Pro-PO Based Immunomodulatory Effect of Glucan and LPS on Tiger Shrimp, *Penaeus monodon* (Fabricius)

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ABSTRACT

While immunostimulants play an important role in enhancing immunity in shrimp, their relative efficiency is still not clear. This study was planned to test the efficiency of the immunostimulants β-1,3-glucan and lipopolysaccharide (LPS) on tiger shrimp, *Penaeus monodon* by incorporating them in the basal shrimp diet. Glucan at 10, 20, 40 and 60 mg/kg levels and LPS at 10, 20, 30 and 40 mg/kg levels were attempted for 10 days. The prophenoloxidase (pro-PO) activity was evaluated in haemolymph (in haemocytes and plasma) of test shrimps using ELISA. The reliability of the pro-PO factor, in terms of its disease resistance capability was tested through challenge studies using *Vibrio parahaemolyticus,* a known pathogen of shrimp. An enhanced pattern of immunity (in terms of pro-PO and survival to challenge) was recorded for Glucan-10 and LPS - 30 mg/kg followed by LPS - 10, LPS - 20, Glucan - 20 and Glucan - 40 mg/kg levels, demonstrating that immunostimulants in feed at the right concentrations could improve disease resistance in shrimp.

INTRODUCTION

Though shrimp culture has undergone rapid development in most parts of the South-East Asian countries, sustained production is increasingly hampered by environmental pollution, poor management and epizootic diseases. Due to the serious disease problems encountered in the shrimp aquaculture sector, several investigators have considered the possibility of adopting immuno prophylactic measures. Shrimps possess only a very primitive specific defense system and therefore non-specific immune system plays a vital role. Itami *et al*. (1989) observed that the immunized shrimps *(Penaeus japonicus)* were better protected when challenged against pathogenic bacteria. Induction of resistance in tiger shrimp, *Penaeus monodon* challenged with *Vibrio vulnificus* after treatment with Glucan was reported by Sung *et al*. (1994). Cellular defensive mechanisms in crustaceans generally rely on haemocytes with several functions such as coagulation, phagocytosis, encapsulation and prophenoloxidase activity. The pro-PO mechanism is one of the main defensive mechanisms as a non-self recognition system (LeMoullac *et al*., 1997). Enhancement of the prophenoloxidase system (pro-PO) in haemocytes, specifically by treatment with b-glucans and LPS was observed by Vargas-Albores *et al*. (1998). Studies on the immune system and

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phenoloxidase activity would certainly be valuable in providing a better understanding of its susceptibility to invading microorganisms and the defense reactions elicited during such infections (Perazzolo and Barracco, 1997).

The pro-PO system acts both as recognition and effector component of the arthropod defense system, since it can specifically be enhanced by polysaccharides from fungal or bacterial cell walls (Vargas-Albores, 1995). In shrimp, pro-PO is activated by two steps: in the first step, degranualation occurs when haemocytes are stimulated by microbes, b-glucans or LPS which enable the inactive form of pro-PO and prophenoloxidase activating enzyme (PPAE) to be released. The second step requires the participation of Ca^{3+} for the conversion of inactive PPAE to an active serine protease that in turn transform pro-PO to active PO (Vargas-Albores *et al*., 1998). Thus both the LPS and b-glucans are capable of stimulating shrimp haemocytes to release cellular components.

MATERIALS AND METHODS

Hatchery produced post larvae (PL-25) of *Penaeus monodon* were acclimatized and reared in 1.5 ton capacity outdoor cement cisterns until they reach 3.07 ± 0.2 g size. b-1,3-glucan from Baker's yeast and lipopolysaccharide (LPS) from *Escherichia coli* (Serotype 055: B5) were the two immunostimulants used in the study (Sigma-Aldrich, Bangalore). The basal shrimp feed was prepared as per the standard formulation of Chen *et al*. (1998). The inclusion levels of immunostimulants in the test feed are shown in the Table 1. The shrimp seeds acclimatized to the laboratory conditions 29 ± 2°C and at 25 ppt were distributed into 36 experimental tanks of 50 L capacity inter connected on a water recirculation system (WRS), at the rate of six shrimps per tank. While 16 tanks each were used for the two immunostimulant-incorporated feeds, the remaining tanks were maintained for the control feed. Shrimps were fed with control diet for one week to acclimatize them to the experimental conditions. Shrimps were fed with immunostimulant incorporated feeds for ten days followed by control feed for the next five days.

For challenge studies, bacterial culture of *Vibrio parahaemolyticus* obtained from the Institute of Microbial Technology (IMTECH, Chandigarh) was maintained by subculturing after testing them on healthy shrimps to reproduce the specific pathogen. After incubation at 25°C for 24 h in Tryptic Soya Broth (TSB), the *Vibrio parahaemolyticus* culture were harvested in sterile saline solution (2% NaCl) and diluted by tenfold serial dilution. The LD_{50} value, was determined by administering the lower concentration of cultures (10^0 - 10^{-4}) to the juveniles of *Penaeus monodon* by intra-muscular injection between the fourth and fifth abdominal segments with 0.05ml from different suspensions (10^0-10^{-4}). Parallel controls with sterile, saline injection (2% NaCl) and no injection were also maintained. The LD_{50} was determined by recording the mortality for 5 days. The shrimp in the immunostimulant trial were challenged with the *V. parahaemolyticus*. Bacterial cell counts approximately to that of LD_{50} values were injected into the experimental animals. Parallel controls with no immunostimulant treatment and saline control were also maintained. The mortality pattern was also observed for the period of 5 days after challenging.

The immunostimulant - incorporated feeds were fed for 10 days to the experimental shrimps followed by control feed for the next 5 days. On 16th day, immune enhancement in treated shrimps (glucan at 10, 20, 40 and 60 mg/kg and LPS at 10, 20, 30 and 40 mg/kg) was detected by challenging them with *Vibrio parahaemolyticus* (LD_{50} = 5.7 x 10^7 cfu/shrimp).

For pro-PO assay, haemolymph was collected from experimental shrimps (after giving 10 days of immunostimulant - incorporated shrimp feed and 5 days of control feed) using a 26 gauge needle of 1 ml syringe (DISPOVAN) by inserting it into the ventral sinus located at the base of the first abdominal segment. Haemolymph was collected 1:1in anticoagulant (30 mM trisodium citrate, 338 mM sodium chloride, 115 mM glucose, 10 mM EDTA, pH 7.0) to carry out pro-PO assay (LeMoullac *et al*., 1997).

Phenoloxidase activity in haemocytes and plasma was measured as detailed by LeMolluac *et al*. (1997) using ELISA. Haemocyte suspension and plasma were separately incubated with zymosan and transferred to microtitre plate (ELISA plate) in duplicate (60 µl each). L-DOPA (L-dihydroxyphenylalanine, 4 mg/ml of cacodylate buffer) was added to both haemocyte suspension and plasma. After 10 min, optical density was measured at 490 nm using ELISA reader (Lab Systems, Finland). Protein content of haemocyte suspension and plasma were measured (Lowry *et al*., 1951) to estimate the PO activity for 0.001/min/mg of protein. ANOVA was carried out for the results to confirm whether there was a statistical significance, using Statistical Package for Social Studies (SPSS). The significance level used was $P < 0.05$.

Table 1. Immunostimulatory effect of shrimp feeds on *P. monodon.*

Dose of immunostimulant (mg/kg)	Percentage survival	Haemocyte-based pro-PO activity (u/min/mg of protein x 10-5)	Plasma-based pro-PO activity (u/min/mg of protein x 10-5)
β - Glucan - 10	75.00 ± 00.00	0.800 ± 0.000	2.100 ± 0.700
β - Glucan - 20	62.50 ± 12.50	0.486 ± 0.080	1.428 ± 0.057
β - Glucan - 40	62.50 ± 12.50	0.449 ± 0.045	0.719 ± 0.000
β - Glucan - 60	37.50 ± 12.50	0.226 ± 0.052	0.730 ± 0.034
LPS - 10	62.50 ± 12.50	0.469 ± 0.025	1.621 ± 0.255
LPS - 20	62.50 ± 12.50	0.505 ± 0.039	1.592 ± 0.194
LPS - 30	75.00 ± 00.00	1.752 ± 0.000	1.686 ± 0.496
LPS - 40	50.00 ± 00.00	0.270 ± 0.016	0.920 ± 0.158
Control	50.00 ± 00.00	0.267 ± 0.033	0.667 ± 0.000

RESULTS

The bacterial concentration of 5.7 x 10^7 cfu/shrimp was estimated as LD_{50} from pathogenicity test and the dose was used to challenge the immunostimulant treated shrimps. The highest survival was recorded in glucan at 10 mg/kg and LPS at 30 mg/kg. While glucan 20 and 40 and LPS 10 and 20 mg/kg recorded 62.5% survival, LPS - 40 mg/kg showed a survival (50%) similar to that of control (Table 1). No significant differences in survival appeared at challenge levels ($P < 0.05$).

The pro-PO activity of haemocytes and plasma were measured for the various immunostimulant incorporation levels. In haemocytes, LPS at 30 mg/kg recorded the highest PO activity (1.752×10^{-5}) followed by glucan at 10 mg/kg (0.800×10^{-5}), recording a percent increment of 199.63 and 556.18 respectively. All the treatments recorded higher PO activity than that of control (Table 1) except glucan at 60 mg in haemocytes.

Glucan at 10 mg/kg recorded the highest PO activity in plasma (2.1×10^{-5}) followed by LPS 30 mg/kg (1.686×10^{-5}) showed a percent increment of 68.2 and 60.4 respectively. All the treatments recorded higher PO activity than that of control (Table 1). However, no significant ($P < 0.05$) difference was established in the prophenoloxidase activity in both haemocyte and plasma.

DISCUSSION

Use of immunostimulants for boosting the defense mechanism in crustaceans in general and shrimps in particular is a new and promising field (Sung *et al*., 1994; Newman, 1996). Itami *et al*. (1989) demonstrated that the immunized shrimps *(P. japonicus)* were better protected against challenge with pathogenic organisms. Induction of resistance of *P. monodon* against challenge with *Vibrio vulnificus* after treatment with β-glucan was reported by Sung *et al*. (1994) and recorded that the preferred route of delivery in aquaculture system would be the oral route.

Newman (2000) administered LPS at different doses to penaeid shrimps viz., 20, 40 and 100 mg/kg and challenged with white spot syndrome virus (WSSV). While higher survival rate was reported at 20 mg/kg (75%) the other two doses showed lower survival (40 mg: 64.7% and 100 mg: 52.9%). In the present study, LPS at 30 mg/kg recorded the highest survival (75%) followed by 10 and 20 mg/kg (62.50%). However, LPS 40 mg/kg recorded a poor survival rate of 50%, suggesting that enhanced LPS level on feed beyond the optimum level would not help the shrimp to develop disease resistance.

Sung *et al*. (1994) used glucan at 0.5 and 1 mg/ml in *P. monodon* and recorded an improved resistance to the challenge by *Vibrio vulnificus*. In the present study, an improved survival rate was recorded with all the treatments (62.5 to 75%), except the dose of glucan 60 mg/kg (37.5%), indicating that the immunostimulants used have positive impact on *Penaeus monodon* to enhance their immunity.

Vibriocidal activity was studied *in vitro* in penaeid shrimps using β-1,3-glucan as immunostimulant by Sung *et al*. (1996), Karunasagar and Karunasagar (1999) and Devaraja *et al*. (1998). In plasma and haemocytes, vibriocidal activity persisted even at 72h in *P. monodon* using *V. harveyi* (Devaraja *et al*., 1998). They observed a maximum of 72.7% inhibition in plasma and 75% in haemocytes when treated with 0.4% glucan incorporated diet. The other doses (0.1, 0.2 and 0.3%) however showed lesser inhibition activity. In this study, glucan at 10 mg/kg recorded a higher survival of 75% than the other doses (20 and 40 mg / kg : 62.50% and 60 mg / kg : 37.50%). However, significant differences could not be established between and among the treatments.

Prophenoloxidase (pro-PO) system is considered to play an important role in the defense mechanism of crustaceans (Soderhall and Cerenius, 1992). Pro-PO activity on shrimp has been carried out in *Penaeus californiensis* (Vargas-Albores *et al*., 1993a; 1996; Hernandez-

Lopez *et al*., 1996; Gollas-Galvan *et al*., 1997), *P. paulensis* (Perazzolo and Barracco, 1997), *P. stylirostris* (Moullac *et al*., 1997) and *P. monodon* (Sung *et al*., 1996; Sritunyalucksana *et al*., 1999; Devaraja *et al*., 1998). Devaraja *et al*. (1998) reported that *P. monodon* fed with 0.2% glucan showed maximum PO activity than other doses viz., 0.1, 0.3 and 0.4% with the peak activity noted at 48 h. In the present study glucan at 10 mg/kg recorded the highest PO activity (2.100×10^{-5}) in plasma, suggesting that glucan at a very low of incorporation can effect desired results in *P.monodon*

It is further confirmed that the PO activity enhanced due to the incorporation of immunostimulants (elicitor) viz., glucan and LPS through the feed. Perazzolo and Barracco (1997) observed similar enhancement of PO activity in shrimp haemocyte lysate suspension (HLS), following pretreatment with different elicitors, viz., LPS and laminarin (β-1,3-glucan). In addition, LPS was also able to stimulate PO activity in the fresh serum of shrimp in contrast to the β-1,3-glucan. LPS was found to be more effective than β-1,3-glucan in stimulating PO activity in both the HLS and in serum of shrimp whereas in the present study both the stimulants enhanced PO activity in haemocytes and in plasma. However, β-1,3-glucan (10 mg/kg) induced relatively a higher PO activity ($2.100 \pm 0.700 \times 10^{-5}$) than the LPS treatment in plasma. LPS at 30 mg/kg recorded a higher PO activity ($1.752 \pm 0.000 \times 10^{-5}$) in haemocyte than β-1,3-glucan treatment.

While the immunostimulantory effect of glucan and LPS has been established in penaeid shrimps (Vargas-Albores *et al*., 1993; 1996; Hernandez-Lopez *et al*., 1996; Perazzolo and Barracco, 1997), the mechanism to measure the enhancement accurately is yet to standardize. The pro-PO assay, one of the effective systems could be used in shrimps to understand the effect as well as the pattern of enhancement of immunity due to immunostimulant incorporation. Standardization of such techniques could held to establish ideal tools to access the relative efficiency of immunostimulatory products available in shrimp aquaculture sector.

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