The Asian Journal of Animal Science (December 2009 to May 2010) Vol. 4 Issue 2 : 161-166

RESEARCH PAPER

Enhancement of phagocytosis, agglutinin and bactericidins in haemolymph of the spiny lobster, *Panulirus homarus* by immunostimulants

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Accepted : August, 2009

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ABSTRACT

The non-specific immunity level of the Indian spiny lobster, *Panulirus homarus* was enhanced by administration of immunostimulants such as chitosan, levamisole and *Ulva* extract. The total haemocyte count enhanced to 60.25% among the levamisole treated group. The differential haemocyte values also enhanced in the immunostimulated group were noted. The increase in phagocytic rate of the haemocytes could be correlated to the haemogram profile of the experimental group. Serum agglutinins towards *Vibrio fischeri* and *Micrococus luteus* and the bacterial clearance ability of the haemolymph were enhanced among the treated group as against the control. The possible use of marine natural products in enhancing the disease resistant capability of lobsters has been discussed.

Key words : Lobster, Chitosan, Levamisole, *Ulva,* Immunostimulant, Agglutinin, Haemogram.

The lobster culture and fattening are emerging as lucrative and remunerative ventures owing to the high export value. Mortalities due to stress and diseases in lobster holding facilities are frequently noted, causing serious setbacks. As vaccines are impractical and chemaotherapeutants have certain inherent setbacks, strengthening the host's defense system forms the best suitable alternate management strategy.

Crustaceans have the capability to clear a variety of exogenous materials entering into their haemolymph including viruses (McCumber and Clem, 1981), bacteria (Cornick and Stewart, 1968; Mckay and Jenkins, 1970; Smith and Ratcliffe, 1978; Adams 1991) and even dyes and latex particles (Merrill *et al.,* 1979; Mullainathan *et al.*, 1984, Factor and Beekman, 1990). By phagocytosis, the foreign particles in the haemocoel are removed (McKay and Jenkin 1970; Fontaine and Lightner, 1974; Paterson *et al.,* 1976; Smith and Ratcliffe, 1978; Goldenberg *et al.*, 1984). Antibacterial components, such as bactericidins, lectins and agglutinins are also reported to be effective against the invading bacteria (Cornick and Stewart, 1968).

Enhancing the non-specific defense mechanism and resistance against the invading organisms can be achieved by immunostimulants. They were reported to be successful in preventing microbial diseases in cultured shellfishes (Sung *et al.,* 1994; Song and Hsieh, 1994; Itami *et al.*, 1994). The possible development of immunostimulants from seaweeds and other sources were also reported by Hanazawa *et al.*(1982) and Manaicino and Minucci (1983).

Inspite of the natural and synthetic analogues of immune enhancers being developed for fish and shrimp, the works related with lobsters are limited. The present report highlights the efficacy of a novel seaweed (*Ulva*) based natural product and standard immunostimulants on the host defense factors such as haemogram, cellular and humoral immune responses among lobsters.

MATERIALS AND METHODS

Experimental specimens :

Indian spiny lobsters (*Panulirus homorus*) (abw: 75g) were collected from the rocky shore off Vizhinjam coast (Thiruvananthapuram, Kerala) and acclimated to laboratory conditions in 60 L rectangular glass tanks with seawater. The tanks were provided with bio-filter and hiding tubes. They were maintained with adequate aeration and optimum water quality (salinity-35 ± 2 ppt, temperature = 28 ± 2^0C). Lobsters were fed *ad-libitum* with mussel (with shell) and clam meat.

Immunostimulants :

Chitosan:

De-acetylated form of muco-polysaccharides isolated from the shell of crustaceans was obtained from CIFT, Kochi. 100 mg of chitosan dissolved in 10 ml of diluted acetic acid was set to pH 7 with 0.1M NaOH solution. The final desired concentration was obtained in PBS.

Levamisole :

The synthetic antihelminthic drug, vermisole tablets

(Kantenwal Lab, Mumbai) was used.

Ulva :

A seaweed based natural product was extracted in the laboratory. 500 mg of crude *Ulva* extract (prepared, as per methods of Lipton, 2001) dissolved in 10 ml of normal saline was diluted to get the desired concentration of 500-mg/kg-body weight.

Single dose of 50 mg of *Ulva* and o.1 mg each of Chitosan and Levamisole per lobster were administered separately to a group of 5 lobsters.

Preparation of bacterial suspension :

To evaluate the bacterial clearance potential, phagocytic assay and agglutination titre, appropriate bacterial suspensions were prepared prior to the experiment. *Vibrio fischeri,E. coli, Micrococcus luteus* strains were cultured over night in nutrient broth at 28° C. To prepare the solution for injection, cultures were pelleted at 3000 rpm for 10 min, washed thrice in 0.85% saline and serially diluted in sterile saline to obtain the optimum concentration. The bacterial counts were determined using the hemocytometer. For the determination of phagocytosis and agglutination index, the bacterial suspension was inactivated by to overnight exposure in 0.3% formalin.

Haemolymph sampling :

Haemolymph was obtained from the orthroidial membrane of leg joints using a 25-gauge needle and a 1 ml syringe filled with 0.2 ml of cold modified Alsever'sSolution (AS) (19.3 mM sodium citrate, 239.8 mM NaC1, 182.5 mM glucose, 6.2 mM EDTA at PH 7.3) as an anticoagulant. As bleeding by syringe was found not effective, haemolymph was collected by cutting one of the chelate legs, Sears were made in slide immediately and air-dried. The rest of the free flowing haemolymph was collected in 1.5 ml eppendorf tubes and store on ice for THC, DHC and other experiments.

Determination of Haemogram :

Total haemocyte counts (THC) :

THC was determined as usual method. The total haemocytes counts (THC) cell per mm ^{3} of haemolymph were calculated as follows:

$$\text{THC} = \frac{\text{Haemocytes in four - 1mm3 x depth factor x dilution factor}}{\text{Number of squares counted}}$$

Differential haemocyte counts (DHC):

Haemolymph smears were stained with May Grunwald-Giemsa and counted. Cells were observed at a magnification of 1000x. Haemocytes were characterized and relative abundance of the cell type were determined by counting 100 cells. The percentage of each type of haemocytes was calculated on the basis of total number of all the haemocytes in a single observation. The DHC was expressed as follows :

$$\text{DHC} = \frac{\text{Number of specific type of cells counted}}{\text{Total number of cells counted}} \text{x100}$$

Phagocytic assay :

Formalin inactivated *V. fischeri* was used for the phagocytic assay. A drop of freshly collected haemolymph was mixed $1x10^7$ cells of bacterial strain PBS on a glass cover slip and placed in a humidified chamber for 30 minutes at 20^0C. After incubation, and washing with PBS, the cells were fixed with methanol. They were stained with May Grunwald-Giemsa (HiMedia) for 15 min. and washed thrice with distilled water. The cover slip was turned upside down on to a drop of mounting fluid on a glass microscope slide, and the results were read via oil immersion at 1000x magnification. Phagocytic cells, engulfing more than three bacteria were counted and their number were compared with the counts of the control group of lobsters.

Bacterial clearance :

To determine how rapidly bacteria were cleared form the haemolymph, lobsters were injected with 0.1 ml V. fischeri suspension containing 10^5 cells/ml and haemolymph were sampled from the animal after 10 and 90 min. One hundred ul of haemolymph diluted with sterile saline was spread on to triplicate TCBS agar (HiMedia) plates. Bacterial colonies per plate were counted and divided by the volume of haemolymph extracted to determine the number of colony forming units (CFU) per milliliter of haemolymph.

Agglutination titre:

Initially plasma was prepared by bleeding haemolymph directly in clean dry Eppendorf tube. The Eppendorf tube was incubated in a sliding position at 20^0C for l h. After incubation, the plasma layer was separated by centrifugation at 5000 rpm for 10 min. The resultant plasma was diluted with PBS in a 74 well plate. A drop of different dilutions were mixed with a drop of heat or formalin killed bacteria and the agglutination were observed under 100x magnification. The index was estimated by the dilution factor.

RESULTS AND DISCUSSION

An increased level of THC's among experimental lobsters compared to the control group was noted (Table 1). The THC of the chitosan treated group increased to 20.25% over the control group on the day 1 and which decreased in the day 21 to the extent of 11.02. The value recorded for the intermittent days were 11.56% for 7th day and 19.10% for 14th day, respectively. More or less same trend was noted in all other experimental groups of levamisole and *Ulva*. Interestingly, drastic increase of THC to the rate of 60.25 was noted in the levamisole group on the first day. However, it was declined considerably to 28.24% 21st day after treatment. But in the *Ulva* group, the THC showed a peak value after 14 days of treatment (22.19%), which decreased to 8.00% on the 21st day. The percentage increment of THC over control is shown in Fig.1.

The DHC values are also expressed in Table 1. In the control group, the most abundant type was agranular hyalinocytes whereas in experiment groups, the granulocytes were abundant.

The trend of influence of the tested immunostimulants on the *in vitro* phagocytic rate (Table 2) of lobsters is shown in Fig. 2. It could be noted that the phagocytic rate increased concomitant to the haemogarm. The rate of increase in levamisole treated group was 15.25, 18.73, 13.13 and 10.95% over the control group on the 1,7,14 and 21st day of post-treatment respectively. In the case of *Ulva*, the rate of phagocytosis had the peak in day 14th and then decreased to 1.54% on the 21stday of treatment.

The serum agglutination titre of lobsters revealed that the level agglutinins increased considerably against bacterial pathogens in the experimental group over the

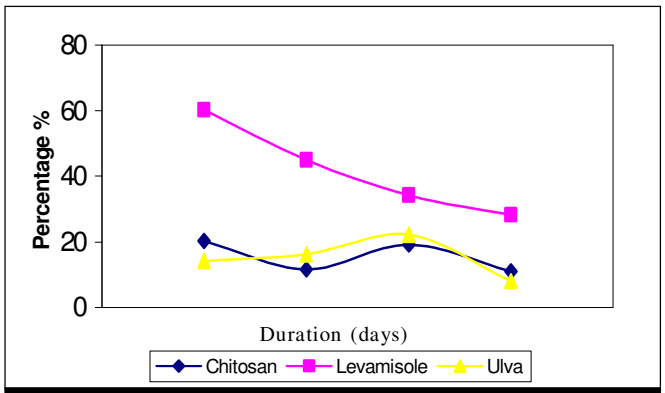

Fig. 1 : Percentage increment of THC over the control *P. monodon*

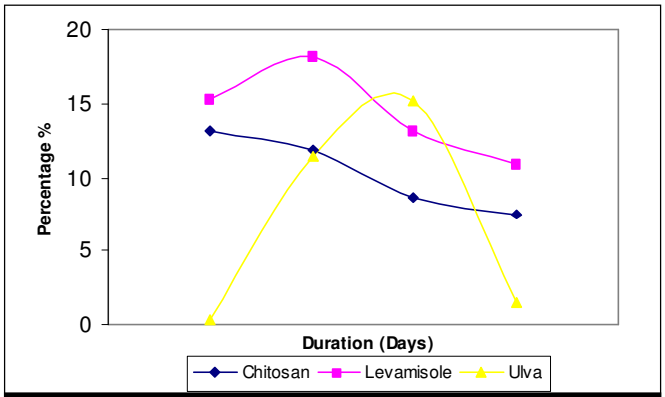

Fig. 2 : Percentage increment of phagoctic index of *P. monodon* over control

Table 1 : Haemogram of *P.homarus*

Experiment	Total hemocyte counts (cells/mm)^3					Differential hemocyte counts (%)			
Days	1	7	14	21	Type	1	7	14	21
Control	9875±892	10700±818	8900±318	8275± 318	PH	05.79±0.51	06.92±0.59	06.92±0.77	06.13±0.97
					HC	46.61±1.04	45.48±1.24	54.29±1.44	42.72±1.61
					EG	42.41±0.92	42.25±0.36	35.96±3.28	38.63±0.48
					CG	05.19±0.53	05.35±0.53	04.92±0.95	06.49±0.16
Chitosan	11875±877	11937.5±896	10600±544	9187± 1016	PH	10.91±2.36	10.95±0.77	07.72±1.02	07.19±0.60
					HC	36.15±2.91	36.54±0.75	43.83±0.24	46.00±4.45
					EG	39.58±5.91	39.89±2.10	41.25±0.36	39.54±1.99
					CG	13.32±1.32	12.20±4.24	07.16±1.18	07.25±1.86
Levamisole	15825±2496	15512.5±975	11937.5±3005	10612±380	PH	09.00±0.55	06.83±0.12	05.06±1.15	05.90±0.49
					HC	35.15±3.70	38.15±1.53	44.52±4.73	52.37±1.88
					EG	45.50±3.46	44.95±1.56	42.79±1.85	35.70±1.88
					CG	09.84±2.11	10.05±0.07	07.60±1.72	06.02±3.27
Ulva	11275±1072	12437.5±830	10875±1197	8937± 750	PH	08.69±2.56	08.57±0.81	05.97±1.07	05.97±1.07
					HC	39.13±2.11	38.93±1.31	57.93±1.67	57.93±1.67
					EG	44.66±2.90	44.54±0.77	27.85±4.24	27.85±4.24
					CG	07.50±1.49	07.96±2.96	08.23±0.53	08.23±0.53

Table 2 : Phagocytic index, agglutination and bacterial clearance

Experiment	Phagocytic index (%)					Agglutination titre				Bacterial clearance (%) after 90min			
Days	1	7	14	21	Bacteria	1	7	14	21	1	7	14	21
Control	49.42	49.31	42.85	40.00	*V. fischeri*	1:14	1:14	1:14	1:14	77.40	74.78	72.91	73.44
					E. coli	1:14	1:15	1:15	1:13				
					M. luteus	1:15	1:16	1:16	1:15				
Chitosan	55.91	55.17	48.57	45.16	*V. fischeri*	1:18	1:20	1:18	1:16	91.94	81.93	80.89	78.33
					E. coli	1:19	1:21	1:17	1:17				
					M. luteus	1:21	1:22	1:20	1:18				
Levamisole	56.96	58.55	48.48	49.60	*V. fischeri*	1:19	1:23	1:19	1:17	96.63	91.93	90.76	76.88
					E. coli	1:18	1:23	1:21	1:18				
					M. luteus	1:21	1:26	1:21	1:18				
Ulva	49.60	54.96	49.35	40.65	*V. fischeri*	1:17	1:19	1:18	1:15	92.99	89.32	83.44	79.19
					E. coli	1:16	1:19	1:18	1:15				
					M. luteus	1:18	1:21	1:19	1:16				

control (Table 2). The titre varied significantly between the bacterial strains. In the Chitosan treated group, the titre value recorded against *V. fischeri* was 1:18, 1:20, 1:18 and 1:16 and in *M. luteus* the rate had increased to 1:21, 1:26, 1:21 and 1:18, respectively for the interval days of 1,7,14 and 21. In the control group, the titres were more or less similar or it was recorded between the ranges of 1:14-1:16. Levamisole treatment also enhanced the titre to the extent of 1:17 to 1:23 whereas in the case of *Ulva*, the influence was moderate.

The clearance rate of bacteria injected in to the haemocoel of the experimental lobsters is depicted in Fig.3. The clearance rate was high in the levamisole treated group to the extent of 24.84% over the control followed by *Ulva* (20.14%) and Chitosan (18.78%) on the first

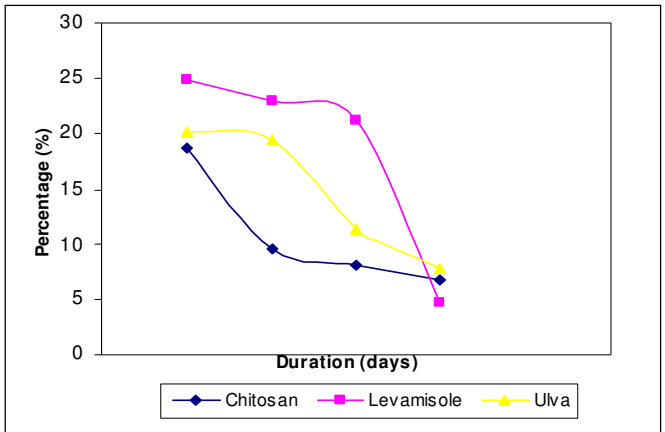

Fig. 3 : Percentage increment of Bacterial clearance *P. monodon* over control

day. However, the rate declined subsequently.

The crustacean haemocytes have been implicated in diverse functions including the active role in internal defense against invading biotic and abiotic foreign materials (Evans and Brock, 1994). In the present study, the haemogram profile of immunostimulated group indicated that THC's of lobster haemolymph increased considerably over the control group on the day 1 and declined after 21 days. THC could provide as useful clue to assess the physiological status of the animal (Martin and Graves, 1985). The results of higher THC obtained in immunostimulated group could represent the increased healthy status of lobsters.

It is well established that the foreign particles in the crustacean haemocoel are removed by phagocytosis (McKay and Jenkin, 1970). The *in vitro* phagocytosis of erythrocytes by hemocytes of the crayfish, *Parachaeraps bicarinatus*, required specific opsonins (Fontaine and Lightner, 1974, Paterson *et al.,* 1976, Smith and Ratcliffe, 1978, Goldenberg *et al.,* 1984). Opsonins appeared to be hemagglutinins, which enhanced adhesion of erythrocytes to hemocytes (McKay *et al.,* 1969). According to Takahashi *et al.*(1995), high phagocytic activity in crustaceans was noticed through the administration of B 1-3 glucan and peptidoglucan. In the case of *Ulva*, the phagocytosis was found to be peak on 14th day. The bacterial clearance potential was increased considerably in the levamisole treated group followed by *Ulva* and Chitosan. It was obvious that the bactericidal activity was reduced after 21 days of post-treatment. *E. coli* and *M. luteus* were more sensitive compared to *V. fischeri*.

The parallel increase of defense factors on the initial days and their drastic decrease after 21 days of post-treatment could reveal that the memory capability was short and almost lost in the final phase of the experimental period. Thus, could be correlated with poorly-developed specific immune system of crustaceans, particularly in

the present instance, the lobsters (Lightner, 1988).

Among the common synthetic analogues, levamisole was found to be more potent. The efficacy of immunostimulatory compounds such as chitosan and levamisole were least in crustaceans although there are few work in fish. Chitin/chitosan which is acetylated and de-acetylated form of muco-polysaccharides is isolated from the shell of crustaceans, insect exoskeletons and fungal cell walls (Sakai *et al.,* 1992). It is highly chelating and complex forming molecule. Though the novel marine natural product of *Ulva* was promising, its potency is less compared to chitosan and levamisole. This could be attributed to the crude form of product, which could have contained lower amount of their active principles. Similar report on algal compared from *Undaria pinnaftifida* was found in seaweeds, sulfated polysaccharides were reported to be immunostimulatory (Hanazawa *et al.,* 1982, Manicino and Minucci 1983), Polysaccharide fractions from the marine alga, *Porphyra yezoensis* were found to stimulate murine phagocytic function *in vivo* and *in vitro* (Yoshizawa *et al.,* 1995). However, very little information is available to draw a comparative status of their extracts and their activation among lobsters.

Controlled laboratory studies have demonstrated that other immunostimulants such as Vibrio bacterin, yeast glucan, peptidoglucan, schizophyllam and lipopolysaccharide, have the potential to reduce the impact of diseases in shrimp (Sung *et al*., 1994; Song and Hsech 1994; Itami *et al.*, 1994). The dietary incorporation of beta-1, 3-glucan from *Schizophyllum commune* was reported to enhance the resistance of post larval, juvenile and adult *Penaeus monodon* to White Spot Syndrome Virus (WSSV) (Chang *et al*.,1999, Dugger and Jory, 1999) and against *Vibrio* sp. (Rao *et al.,* 1996). The phenoloxidase (PO) activity of hemocyte lysate supernatant (HLS) from both tiger shrimp *Penaeus monodon* and giant freshwater prawn, *Macrobrachium rosenbergii* was enhanced significantly by treating with beta-1, 3-1,6- glucan at rate of 1 mg/ml (Sung *et al.,* 1998). The bacterial clearance ability of haemolymph of tiger shrimp, *P. monodon* immersed with *V. vulnificus* shows good result within 12 h following invasion and completely at 24 h. The anti-*E coli* activity of plasma, phenoloxidase (PO) activity, as well as the production of superoxide anion (O_2) were also significantly enhanced by glucan treatment (Sung *et al.,*1996).

From the findings of present study, it could be informed that though the immunostimulation in lobster is short, the resistance over disease causing agent could be enhanced. However, continuous administration of marine natural products such as chitosan and *Ulva* may be required to obtain sustained immunostilanting activity.

Acknowledgements :

The authors are thankful to the Dept. of Biotechnology for providing financial assessments in the form of sponsor project. Thanks are expanded to Dr. Mohan Joseph Modayil, former Director, Dr. R. Paul Raj, former Head, PNP Division, CMFRI for the encouragement.

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