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Immunomodulatory potential of marine secondary metabolites against bacterial diseases of shrimp

Joseph Selvin[a,*], A.J. Huxley[a], A.P. Lipton[b]

[a] *Department of Biotechnology, Malankara Catholic College, Mariagiri, Kaliakavilai-629 153, Kanyakumari District, India*
[b] *Marine Biotechnology Laboratory, Central Marine Fisheries Research Institute, Vizhinjam-695 521, Thiruvananthapuram, India*

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Abstract

Shrimp disease management using bioactive marine secondary metabolites (MSMs) was developed as a package of practice for the sustainable shrimp farming. Therefore, the effect of MSMs on the host defense factors of shrimp was evaluated in the present study. Findings indicated that *Ulva* diet significantly increase the defense factors such as haemogram, agglutination index, phagocytic rate, bacterial clearance and serum bactericidal activity of treated shrimps over the control group. Based on the gut bacterial load, *Ulva* diet was considered as proactive drug whereas *Dendrilla* diet was determined as a curative agent.

Keywords: Disease-management; Immunostimulant; Shrimp disease; *Penaeus monodon*; *Ulva fasciata*; *Dendrilla nigra*; Marine secondary metabolites

1. Introduction

In shrimp, the first lines of defense are elicited by haemocytes through phagocytosis, encapsulation and nodule formation. The phagocytic activity is enhanced considerably by the activation of prophenoloxidase (Pro-PO) system localized in the semigranular and granular haemocytes (Hose et al., 1990). Among the factors concerning the humoral defense system, the phenoloxidase (PO), bactericidin and lectins are considered as

* Corresponding author.
E-mail address: selvinj@rediffmail.com (J. Selvin).

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important (Azad et al., 1995). These defense factors in the cellular systems cooperatively provide a defense barrier against invading pathogenic organisms found in the environment. These systems have a mutual interaction and construct an elaborate network of host immunodefense. Reports on the microbicidal activity of crustacean haemolymph clearly indicated that about 75% of the encountered bacteria are eliminated within 10 min of exposure (Sung et al., 1996). The effect of agglutinins, which cause agglutination of foreign particles, is less, short-lived and non-specific (Adams, 1991). Therefore, elicitation of non-specific defense factors against bacterial infection is emerging as an effective proactive management strategy. Albeit MSMs have been found to be effective for controlling bacterial diseases of shrimp (Selvin, 2002; Selvin and Lipton, 2003a,b), the mechanism of action has not been established. The present paper addresses the findings of preliminary experiments carried out to evaluate the influence of MSMs on the host defense system of shrimp.

2. Materials and methods

2.1. Preparation of medicated feed

MSMs, which showed maximum activity in an in vitro antibacterial study (Selvin, 2002), were used in the present study. The commercial pelleted shrimp grower feed No. 1 (C.P. feeds, Cochin) was used for the preparation of sprayed medicated feed. Based on earlier findings (Selvin and Lipton, 2003a,b), the effective doses of marine alga *Ulva fasciata* and sponge *Dendrilla nigra* were 1000 and 500 mg/kg, respectively, and were used for the preparation of medicated feed. The doses were incorporated in the feed by spraying appropriate secondary metabolites on the surface of the feed at a rate of 3.2% of the shrimp body weight daily (Selvin and Lipton, 2003a).

2.2. Treatment schedule

Shrimps with an average body length of 5–6.5 cm range were reared in the circular high Density Polymer (HDP) tanks at a stocking density of 20 individuals per 200 l of seawater. The tanks were aerated and maintained at $30^{\circ} \pm 2$ °C and 35‰ salinity. The animals were fed with appropriate medicated feed in three equal installments at a rate of 3.2% of their body weight for a period of 15 days. On the 16th day of post-treatment, the shrimps were sampled randomly for analysis of defense factors. All analyses were carried out using pooled haemolymph of 20 shrimps and results were expressed as average of triplicate experiments.

2.3. Determination of host defense factors

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment using a 25-gauge needle and a 1-ml syringe filled with 0.2 ml cold modified Alsever's Solution (19.3 mM sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA in pH 7.3) as an anticoagulant (Braak et al., 1996).

Total haemocyte count (THC) and differential haemocyte counts (DHC) were determined after Jones (1962). The phagocytic assay was carried out using formalin-killed bacterial cells of *Vibrio fischeri*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Micrococcus luteus*. The assay mixture containing 100 μl freshly collected haemolymph and 10 μl formalin-killed bacterial strains (1×10^7 cells/ml) in PBS (pH 7.2) were mixed on a glass coverslip and incubated in a humidified chamber at 20 °C for 30 min. The cells were fixed in methanol and stained with May Grunwald-Giemsa for 15 min. The coverslip was turned upside down on a glass microscope slide, and the results were observed under 1000× magnification. Phagocytic cells (engulfing more than three bacterial cells) were counted over the whole slide and the results were expressed as relative percent of phagocytosis over the control group of shrimps. Agglutination index was evaluated using plasma of haemolymph collected directly in clean dry Eppendorf cups by cutting the telson. The Eppendorf cup was incubated in a sliding position at 20 °C for 1 h. After incubation, the plasma layer was separated by centrifugation at 5000 rpm for 10 min. The resultant plasma was diluted with phosphate buffered saline (PBS) in a 74-well plate and subsequently, the dilutions was mixed with 10 μl of heat or formalin-killed bacterial suspension and the agglutination was observed under 100× magnification. The index was estimated by the dilution factor.

Antibacterial activity of bactericidins in the plasma was determined by viable plate count method (Sung et al., 1996) and as expressed as the ratio of survival index (RSI) for the treated shrimps to that of the control group. The results were considered positive if the ratio was less than 1. To determine the rate of bacterial clearance from the haemolymph, shrimp were injected with 0.1 ml *V. fischeri* suspension (containing 10^6 cells/ml) and haemolymph were sampled after 5, 10, 30 and 60 min. The samples were immediately mixed with 7 ml of melted TCBS agar, poured into petri dishes and incubated at room temperature for 18 h. Number of bacterial colonies per plate was counted and divided by the volume of haemolymph collected to determine the number of colony forming units (cfu) per milliliter of haemolymph.

The effect of antibiosis on the normal gut microflora was studied by Total Viable Count (TVC) method. On the 7th day of oral dosing of appropriate MSMs and OTC, the shrimps were randomly sampled for bacterial count analyses. Shrimps were surface sterilized with 70% ethanol and cut through the mid-ventral line using a pair of sterile scissors. The entire gut was carefully removed and ground in a mortar and pestle (surface sterilized) with 1 ml of sterile PBS. The resultant aliquot was serially diluted with sterile PBS and plated in triplicate in nutrient agar plates. After incubation at 32 °C, the TVC was determined.

3. Results and discussion

The haemogram profile of different experimental groups of shrimps varied considerably (Table 1). In the *Ulva* and *Dendrilla* treated group, the THC enhanced to 6725 and 6200 cells/ml, respectively, when compared to that of control group (5437 cells/ml). The DHC also fluctuated widely among the treated and control groups. From Table 1, it could be noted that the infected shrimps had higher percent of eosinophilic granulocytes (EG) (41.84%) with a parallel decrease in the percent of prohaemocytes (PH) (6.21%). In the

Table 1
Haemogram profile of control and treated shrimps

Haemolymph count	Experimental groups			
	Control $n=20$	Infected $n=5$	Treated $n=20$	
			Ulva	Dendrilla
THC (cells/ml)	5437.50	9185.71	6725.00	6200.63
DHC (%)				
(i) PH	15.57	6.21	12.38	15.68
(ii) HH	20.49	17.35	24.76	25.58
(iii) IG	39.34	34.69	33.33	35.51
(iv) EG	24.59	41.84	29.52	23.25

n = No. of shrimp sampled for haemolymph collection.

apparently normal shrimp, the EG and PH were 24.59% and 15.57%, respectively. The group of shrimps treated with *Dendrilla* extract did not show variations (which is comparable to the control group) while the *Ulva*-treated group had EG and PH of 29.52% and 12.38%, respectively. The percent of hyaline haemocytes (HH) enhanced in the *Ulva*- and *Dendrilla* medicated shrimps to 24.76% and 25.58%, respectively. The HH value of control group was accounted for 20.49%. In the case of intermediate granulocytes (IG), the treated shrimps showed declined values (33.33% for *Ulva* and 35.51% for *Dendrilla*) over the control group (39.34%).

Crustacean haemocytes play important roles in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell to cell communication (Johansson et al., 2000). The semigranular haemocytes were reported to be the primary cells involved in the phagocytosis of foreign particles in shrimp (Bachere et al., 1995; Soderhall and Cerenius, 1992). Granular haemocytes were also reported to be capable of phagocytosing foreign material but with less frequency than the smaller ones (Hose and Martin, 1989). However, granular cells have been proven to play a significant role in the shrimp defense system due to their antibacterial activity (Chisholm and Smith, 1995). The smallest and least numerous hyaline cells were also considered as phagocytes (Soderhall and Cerenius, 1992). It was reported that the immune system of *Penaeus chinensis* could apparently be activated by oral administration of immunodrugs derived from the land and marine plants (Wang et al., 1995). The immune factors of shrimp haemolymph were successfully stimulated and enhanced resistance was achieved against infectious diseases by the administration of glucans (Song et al., 1993, 1997; Itami et al., 1994; Sung et al., 1994; Chang et al., 2000), peptidoglycans (Takahashi et al., 1995; Itami et al., 1998; Henning et al., 1998) and lipopolysaccharides (Karunasagar et al., 1996).

Agglutination titre was considerably increased in the *Ulva* treated shrimp. A very high titre of 8192 each was obtained for formalin-killed *V. fischeri* and *A. hydrophila* antigens (Table 2). In the case of normal shrimp, the titre was 2048 and 4096, respectively. The enhancement was found to be moderate against *E. coli* (4096), *M. luteus* (4096) and *P. aeruginosa* (2048) antigens. It was found that the *Dendrilla* medication did not induce any changes in the titre against *E. coli* (2048), *P. aeruginosa* (1024) and *A. hydrophila* (4096). However, the medication showed moderate enhancement against *V. fischeri* (4096) and *M. luteus* (4096). As the agglutination titre values were the index of level of agglutinin in the

Table 2
Agglutination titre of normal and treated shrimp

Bacterial species	Experimental groups		
	Control $n=20$	*Ulva*-treated $n=20$	*Dendrilla*-treated $n=20$
V. fischeri	2048	8192	4096
E. coli	2048	4096	2048
P. aeruginosa	1024	2048	1024
A. hydrophila	4096	8192	4096
M. luteus	2048	4096	4096

circulation, the enhanced level achieved by the MSMs treatment might elicit a high defense against bacterial pathogens (Selvin, 2002).

The phagocytic rate was significantly increased in the *Ulva*-treated shrimp (Table 3). The rate of phagocytosis was increased to 5.8%, 2.8%, 1.56%, 1.38% and 0.66%, respectively, over the normal shrimp against *E. coli*, *V. fischeri*, *P. aeruginosa*, *A. hydrophila* and *M. luteus*. The *Dendrilla*-medicated group exhibited enhanced phagocytosis against *E. coli* (3.8%) over the control group. However, only a meagre enhancement was observed against *V. fischeri*, *P. aeruginosa* and *A. hydrophila*. The phagocytic cells reported to remove foreign particles in the crustacean haemolymph (Mckay and Jenkin, 1970; Fontine and Lightner 1974; Paterson and Stewart 1974; Paterson et al., 1976; Smith and Ratcliffe 1978, 1980; Goldenberg et al., 1984). The index of phagocytosis indirectly measures the level of pro-phenoloxidase activating system, which initiates haemocyte encapsulation (Hose et al., 1990).

Results of bacterial clearance showed that all the control shrimps cleared 12.0% of the challenged *V. fischeri* cells within 10 min. During the same interval, the clearance was high, to the extent of 24.0% and 22.0% in the *Ulva*- and *Dendrilla*-treated groups, respectively (Table 4). The results of viable plate count indicated that 46.0% was reduced in 30 min, which progressed to 24.46% in 1 h in the control group. The clearance was very high to the extent of 64.0% within 30 min and 85.0% in 60 min in the *Ulva*-treated group. In the *Dendrilla*-treated group, a lower clearance of 58.0% in 30 min and 82.0% in 1 h were recorded. *Ulva* medication cleared *Vibrio* cells to the extent of 64% at 30 min and 88% at 60 min, respectively. The plasma bactericidin was reported to effectively combat the bacterial infection in lobsters (Cornick and Stewart, 1968; Mori and Stewart, 1978).

Table 3
Percentage of phagocytosis in the normal and treated shrimp

Bacterial species	Experimental group		
	Control $n=20$ (%)	*Ulva*-treated $n=20$ (%)	*Dendrilla*-treated $n=20$ (%)
V. fischeri	49.20	52.00	49.90
E. coli	48.80	54.60	52.60
P. aeruginosa	42.80	44.36	42.89
A. hydrophila	44.90	46.28	45.56
M. luteus	45.54	46.20	45.26

Table 4
Rate of bacterial clearance in the normal and treated shrimp

Time (min)	Experimental group ($n=20$)		
	Control (cfu/ml)	*Ulva*-treated (cfu/ml)	*Dendrilla*-treated (cfu/ml)
0	1.0×10^6	1.0×10^6	1.0×10^6
10	8.8×10^5	7.6×10^5	7.8×10^5
30	4.6×10^5	3.6×10^5	4.2×10^5
60	2.46×10^5	1.2×10^5	1.8×10^5

According to Sung et al. (1996), about 10% of the exposed *V. vulnificus* cells were cleared-off within 5 min and they were completely cleared-off at 24 h. Adams (1991) reported that more than 99% of heat-killed *V. alginolyticus* were cleared from haemolymph of *P. monodon* within 48 h after exposure. In the present experiment, 88% of viable *V. fischeri* cells were cleared-off from the haemolymph within 1 h in the *Ulva*-treated group. The rapid bacterial clearance rate of shrimp haemocytes was found to be stimulated by *Ulva* treatment. Therefore, it was conjectured that bacteridins found in shrimp plasma might be inducibly released from haemocytes by *Ulva* medication.

The results of in vitro broad-spectrum antibacterial activity of serum are presented in Table 5. The survival index ratio of *E. coli*, *V. fischeri*, *P. aeruginosa*, *A. hydrophila* and *M. luteus* were <1 following treatment with *Ulva*. However, the index was>1 for *P. aeruginosa* and *A. hydrophila* in the shrimps treated with *Dendrilla*. In addition, the survival index (SI) of *E. coli* and *M. luteus* was greater than 1 in normal and *Dendrilla*-treated shrimp while the same was always less than 1 in the *Ulva*-treated shrimp. The nature of the bacterial cells also influenced the level of serum bactericidal activity. Among the five bacterial species, the relative rate of clearance in the shrimps treated with *Ulva* diet were graded as *E. coli*>*M. luteus*>*P. aeruginosa*>*V. fischeri*>*A. hydrophila* in decreasing order. This may be one of the reasons for the consideration of *V. fischeri* and *A. hydrophila* as shrimp pathogens. The results showed that the normal and *Dendrilla* shrimp serum did not exhibit antibacterial activity against *P. aeruginosa* and *M. luteus*. These findings suggest the quick production of bactericidins in the haemolymph of *Ulva*-treated group.

The TVC of gut was drastically reduced following *Dendrilla* feeding (6.36×10^3 cfu/ml) and it was almost similar to that of the OTC-treated group (2.84×10^3 cfu/ml) (Table 6). The TVC of *Ulva* fed shrimps was 1.06×10^4 cfu/ml and it was near to that of the control group (4.08×10^4 cfu/ml). Findings of the present study indicated that *Ulva* diet

Table 5
Serum bactericidal activity of normal and treated shrimp

Group	*V. fischeri*		*E. coli*		*P. aeruginosa*		*A. hydrophila*		*M. luteus*	
	SI[a]	RSI[b]	SI	RSI	SI	RSI	SI	RSI	SI	RSI
Control	0.82	1.00	1.52	1.00	0.62	1.00	0.94	1.00	1.29	1.00
Ulva	0.76	0.92	0.89	0.58	0.56	0.90	0.89	0.94	0.92	0.71
Dendrilla	0.78	0.95	1.38	0.90	0.68	1.09	0.96	1.02	1.16	0.89

[a] SI—Survival Index.
[b] RSI—Ratio of Survival Index.

Table 6
Total viable count (TVC) of gut content of normal and treated shrimp

Experimental group	TVC (cfu/ml)
Control	4.08×10^4
OTC fed	2.84×10^3
Ulva fed	1.06×10^4
Dendrilla fed	6.36×10^3

could be used as prophylactic agent while the *Dendrilla* diet could be used as therapeutic agent.

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