Immunostimulation of Shrimp Through Oral Administration of *Vibrio* Bacterin and Yeast Glucan

T.N. Devaraja, S.K. Otta, G. Shubha, *Indrani Karunasagar P. Tauro, Iddya Karunasagar

Department of Fishery Microbiology, University of Agricultural Sciences, College of Fisheries Mangalore - 575 002, India.

ABSTRACT: The effect of oral administration of Vibrio bacterin and yeast glucan to black tiger shrimp, *Penaeus monodon* was studied. Vibriocidal activity was detectable in hemolymph and hemocyte lysate. The activity peaked at 48 h post treatment but persisted even at 72 h. Generation of reactive oxygen species in hemocytes could be detected by nitroblue tetrazolium (NBT) assay. Enhanced phenol oxidase activity was observed in hemocytes of treated shrimp. When a combination of bacterin and glucan was administered, the response was much higher compared to individual treatments. The results suggest that immunostimulation of shrimp can be achieved through oral administration of a combination of Vibrio bacterin and yeast glucan.

KEY WORDS: Immunostimulants, *Penaeus monodon*, *Vibrio* bacterin, glucan.

INTRODUCTION

Shrimp aquaculture has been facing serious disease problems and therefore, several investigators have considered the possibility for immunoprophylaxis. Itami et al. (1989) noted that immunised shrimps *(Penaeus japonicus)* were better protected against challenge. Adams (1991) observed that bactericidins and other humoral factors were induced in *P. monodon* following treatment with vibrio bacterin. Sung et al. (1991) reported that *P. monodon* larvae treated with Vibrio bacterin grew faster. Induction of resistance in *P. monodon* against challenge by *Vibrio vulnificus* after treatment with beta-glucan was reported by Sung et al. (1994). They further noted that beta-glucans stimulated phenoloxidase activity in hemocytes of *P. monodon*. Activation of hemocytes as indicated by increased respiratory burst activity following treatment with beta-glucans has been recorded by Song and Hsieh (1994). Enhancement of the pro-phenol oxidase system (proPO) in hemocytes by treatment with beta-glucans was observed by Sung et al. (1994). However, most of these reports examined the response after treatment by immersion or injection. The preferred route of delivery in aquaculture systems would be the oral route. Further, interactions between immunostimulants have not been examined. In this study the response of black tiger shrimp, *Penaeus monodon* to oral administration of immunostimulants was evaluated and the combined effect of bacterin and glucan was also investigated.

MATERIALS AND METHODS

Shrimp

Healthy *P. monodon* weighing 22-27 g were obtained from local farms. They were maintained in 300 l tanks containing 150 l sea water and fed with commercial diet at 4% body weight three times a day.

Treatment with immunostimulants and assessment of immune response

Vibrio bacterin contained heat killed cells of *V. harveyi* (local strain, isolated from moribund shrimp) at a density of 10^9/ml. Yeast B-1,3 glucan was a product obtained from Mangalore Biotech Laboratory. Immunostimulants were added to the feed at following levels before feeding: bacterin, 5, 10, 15 and 20%; glucan, 0.1, 0.2, 0.3 and 0.4%. The following combinations of bacterin + glucan were also tried : 5+0.1, 10+0.2, 15+0.3 and 20+0.4%. Diet containing immunostimulants was given at the rate of 4% body weight for three successive feedings in one day at the start of the tests. Thereafter, normal diet was given. At 24, 48 and 72 h after feeding with immunostimulant containing diet, the immune response was assayed. For immunological assays, hemolymph was collected from the sixth abdominal segment using a 26 gauge needle on a 2 ml syringe containing 0.2 ml anticoagulant (0.0l M Tris - HCl, 0.25 M sucrose, 0.1 M sodium citrate, pH 7.6). The hemolymph was centrifuged at 300xg for 10 min at 4ºC and the supernatant was used as plasma supernatant (PS). The pellet was resuspended in Hanks Balanced salt solution (HBSS). Hemocyte lysate supernatant (HLS) was prepared by a modification of the protocol described by Sung et al. (1994). The hemocytes were washed in HBSS (instead of cacodylate buffer) and lysed by sonication.

Vibriocidal activity was measured by modification of procedure of Adams (1991). Briefly, *V. harveyi* was cultured overnight in tryptic soy broth (TSB) at 30ºC. Bacteria were

Devaraja, TN, Otta SK, Shubha G, Karunasagar I, Tauro P, Karunasagar I (1998) Immunostimulation of shrimp through oral administration of *Vibrio* bacterin and yeast glucan. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail:mircen@giasbg01.vsnl.net.in

pelleted by centrifugation, washed in phosphate buffered saline (PBS) and resuspended in it to the original volume. To 100µl PS or HLS, 1µl bacterial suspension was added and incubated for 1 h. Serial tenfold dilutions were then prepared in PBS and plated on tryptic soy agar (TSA). After 24 h at 30°C, colonies were counted. Percentage inhibition was calculated as follows :

$$\% \text{ inhibition} = 100 - \frac{\text{cfu/ml in treated shrimp}}{\text{cfu/ml in untreated shrimp}} \times 100$$

Production of reactive oxygen species was measured by nitroblue tetrazolium (NBT) assay described by Song and Hsieh (1994). Briefly 100µl aliquots of hemocyte suspension in HBSS were transferred to flat bottom 96 well microtitre plates and incubated at 37°C for 30 min. NBT solution was removed and 100µl methanol was added to each well. Methanol was removed and wells washed thrice in 70% methanol, air dried and coated with a solution of 120µl KOH (2M) and 140µl DMSO to dissolve cytoplasmic formazan.The optical density was then measured at 630 nm using a Precision Microplate Reader (Emax, Molecular Device, USA).

Phenoloxidase activity was measured as detailed by Sung et al. (1994). HLS (50µl) was transferred to a flat bottom microtitre plate and 100µl L-DOPA (3-4 Dihydroxyphenyl L alanine, 1.6 mg/ml in HBSS) was added. After 10 min, OD was measured at 490 nm using a microplate reader. Protein content of HLS was measured by Folin reaction (Lowry et al. 1951). Units of enzyme activity were calculated based on the definition that increase in absorbance of 0.001/min/mg protein was one unit (Söderhäll & Unestam 1979). All experiments were repeated thrice and mean values are presented. Friedman's non-parametric test (Weber 1973) was used to analyse the statistical significance of the results.

RESULTS AND DISCUSSION

Results in Table 1 show that vibriocidal activity was induced in the plasma and hemocyte fractions of *P. monodon* treated orally with vibrio bacterin. Peak response was recorded at 48 h after treatment and the activity persisted even at 72 h. A maximum of 85.8% inhibition was recorded in the plasma of shrimp groups fed at 20% level. The maximum inhibition in hemocyte fractions was 79.2% in groups fed at 10% bacterin. Even at 72 h after treatment, there was more than 75% inhibition in all batches. Induction of bactericidal activity in *P. monodon* following bacterin treatment has been reported by Adams (1991) who administered bacterin by immersion and injection and by Sung et al. (1996) who administered bacterin by immersion. The results presented here show that administration of *Vibrio* bacterin through feed is also effective in inducing vibriocidal activity in *P. monodon* and that the activity persists for over 72h after treatment.

Table 1. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with *Vibrio* bacterin at different levels.

Treatment (% bacterin)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
5.0	70.1	80.9	79.6	65.5	76.5	75.5
10.0	69.5	82.9	81.8	67.2	79.2	79.0
15.0	72.0	84.9	82.8	70.1	78.7	76.3
20.0	71.9	85.8	83.9	69.5	76.9	76.0

Treatment of *P. monodon* with yeast glucan at different levels also induced vibriocidal activity in plasma and hemocyte fractions (Table 2). Peak activity was noted at 48h and maximum activity was recorded in the groups treated with 0.4% glucan. Only marginally higher activity (75% inhibition) was noted in the hemocyte lysate fraction when compared to plasma (72.2 % inhibition). Results of Sung et al. (1996) indicated that the treatment of *P. monodon* with glucan by immersion brought about induction of anti-bacterial activity in the plasma which could be recorded up to 24 h. Results presented here show that vibriocidal activity was detectable in both the plasma and the hemocyte fractions of *P. monodon* treated with the glucan by the oral route and that the activity persisted for more than 72 h.

Table 2. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with glucan at different levels.

Treatment (% bacterin)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
0.1	55.2	67.8	66.1	60.8	69.1	67.8
0.2	60.2	66.2	65.9	63.3	70.9	70.1
0.3	58.8	71.1	68.2	64.2	73.0	69.5
0.4	61.9	72.2	67.9	63.2	75.0	69.3

Itami et al. (1994) suggested that oral administration of beta glucan enhanced disease resistance of Kuruma prawn, *P .japonicus*. They noted high phagocytic activity in the hemocytes of *P. japonicus* treated with 0.01% glucan for 3 days or 0.005% glucan for 10 days. Results of the present study indicate that oral administration of 0.1% glucan for one day can induce vibriocidal activity in both hemocytes as well as plasma.

Results in Table 3 indicate the combined effect of bacterin and glucan. It can be seen that in combination, the activity is significantly ($p < 0.05$) enhanced. Over 90% inhibition was noticed in all batches at 48h and even at 72h, over 83% inhibition was still recorded. This suggested that the bacterin and glucan combination was more effective than either of the components used individually.

Generation of reactive oxygen species as indicated by NBT assay has been used by a few investigators to measure immunostimulation in shrimp. Song and Hsieh (1994) used the NBT assay to study the effect of treating shrimp hemocytes with glucan *in vitro*. Sung et al. (1996) noted enhanced production of reactive oxygen (O_2^-) in shrimp treated by immersion with the *Vibrio* bacterin and glucan. However, they noted that levels dropped to baseline levels within 12 h. In the present study, treatment was by the oral

Table 3. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with a combination of the *Vibrio* bacterin and yeast glucan.

Treatment (% bacterin + % glucan)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
5.0+0.1	77.5	90.6	87.4	78.2	90.0	83.1
10.0+0.2	80.0	92.9	87.4	80.6	92.7	84.8
15.0+0.3	78.1	92.4	83.8	77.1	92.7	83.1
20.0+0.4	75.6	91.8	83.1	76.4	92.0	87.5

route and an enhanced response was observed in all groups, with a peak at 48 h (Table 4). Maximum response was noted at 5% bacterin and 0.2% glucan when they were used separately. Enhanced levels persisted even at 72h after the treatment. The longer duration of the response than that reported by Sung et al. (1996) could have been due to differences in the concentration of immunostimulants and/or the route of administration. Results in Table 4 further show that the treatment with a combination of 10% bacterin and 0.2% glucan resulted in much higher levels compared to individual immunostimulants. These observations show a partially additive effect in stimulating the generation of reactive oxygen in hemocytes.

Table 4. Respiratory burst activity in hemocytes of *P. monodon* measured by NBT assay after feeding with or without immuno-stimulants.

Treatment	OD_{630} at time post-treatment		
	24h	48h	72h
Nil	0.5	0.5	0.5
Bacterin			
5 %	1.1	2.2	1.9
10%	1.1	2.2	1.6
15%	1.6	2.2	1.8
20%	1.4	1.2	1.8
Glucan			
0.1%	0.8	1.2	1.1
0.2%	1.1	1.4	1.2
0.3%	1.2	1.4	1.4
0.4%	1.1	1.5	1.4
Bacterin+Glucan			
5 +0.1%	1.4	2.5	1.8
10+0.2%	1.5	2.8	2.0
15+0.3%	2.1	2.7	2.1
20+0.4%	1.9	2.6	2.0

The pro-phenol oxidase (proPO) system has been considered to play an important role in the defence system of crustaceans (Söderhäll & Cerenius 1992). Activation of the proPO system (measured in terms of the phenol oxidase activity) has been used by some investigators to measure immunostimulation in shrimp (Sung et al.1994 1996). The results in Table 5 show significant enhancement ($p < 0.05$) in PO activity in the hemocytes from all the treated batches. Peak activity in all the cases occurred 48h after treatment and the elevated levels persisted up to 72h after treatment. In the case of the bacterin treatment, highest activity (33.4 u/min/mg protein) was in the groups treated with 15% bacterin. Even at 72h after treatment, activity in this batch was 10 times greater than that of the untreated control. Glucan treatment also brought about increased PO activity and the highest activity (21.56 u/min/mg protein) was noticed with 0.2% glucan. In general, the activity elicited by the glucan was lower than that elicited by *Vibrio* bacterin. Sung et al. (1996) recorded PO activity in shrimp hemocytes treated by immersion with the *Vibrio* bacterin and glucan. They noted peak activity 3 h after treatment and a drop by 24 h. We found that treatment by the oral route resulted in a prolonged enhancement of PO activity. Further, our results showed that bacterin and the glucan in combination were most effective in eliciting PO activity. The highest level of 48.78 u/min/mg protein was obtained with 15% bacterin + 0.3% glucan. In these treatment groups the PO activity was higher than that of the untreated controls by a factor of 14, even 72 h after treatment.

Table 5. Phenol oxidase activity in hemocytes of *P. monodon* after feeding with or without immunostimulants.

Treatment	Phenol oxidase activity (u/min/mg protein)		
	24h	48h	72h
None	3.53	3.66	3.13
Bacterin			
5 %	22.21	25.33	22.62
10%	28.65	32.41	30.11
15%	28.71	33.38	31.12
20%	26.69	32.74	30.47
Glucan			
0.1%	12.98	17.12	13.93
0.2%	13.65	21.56	17.34
0.3%	12.71	19.18	15.23
0.4%	13.93	19.91	17.31
Bacterin+Glucan			
5 +0.1%	33.16	38.49	37.18
10+0.2%	37.46	46.51	43.46
15+0.3%	38.19	48.78	44.39
20+0.4%	36.93	47.65	43.56

The results of this study show that immunostimulation of shrimp, *P. monodon*, is possible through oral administration of *Vibrio* bacterin and the yeast glucan. The results also demonstrate that a combination of these two immunostimulants is much more effective than either of them used individually.

Acknowledgements. This research was supported by grants from the Department of Biotechnology, Govt. of India and this financial assistance is gratefully acknowledged.

LITERATURE CITED

Adams A (1991) Response of penaeid shrimp to exposure to Vibrio species. Fish & Shellfish. Immunol 1: 59-70.

Itami T, Takahashi Y, Tsuchihira E, Igusa H, Kondo M (1994) En-

hancement of disease resistance of Kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn hemocytes after oral administration of B 1-3 glucan (Schizophyllan) In: Chou LM, Munro AD, Lam TT, Chen TW, Leong LKK, Ding JK, Hooi KK, Khoo HW, Phang VPE, Shim KF C.H.Tan (eds) The Third Asian Fisheries Forum, Asian Fish.Soc. Manila, p 375-378.

Itami T, Takahashi Y, Nakamura Y (1989) Efficacy of vaccination against vibriosis in cultured Kuruma prawns *Penaeus japonicus*. J Aquat Animal Health 1: 238-242

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193 : 265 - 275

Söderhäll K , Unestam T (1991) Activation of serum prophenoloxidase in arthopod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of cray fish phenoloxidase. Can J Microbiol 25 : 406 - 414

Söderhäll K, Cerenius L (1992) Crustacean immunity. Ann Rev Fish Dis 2: 3-23

Song Y, Hsieh Y (1994) Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev Comp Immunol 18: 201-209

Sung HH, Song YL, Kou GH (1991) Potential uses of bacterin to prevent shrimp vibriosis. Fish & Shellfish Immunol 1: 311-312

Sung HH, Kou GH, Song YL (1994) Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathol 29: 11-17

Sung HH, Yans YL, Song YLJ (1996) Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulations. J Crustacean Biol 16: 278 -284

Waber E (1993) Non parametric methods. In : Delaunnois AL (ed) Biostatistics in Pharmacology - II. Pergamon Press Ltd. Oxford , p 937 - 942