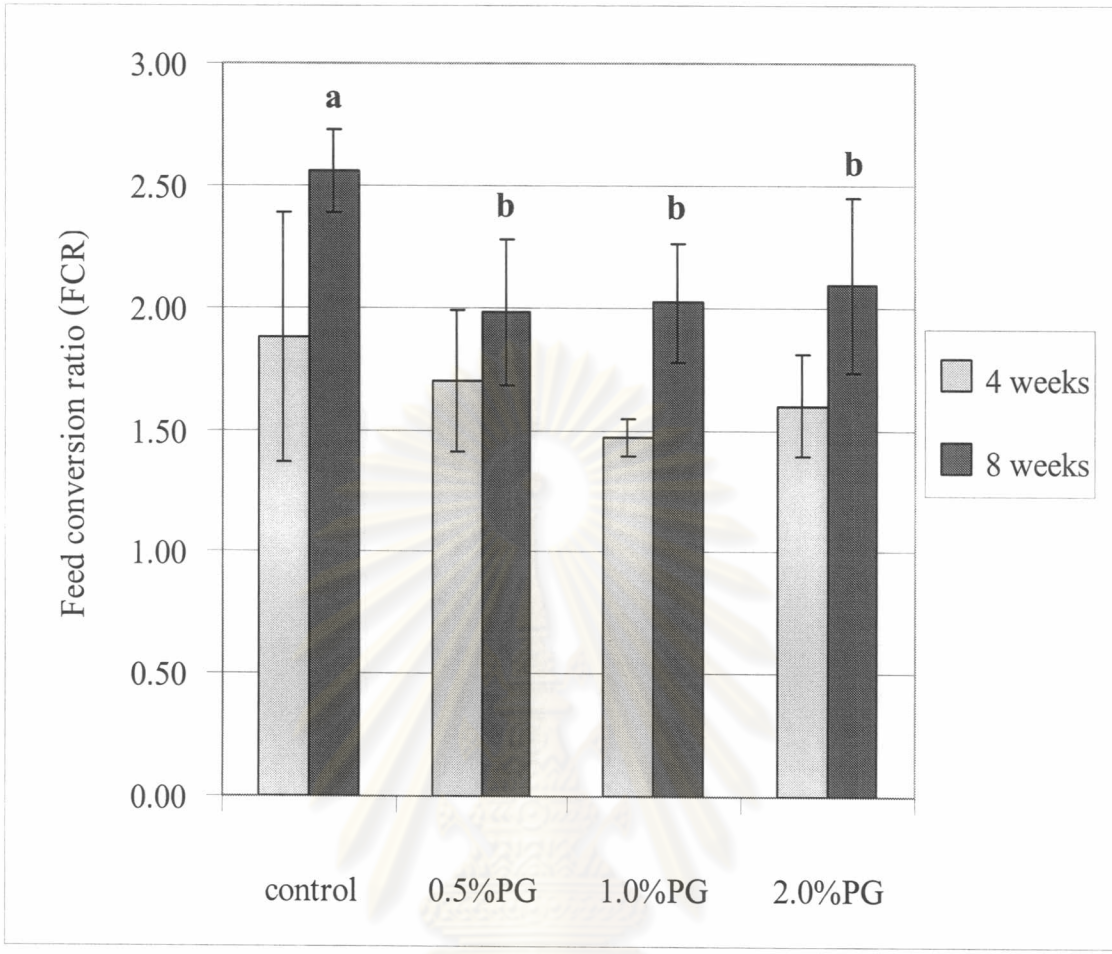


Figure 25. Feed conversion ratio (FCR) of *Penaeus monodon* juvenile in the trial rearing after 4 weeks and 8 weeks feeding with diets containing polysaccharide gel from durian fruit-hulls. Values are mean ± SD. control = 0% PG. a, b = significant difference between groups, ($P<0.05$).

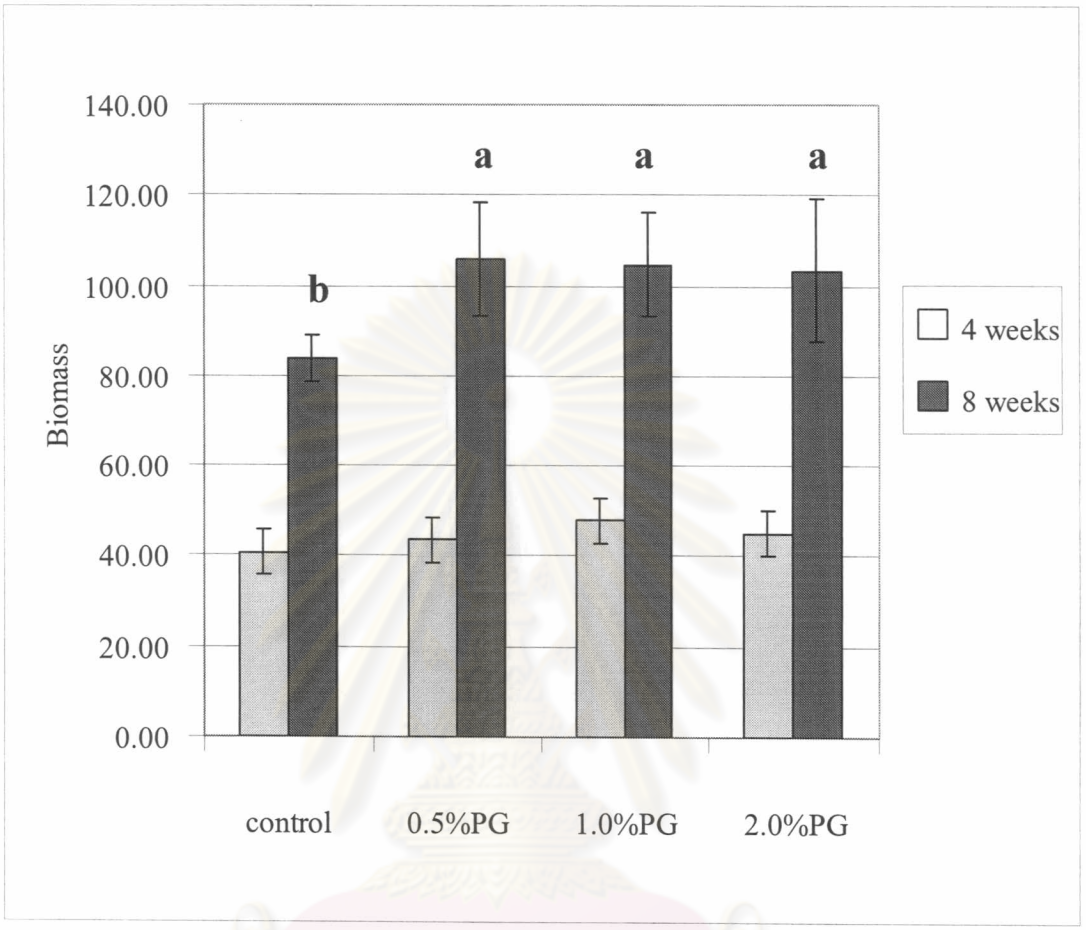
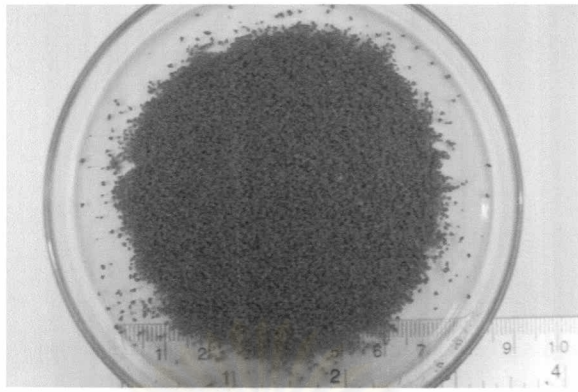
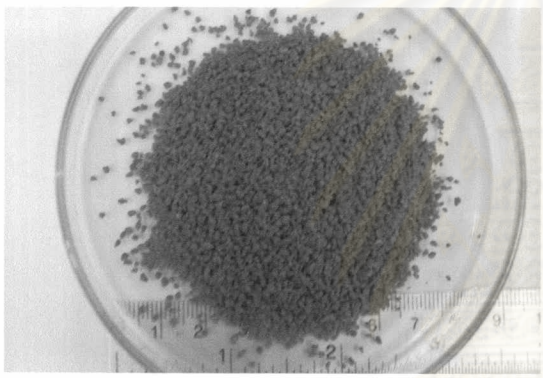
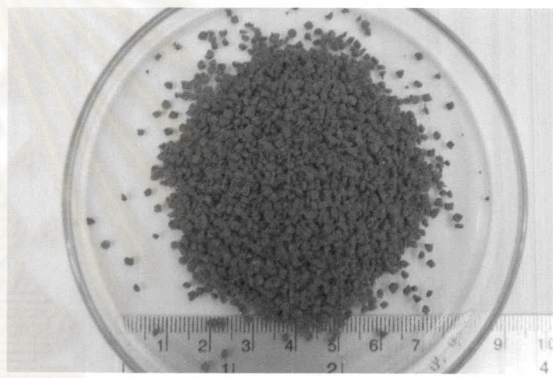


Figure 26. Biomass of *Penaeus monodon* juvenile in the trial rearing after 4 weeks and 8 weeks feeding with diets containing polysaccharide gel from durian fruit-hulls. Values are mean ± SD. control = 0% PG. a, b = significant difference between groups, ($P<0.05$).



(a)





(b) (c)

Figure 21. The shrimp diets in various feed size were prepared from common diet supplemented with durian polysaccharide gel (PG). Shrimp in the rearing trial fed with the different feed size of shrimp diets due to feeding period, using the small size of shrimp diet in the early of trial rearing (a) and the large size of shrimp diet (b and c) in the bigger shrimp, respectively.

There is little information about the carbohydrate nutrition of shrimp (New, 1976 and 1980, Kanazawa, 1984). The concentration levels and sources of carbohydrate in the shrimp diet have also been reported to effect the growth performance of *P. aztecus* (Andrews et al., 1972). The 20% glucose diet has contrary found reduce the growth rate of *P. aztecus*. However, 30% starch in diets with lower protein content have increased growth rate. Andrews and Sick (1972) reported that dietary glucose was rapidly absorbed but inefficiently utilized for energy metabolism, whereas the glucose from digested polysaccharides was absorbed more slowly and effectively utilized. C-14 glucose in intact starch is incorporated into the tissues of *P. setiferus* at a higher rate than free glucose.

The mechanism of the energy utilization from glucose was reported by Abdel-Rahman et al. (1979) that serum glucose levels in *P. japonicus* increased rapidly at the hour 1 after administration of glucose and remained at high levels for 24 hr. However, serum glucose did not increase as rapidly in shrimp fed with fructose. Whereas, after fed with the diet contained disaccharides (maltose) and polysaccharides (soluble starch), serum glucose was found to increase to a maximal level at the hour 3 and then decrease to a low level after 24 hr. Dietary glucose utilization as an energy source was quickly absorbed from the alimentary canal and released into the hemolymph. Disaccharides such as sucrose, maltose and trehalose have a high nutritive value as carbohydrate sources for *P. japonicus*. The sucrose sugar at 10 and 20% has found promote survival rate of *P. japonicus*. Polysaccharides such as dextrin and starch have also been found effective to increasing the growth rate in shrimp, in contrast to the less effective has been found in monosaccharides such as glucose, galactose and fructose. The carbohydrate source of monosaccharide showed poor ability in utilization by shrimp, which was similar to the finding by the separate studies. For example, the inclusion of glucose in diets for the brown shrimp *P. aztecus* (Andrews, Sick et al., 1972), pink shrimp *P. duorarum* (Sick and Andrews, 1973), kuruma prawn *P. japonicus* (Kitabayshi et al, 1971; Deshimaru and Yone, 1978), common prawn *Palaemon serratus* (Forster and Gabbott, 1971; Andrews et al., 1972), white shrimp *P. setiferus* (Andrews, Sick et al., 1972) and black tiger shrimp *P. monodon* (Shiau and Peng, 1992; Alava and Pascual, 1987).

Shiau and Peng (1992) reported that serum glucose level in *Penaeus monodon*, fed with glucose-containing diets, peaked within 1 hr after the meal. After administration the blood-sugar level peaked earliest in shrimps receiving glucose, followed by those fed with dextrin and finally by those fed with starch. Protein deposition was high in prawns fed with starch, intermediate in prawns fed with dextrin and low in prawns fed with glucose. The carbohydrate utilization in *Penaeus monodon* is highest when the carbohydrate source is starch followed by dextrin and finally by glucose. Fox (1992) has reported that using chitin derived from crab shell have found incorporated into commercial diet for *P. monodon* juvenile shrimp. Chitin dietary will not be directly utilized by the shrimp but indirectly utilized via gut or sediment bacteria. In addition, proteins, lipids and minerals associated with native chitin will be useful to the shrimp.

Ali (1982) reported that increasing the carbohydrate (corn starch) content in the shrimp diet of *P. indicus* juvenile shrimp from 10 to 40% has increased the food-conversion efficiency, growth rate and survival rate. Sick and Andrews (1973) reported that corn starch in shrimp diet promoted growth rate in *P. duorarum*. The shrimp fed with 40% corn starch in casein-based diet increased higher the growth rate and survival rate than shrimp group fed with 10 or 40% glucose. High levels of dietary starch have been used for their binding qualities in several experimental and commercial diets (Balazs et al., 1973, Balazs et al., 1974). Shiau and Chou (1991) reported the effects of dietary protein and energy on growth performance of black tiger shrimp. The carbohydrate (dextrin) was used to adjust the dietary energy level. The weight gain, feed conversion ratio (FCR), and protein gain of shrimp improved as dietary energy was raised up in the black tiger shrimp under seawater rearing conditions. Pascual et al. (1983) reported that *P. monodon* juvenile fed with 10 or 40% maltose, sucrose, dextrin, molasses, cassava starch, corn starch or sago palm starch for 56 days, the highest survival rate was obtained in juvenile shrimp fed with a diet containing 10% sucrose.

Alava and Pascual (1987) reported that *P. monodon* juvenile fed the trehalose diet and sucrose diet exhibited better weight gains than those fed with glucose diets. Shrimp fed with the diet containing 20% trehalose had the highest weight gain. Among the three

levels of dietary carbohydrate sugars at level of 20% resulted in the best weight gain, followed by 10% whereas the 30% level gave the lowest weight gain. Trehalose and sucrose diets showed higher survival rate than glucose diet. The sucrose and trehalose were found to be a suitable of carbohydrate source of better utilized than glucose. Glucose was found to inhibit the growth of *P. monodon* in those studies. The reason for poor utilization of monosaccharide such as glucose by shrimp may be related to the rate of absorption across the digestive tract. The poor performance of glucose diets was due to its sudden flux into the metabolic system as compared with a more even influx of more complex sugar, due to time lag caused by the necessity of digesting di- or polysaccharides (Piefer and Pfeffer, 1980). The rapid absorption of glucose across the intestine and concomitant slow response in insulin secretion is responsible for the poor utilization of glucose by carp (Furuichi and Yone, 1982, Murai et al., 1983).

Kanazawa (1984) reported that trehalose in the diet promoted the survival rate and growth rate in *P. japonicus* compared to glucose and fructose. The suggestion in *P. japonicus*, the di- or polysaccharides have a higher nutritive value than monosaccharides because they are not absorbed from the stomach but instead are converted to glucose and trehalose in the midgut and hepatopancreas, and then released gradually into the hemolymph. Dietary monosaccharides such as glucose are quickly absorbed from the stomach and released rapidly into the hemolymph, resulted in physiologically abnormal elevation of serum glucose concentration, making their difficult in utilization as energy sources.

Deshimaru and Yone (1978) reported the effect of dietary carbohydrate source on the growth and feed efficiency of *Penaeus japonicus*. The various sources of dietary carbohydrate, glycogen, starch, dextrin, glucose and sucrose on the growth and feed efficiency of shrimp have been examined by using a purified diet. The highest gain in percentage has obtained with the group fed with the diet containing 10% sucrose and a slightly lower with the diet containing glycogen, whereas the diet containing glucose resulted in the lowest weight gain of shrimp. The sucrose is a suitable source of dietary carbohydrate while starch, dextrin and particularly glucose are less desirable for *Penaeus*

japonicus. A suitable source of dietary carbohydrate is necessary to spare or preclude the use of carbon chains from amino acids for chitin synthesis (Cowey and Forster, 1971). Chitin synthesis is important in the exoskeleton, gut lining and peritrophic membrane of most crustacean species.

Carbohydrate digestion in crustacean has been demonstrated by Kooiman (1964), the presence of many enzymes for carbohydrates including α- and β-amylase, maltase, saccharase, chitinase and cellulase. Enzyme activity reflects dietary modifications in shrimp. Metabolically, carbohydrates are important in the Krebs cycle, glycogen storage, chitin synthesis and formation of steroids and fatty acids. Strong carbohydrase activity has been showed in the herbivorous shrimp *Macrobrachium dayanum* (Tyagi and Prakash, 1967). Extracellular chitinases, originating from the gut bacteria of *Penaeus setiferus* have been detected by Hood and Meyers (1973). Fox (1993) has reported that chitin from shrimp head meal, which is incorporated into commercial diets for *P. monodon* juvenile shrimp will not be directly utilized by the shrimp. Chitin diets will be indirectly digested via gut or sediment bacteria. Chitin diets containing levels of chitin between 0 and 16% did not significantly effect on the growth, survival and chitinase levels in the digestive gland of *P. monodon* juvenile shrimp with increasing level of dietary chitin. The numbers of chitinoclastic bacteria were low in shrimp digestive glands and unrelated to received level of the dietary chitin. The level of chitinase activity decreased with increasing levels of dietary chitin. The synthesis of endogenous chitinase in the digestive gland of shrimp occurs at a slow rate that the juvenile shrimps are able to digest small amounts of dietary chitin in the absence of bacterially produced chitinase. Clark et al. (1993) have reported that apparent digestibility of chitin is found in three species of penaeid shrimp, *L. vannamei*, *P. setiferus* and *P. duoraru*. Thus, chitin is digested by these penaeid shrimp.

Cellulose is partly digested it is suggesting that shrimp have the ability to use marine algae. Shrimp can utilize carbohydrates with varies efficiency according to their sources. The ability to digest specific source of carbohydrate varies between species.

The starch source seems to be more efficiently utilized carbohydrates source in shrimp better than glucose.

Therefore, in the present study, adding of polysaccharide gel (PG) extracted from durian fruit-hulls in PG additive shrimp diet suggested that oral administration of PG seemed to promote the growth performance which was represented by body weight gain increasing of black tiger shrimp *Penaeus monodon*.

4. Immunomodulatory effects

Durian polysaccharide gel (PG) additive diet showed potential effect on promotion of immune responses in black tiger shrimp *P. monodon* juveniles. PG additive diet feeding in groups of treated shrimps showed the result of higher value of the survival rate and the more resistance to pathogenic bacterium and virus of black tiger shrimps *P. monodon* in treated groups than that of control group in the present study. This result indicated that oral administration shrimp of diet containing PG has benefit in enhancing survival rate and increasing the disease resistance of black tiger shrimp against *Vibrio harveyi* 1526 and WSSV infection. Infectious causing diseases, *V. harveyi* 1526 and WSSV, being an importance mass mortalities pathogens of black tiger shrimp in aquaculture industry (Lightner, 1996; Maeda et al., 1998; Israngkura and Sae-Hae, 2002).

In crustacean, melanization occurs when the cellular defense reactions are initiated. The phenoloxidase, the key enzyme in the biosynthetic pathway of melanin, occurs in hemolymph as an inactive pro-enzyme prophenoloxidase (proPO). The proPO is activated into phenoloxidase (PO) when it reacts with activated serine protease, the enzyme induced in the immune cell system (Söderhäll and Cerenius, 1998 and 2004). In the present study, shrimps fed with 1.0 and 2.0 % PG showed PO activity higher ($P<0.05$) than that of control (Table 10). This result was strongly indicated that oral administration of diet with PG helps enhancing the immunity enzyme activity of the *P. monodon*. The hemocytes have also played an important role in the cellular immune response including clotting, non-self recognition, phagocytosis, melanization, encapsulation, cytotoxicity and

cell-to-cell communication (Söderhäll, 1999). Hemocytes are involved in the production of melanin via the prophenoloxidase (proPO) system. The semi-granular and granular cells carry out the function of the proPO system (Johansson and Söderhäll, 1989). Phenoloxidase is the terminal enzyme in the proPO system and being activated by several polysaccharides substrate (Sritunyalucksana, et al., 1999). Shrimps fed with 1.0 and 2.0% PG in diet showed elevating of total hemocyte counts and phenoloxidase activity higher ($P<0.05$) than that of control as indicated in Table 10. These results suggest that oral administration of PG effected the enhancing of the immune response in black tiger shrimp *P. monodon* represented by increasing total hemocytes count and phenoloxidase enzyme activity in this study.

The study of the carbohydrate immunomodulator in shrimp, Boonyaratpalin et al. (1995) has been demonstrated the immunostimulant properties of peptidoglycan in *P. monodon* shrimp. The black tiger shrimps fed peptidoglycan derived from *Brevibacterium lactofermentum* showed increasing phagocytosis activity of shrimp hemocyte. Vargas-Albores et al. (2000) reported the mechanism of the shrimp immune response to β-glucan and lipopolysaccharide. Shrimp recognizes common characteristics present in bacteria and fungi such as lipopolysaccharide and β-glucan has been studied. The microbial components can directly activate defensive cellular functions such as phagocytosis, melanization, coagulation and encapsulation, the presence and participation of plasma recognition proteins can amplify this activation. Beta glucan binding proteins (BGBP) reacts with β-glucan and then the glucan-BGBP complex induces degranulation and the activation of prophenoloxidase. Together with LPS-binding agglutinin, BGBP stimulates cellular function only after its reaction with LPS or β-glucan, respectively.

Cheng et al (2004) reported the immune stimulatory effect of sodium alginate on the white shrimp *L. vannamei* and its resistance against *V. alginolyticus*. The shrimp, injected with 20 and 50 μg per g shrimp body weight of sodium alginate, increased the phenoloxidase activity significantly after 2 days of injection. Sung et al. (1994) reported that *P. monodon* had been immersed in aerated yeast glucan (β-1,3-1,6-glucan), its phenoloxidase activity increased.

Table 10. Effect of polysaccharide gel (PG) on immunogenic performance of *Penaeus monodon* juvenile after 8 weeks feeding period, each treatment using four groups of shrimp (100 shrimps/group). Values are mean ± SD. Control = 0% PG. a, b, c = significant difference between groups ($P<0.05$).

Tests	Shrimp number (n)	Effect of immunogenesis in shrimp after feeding with shrimp diets with different concentration of PG. (mean ± SD)			
		Control	0.5% PG	1.0% PG	2.0% PG
1.Total hemocyte count ($x10^7$ cell/mm^3)	12	^{c}1.16±0.45	^{bc}1.20±0.45	^{ab}1.56±0.43	^{a}1.68±0.57
2).Phenoloxidase activity (units/mins/mg.protein)	12	^{b}609.37 ±117.71	^{b}705.99 ±208.97	^{a}900.11 ±281.85	^{a}893.30 ±252.58

Sung et al. (1996) reported that the *P. monodon* had been immersed in viable cell suspension of *Vibrio vulnificus*, yeast glucan and zymosan (β-1,3-glucan-protein-lipid compound) showed increased phenoloxidase activity in 24 hr. Takahashi et al. (2000) reported that *P. japonicus* after 7 days feeding with lipopolysaccharide at a dose of 20 μg per kg shrimp body weigh per day resulted in increasing its phenoloxidase activity. The study of feeding diet containing polysaccharide gel (PG) extracted from durian fruit-hulls in shrimp as showed in Table 10 indicated that oral administration of PG additive diet seemed to enhance the immune response represented by increasing the phenoloxidase activity and total hemocyte count in black tiger shrimp *(Penaeus monodon)*.

In hemolymph of shrimp and decapod crustaceans are generally composed of three types of circulating hemocytes including hyaline cells, semi-granular cells and large granular cells, which are associated with cellular defense (Bauchau, 1981; Martin and Graves, 1985; Ratcliffe et al., 1985; Tsing et al., 1989; Le Moullac et al., 1997). Crustacean hemocytes are thought to be functionally analogous to vertebrate leukocytes, involved primarily with the recognition and removal of foreign materials. The total hemocyte count (THC) in other study, Van de Braak et al. (2002) reported the roles of hemocytes in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. During the period of the major bacterial clearance from circulation, THC was low in the bacterial-injected group compared to control group, phosphate buffered saline (PBS) injected. THC in crustaceans rapidly drops following injection of foreign material, while THC often increases after PBS injection. The decrease in THC is attributed to defense activities, hemocyte migration to the injection site and reduced concentration of bacteria in the hemolymph. In addition, hemocytes aggregated into non-circulating clumps after acute bacterial infection and injection of foreign material.

Lee and Shiau (2002) reported that dietary vitamin C and its derivatives effect immune responses in grass shrimp *Penaeus monodon*. The vitamin C supplemented diets fed to black tiger shrimp for 8 weeks, showed significantly ($P<0.01$) higher weight gain, survival rate, total hemocyte count (THC) and phenoloxidase (PO) activity was also higher than control group of shrimp fed a diet without vitamin C. These data suggest that

dietary ascorbate enhances immune responses in *P. monodon*. Lee and Shiau (2004) reported that vitamin E requirements of juvenile grass shrimp *Penaeus monodon* and effects on non-specific immune responses. The vitamin E diets supplemented with 75 and 100 mg vitamin E per kg diet fed to black tiger shrimp for 8 weeks, showed significantly ($P<0.05$) higher weight gain and total hemocyte count (THC) is higher than that of shrimps fed diets supplemented with $\leq$ 50 mg vitamin E per kg diet. These data suggest that dietary vitamin E is required for growth and non-specific immune responses in *P. monodon*. The values of the total hemocyte count (THC) of the black tiger shrimp have been reported by using haemocytometer, Rukpratanporn (1999) measured the THC values were at $1.35 \pm 0.56 \times 10^7$ cells/ml, rearing period for 90 days and temperature at 24.2-27.5 °C in closed-recirculating pond. Lee and Siau (2004) measured the THC values were at $1.52 \pm 3.69 \times 10^7$ cells/ml, rearing period for 49 days and temperature at 24.2-27.5 °C in an aquarium tank.

5. Challenge test

5.1 WSSV Challenge test by cohabitation method

The results in Figures 28 and 29 demonstrated that the survival rate of *Penaeus monodon* juvenile shrimp on challenge test with WSSV 10^6 (1:100 dilution) by cohabitation method after 4 weeks and 8 weeks feeding period seemed to be sustained. The RPS values showed the shrimp resistance, when challenged with WSSV in the period of prefeeding for 4 and 8 weeks with different concentration of PG in shrimp diet, as in Table 11. PG In the present study, treated shrimps in 4 weeks feeding period groups with 0.5, 1.0 and 2.0 % PG additive shrimp diet showed the higher percentage of survival rate than that of control group (Figure 28), after 4 days of infection, shrimps in control group showed rapidly decreased in survival rate to 0% at day 11 compared to the treated groups of prefeeding diets containing 1 and 2% PG which was 57 and 17% survivals as illustrated in Figure 28. However, treated shrimps after 8 weeks feeding period groups with 2% PG additive shrimp diet showed the higher percentage at 33% of survival rate than that of control group which was of 0% after 14 days of infection (Figure 29).

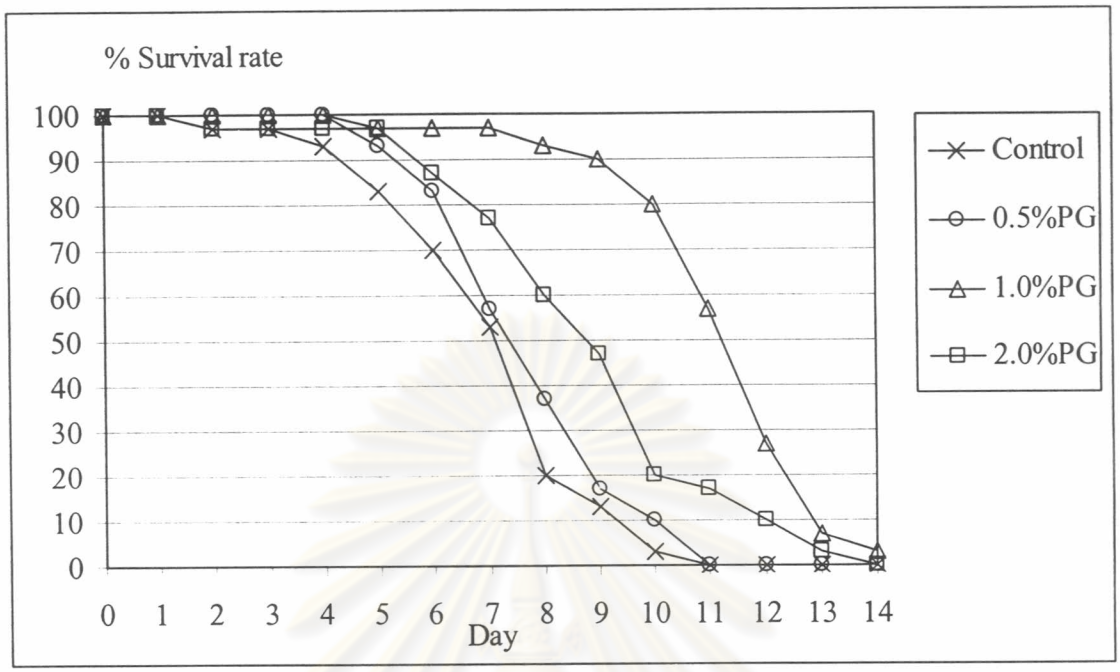


Figure 28. The survival rate of *Penaeus monodon* juvenile on challenge test with WSSV, 10^6(1:100) by cohabitation method after 4 weeks feeding period with shrimp diets containing various concentrations of PG. Values are mean from 3 replicates. Control was a shrimp diet without PG.

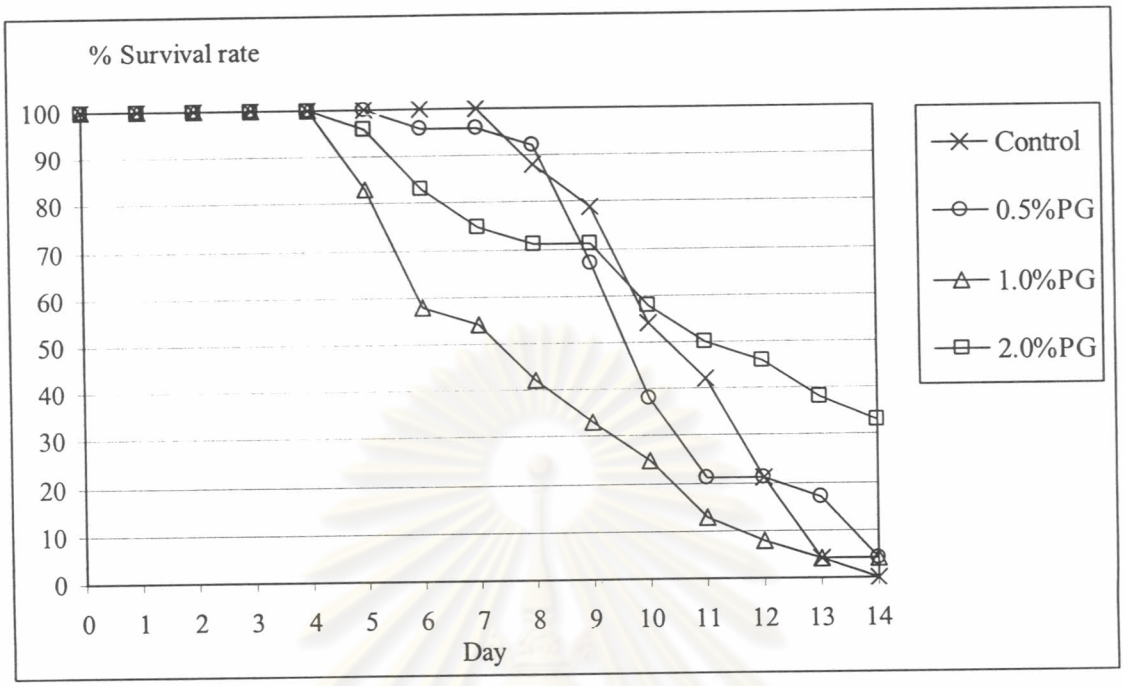


Figure 29. The survival rate of *Penaeus monodon* juvenile on challenge test with WSSV, 10^6 (1:100) by cohabitation method after 8 weeks feeding period with shrimp diets containing various concentrations of PG. Values are mean from 3 replicates. Control was a shrimp diet without PG.

Table 11. Relative percent survival (RPS) of *P. monodon* juvenile shrimp challenged by cohabitation method with WSSV. Cumulative mortality and RPS values were determined at after day 7 of infection for after 4 weeks feeding period and day 10 of infection for after 8 weeks feeding period. Control = 0.0% PG. n = number of shrimp tested per replication.

Shrimp group	Mean of dead shrimp/n		Mortality (%)		RPS (%)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
Control	4.7/10	3.7/8	47	46	0	0
0.5% PG	4.3/10	5.0/8	43	63	9	-37
1.0% PG	0.3/10	6.0/8	3	75	94	-63
2.0% PG	2.3/10	3.3/8	23	41	51	11

The disease resistant results in the challenge experimental survivors of *Penaeus monodon* juvenile shrimps on challenge test with WSSV by cohabitation method, in treated group for 4 weeks prefeeding period with shrimp diets containing 0.5, 1.0 and 2.0% PG showed the percent mortality by 47% mortality of control group at day 7 of infection at 43, 3 and 23, respectively (Table 11). The disease resistant of shrimp after fed with 0.5, 1.0 and 2.0% PG for 4 weeks presented the RPS values of 9, 94 and 51%, respectively, at 47% mortality of control group, after day 7 of infection, whereas 8 weeks feeding period the RPS values were -37, -63 and 11% respectively, at 46% mortality of control group, after day 10 of infection. In the period of prefeeding for 8 weeks with diets contained PG at any concentration showed uneffective compared to the period test. The RPS value was compared with challenge test at 20-50% mortality of control group values not lesser than the criterion 60% has been consider as effective in fish vaccines (Amend, 1981). In this study demonstrated that at prefeeding for 4 weeks with diets contain of 1.0% PG the RPS values was 94% exceed the criterion RPS 60% as effective in fish vaccines after day 10 of infection. The present study demonstrated that the disease resistances of *P. monodon* against the viral pathogen, WSSV, provided the better survival performance by the challenged after 4 weeks of prefeeding diets contain at 1.0% PG in the treated groups better than 8 weeks prefeeding of PG at any concentration. This result indicated that oral administration of diet contain PG seemed to provide the sustained survival rate of shrimp after WSSV infection corresponding to the increasing of the immune response in disease resistance of black tiger shrimp *(Penaeus monodon)*, in this study.

5.2 *Vibrio harveyi* 1526 challenge test by immersion method

The results in Figure 30 illustrated that the survival rate of *Penaeus monodon* juvenile shrimps on challenge test with *Vibrio harveyi* 1526, at 2.15×10^7 CFU/ml by immersion method, in treated group of 8 weeks feeding period with shrimp diets containing 0.5, 1.0 and 2.0% PG, repectively, showed the higher percentage of survival rate than that of control group after day 3 of infection (Figure 30).

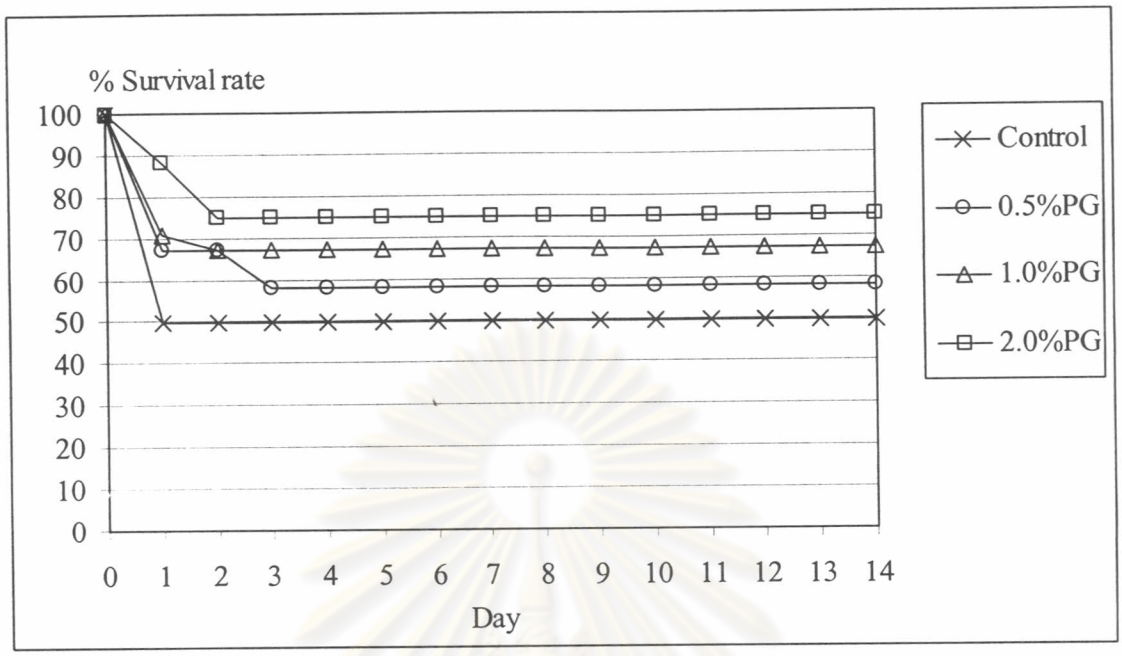


Figure 30. The survival rate of *Penaeus monodon* juvenile on challenge test with *Vibrio harveyi* 1526, 2.15×10^7 CFU/ml by immersion method after 8 weeks feeding period with shrimp diets containing various concentrations of PG. Values are mean from 3 replicates. Control was a shrimp diet without PG.

This result indicated that oral administration of PG additive shrimp diet have beneficial effect in prolong the survival rate of shrimp after *Vibrio harveyi* 1526 infection or the disease resistance of black tiger shrimp *Penaeus monodon* against pathogenic bacterium, *Vibrio harveyi* 1526, in this study.

The RPS values showed the shrimp resistance, when challenged with *Vibrio harveyi* 1526 in the period of prefeeding for 8 weeks with different concentration of PG in shrimp diet, as in Table 12. The disease resistant results in the challenge experimental survivors of *Penaeus monodon* juvenile shrimps on challenge test with *Vibrio harveyi* 1526 by immersion method, in treated group for 8 weeks feeding period with shrimp diets containing 0.5, 1.0 and 2.0% PG showed the percent mortality by 50% mortality of control group at day 3 of infection at 41, 34 and 25, respectively. The disease resistant of shrimp after fed with 0.5, 1.0 and 2.0% PG for 8 weeks presented the RPS values of 18, 32 and 50%, respectively, at 50% mortality of control group, after day 3 of infection. The relative percent survival (RPS) value is compared with challenge test at 20-50% mortality values not lesser than the criterion 60% has been consider as effective in fish vaccines (Amend, 1981). In this study demonstrated that at prefeeding for 8 weeks the RPS values did not meet the criterion RPS 60% for effective in fish vaccines, however, the RPS value in the period of prefeeding for 8 weeks with diets contain at 2.0% PG have a higher resistance to *Vibrio harveyi* 1526 than feeding of PG at any concentration which was RPS 50%, at 50% mortality of control group, after day 3 of infection.

This present results demonstrated that the disease resistances of *P. monodon* against the vibrisis pathogen, *Vibrio harveyi* 1526, provided the better survival performance by the challenged after 8 weeks of prefeeding diets contain at 2.0% PG in the treated groups.

Table 12. Relative percent survival (RPS) of *P. monodon* juvenile shrimp challenged by immersion method with *Vibrio harveyi* 1526. Cumulative mortality and RPS values were determined at after day 3 of infection for after 8 weeks feeding period. Control = 0.0% PG. n = number of shrimp tested per replication.

Shrimp group	Mean of dead shrimp/n	Mortality (%)	RPS (%)
Control	4.0/8	50	0
0.5% PG	3.3/8	41	18
1.0% PG	2.7/8	34	32
2.0 %PG	2.0/8	25	50

Penaeus monodon shrimp can well digest the carbohydrate and used as the energy source. In this situation, carbohydrate from seaweed as well as from plant could be lost during the digestion process effecting the available carbohydrate concentration as immunostimulant but not as a source of energy. This loss would not be important since the shrimp immune system can react with very small amounts of digestible product (Johansson et al., 2000). The group of *Penaeus monodon* shrimp feeding with durian polysaccharide gel (PG) was found the increasing of shrimp survival rate better than that of control against virus and bacteria infection in this study.

The major components of polysaccharide gel contain 2 major polysaccharide chains: long chain acidic sugar of polygalacturonic acid, and long neutral chain of sugars including galacturonic acid, rhamnose, fructose, glucose and arabinose (Pongsamart, 1998). Polysaccharide gel is the mixture of polysaccharides, mainly pectic polysaccharides and a glucan (Hokputsa et al., 2004).

A high amount of glycosidic linkage is mostly α-(1→4)-rhamnogalacturonic acid and rich in neutral sugars side chains of arabinogalactan containing β-(1→6) linkage. Glucose units in PG were mostly (1→4)-linked. Galacturonic acid is an acidic sugar providing carbonyl anionic charge. The polyanionic polymer of PG, may effectively bind and form polyelectrolyte complex with the cationic side chain of lipopolysaccharide present on the bacterial cell surface thus caused the interference and disturbance of bacterial cell surface function, therefore, normal function of bacteria was inhibited (Neu et al., 1992).

Jarp and Tverdal (1997) has suggested that if the cumulative mortalities cases in immunostimulated and unimmunostimulated groups are registered at the end of the observation period, the immunostimulant effect can be measured with the risk ratio, risk difference or the RPS. The RPS is the same as the preventive fraction or the immunostimulant effect (Miettinen, 1974; Amend, 1981). RPS represents the fraction of the mortality that was prevented by the immunostimulant up to the end of the observation period, so RPS is dependent on the experimental conditions and the length of the

observation period. A point estimate of RPS is very often used to present the results from fish vaccination trials, because a single value is clear, precise and easy to understand. Unfortunately, the very precision of this poin estimate gives sometimes the misleading impression that the value presented is the correct answer.

Huang and Song, (1999) has reported that β-1,3-1,6-glucan, derived from baker's yeast *Saccharomyces cerevisiae*, can protect spawners from WSSV, and whether this protection can be passed on the hatchlings via maternal transmission of immunity that showed high values of mean RPS in glucan treatment group, increased when mysis and larvae derived from glucan-injected spawners challenged by immersion with WSSV. Witteveldt et al. (2004) has reported that the WSSV envelope proteins VP19 and VP28 was evaluated the potential to vaccinate *Penaeus monodon* shrimp against WSSV. Shrimp were vaccinated by intramuscular injection of the purified WSSV proteins for 2 and 25 days, and then challenged by injection method with WSSV, the result showed VP19–vaccinated shrimp group significantly ($P<0.05$) better survival as compared to the control shrimp group with a RPS values both after 2 and 25 days vaccination. Also, in the groups vaccinated with only VP28 and group mixture between of VP19 and VP28 the result showed a singnificantly better survival when challenged 2 days after vaccination, but not after 25 days. Namikoshi et al. (2004) has reported that the inactivated WSSV (formalin-inactivated WSSV and heat- inactivated WSSV) with or without immunostimulants (β-1,3-glucan or killed *Vibrio penaeicida*) and of recombinant proteins of WSSV (rVP26, rVP28) were induced the resistance in vaccination trail of *Penaeus japonicus* shrimp against WSSV. Shrimp were vaccinated by intramuscular injection for 10 and 30 days, and then challenged by injection method with WSSV that the result showed the shrimp vaccinated with formalin-inactivated WSSV increased a resistance to the virus on 10 days post-vaccination but not on 30 days. Heat-inactivated WSSV did not induce a resistance in the shrimp even on 10 days post-vaccination. Injections with rVP26 and rVP28 induced a higher resistance with RPS values exceed 60%, on 30 days post-vaccination, that contact with injection with glucan or *Vibrio penaeicida*.

Mastromario et al. (1997) has reported that antiviral activity of natural and semisynthetic polysaccharides on the early steps infection of enveloped viruses, rubella virus. The natural and semisynthetic carbohydrates scleroglucan, locust bean gum, alginic acid, tamarind gum (glyloid) and its three sulfate derivatives, glycogen and its two sulfate derivatives and dextran sulfate, were demonstrated the inhibitory effect on rubella virus infection of Vero cell. The neutral polymer scleroglucan and two highly negatively charged compounds, glyloid sulfate derivatives and dextran sulfate, had the highest inhibitory effect on rubella virus infection antigen synthesis. The antiviral properties of active molecules appeared to depend on the shape of the macromolecule and/or on the electric charge, while saccharide units play a minor role. The polysaccharides blocked a step in virus replication subsequent to virus attachment, such as internalization and/or uncoating. The other anionic polysaccharide polymers have showed the replication inhibition of a wide variety of enveloped viruses including HIV, herpes viruses, poxviruses, togaviruses, arenaviruses, paramyxoviruses and rhabdoviruses such as curdlan sulfate (Osawa et al., 1993; Jagodzinski et al., 1994), carrageenan (González et al., 1987; Carlucci et al., 1999), glycosaminoglycan, galactan sulfate (Witvrouw and De Clercq, 1997; Curatella et al., 2005), polymannuroguluronate sulfate (Miao et al., 2004), chitin sulfate (Ishihara et al., 1993), cellulose sulfate (Yamamoto et al., 1990), lentinan sulfate (Yoshida et al., 1988) and pentosan polysulfate (Baba et al., 1988). Schols et al. (1990) reported the sulfated polymers being potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, respiratory syncytial virus, and toga-, arena- and retroviruses.

Most polyanions are water-soluble, a property which is significant not only for transport, but also for systemic administration. Water soluble polyanionic polymers can distribute themselves in a living system by blood or lymphatic circulation, by cellular transport through the involvement of mobile phagocytic cells, or by absorption on cell surfaces. The role of polymers in the biological processes of organisms usually relates to their soluble or insoluble forms in tissue and tissue fluid, some of which resemble blood protein or nucleotides. Polyanions that enter into biological function by distribution throughout the host behave similarly to certain protein, glycoproteins, and

polynucleotides that modulate a variety of biological responses related to bacteria and fungi, enhance immune responsiveness, inhibit adjuvant arthritis and depending on polymer size, either depress or stimulate the functional phagocytic activity of the reticuloendotelial system. In relation to these immunological and hormonal responses, inflammation, wound repair, blood clotting, and tissue damage are subject to the action of these macromolecules (Regelson, 1968).

Naturally occurring and synthetic polyanions have been investigated for antiviral activity. The mechanism of antiviral activity has not been completely defined but may involve direct inactivation of viruses in some situations. In contrast to standard antiviral chemotherapeutic agents, polyanions can provide prolonged protection. This protection may be related to the slow degradation of most polyanions. The major modes of antiviral action that have been directed inactivation of virus, inhibition of virus replication, induction of interferon, stimulation of phagocytosis and inflammation, specific immuno-enhancement of humoral or cell-mediated immune responses, non-specific immune stimulants in fish and/or shrimp and enhancement of macrophage antiviral functions (Uglea and Ottenbrite, 1996). The mechanism of inhibition the enveloped viruses are uncertain, although most interfere with the binding of virus to cell membrane receptors. The presence of the drugs during HIV adsorption to host cells has been reported as necessary for effective inhibition although a direct antiviral action of dextran sulfate has also been widely described (Bagasra and Lischner, 1988; Parish et al., 1990; Schols et al., 1990; Callahan et al, 1991; Mc Clure et al., 1992). The PG mechanism of inhibition the WSSV and antibacterial activity the *Vibrio harveyi* 1526 is unclear in this study. It is required to be further studied.

In another carbohydrate study to enhance the disease resistance in shrimp, Itami et al. (1998) have been demonstrated to have disease resistance in *Penaeus* shrimp after fed peptidoglycan. The kuruma shrimp (*Penaeus japonicus*) fed with peptidoglycan derived from *Bifidobacterium thermophilum* has increased shrimp survival rate, compared with control groups on WSSV challenge test. Cheng et al. (2004) reported that the *Litopenaeus vannamei* juvenile injected with sodium alginate at a dose of 10 μg per g

shrimp body weight or more has been showed to increase the survival rate on challenge test by injection method with *Vibrio alginolyticus*, $2.0x10^5$ CFU/ml. In those present studies, the survival rate of injected sodium alginate in shrimps being higher than control at the 6 days after the *Vibrio alginolyticus* challenge test. Thus, the white shrimp (*Litopenaeus vannamei*) received sodium alginate has increased immune response and resistance against *Vibrio alginolyticus*.

Sung and Kou (1994) has been reported to increase the resistance of the black tiger shrimp *Penaeus monodon* against *Vibrio vulnificus* after immersion of β-1,3-1,6-glucan extracted from yeast *Saccharomyces cerevisiae* and oral administration of glucan against WSSV infection (Song et al., 1997). Dietary oral administration of schizophyllan, a β-1,3-glucan extracted form fungus *Schizophyllum commune* has been reported to increase the resistance of the black tiger shrimp (*Penaeus monodon*) against *Vibrio damsela* (Liao et al., 1996), and WSSV infection (Chang et al., 1999). The survival rate of *P. monodon* brooder fed with a diet containing β-1,3-glucan at 2 g/kg for 10 days, was significantly higher than that of shrimp brooder fed a diet without β-1,3-glucan (Chang et al., 2000).

Chang et al. (2003) reported that the effectiveness of dietary β-1,3-glucan, derived from *Schizophyllum commune*, in modulating the non-specific immunity of grass shrimp *Penaeus monodon* and its resistance to WSSV have been investigated. The black tiger shrimp juveniles fed with a shrimp diet containing β-1,3-glucan at 10 g per kg diet for 20 days has significantly higher the survival rate by day 9 after challenged by injection of WSSV than that of the other fed groups (β-1,3-glucan 0, 1, 2 and 20 g per kg diet). Therefore, in this study the polysaccharides additive diet using polysaccharide gel (PG) extracted from durian (*Durio zibethinus* L.) fruit-hulls suggested that oral administration of PG additive diet seemed to effect immuno-stimulation, and resulted in increasing the survival rate or producing disease resistance in black tiger shrimp *Penaeus monodon*.