Immunomodulation by ‘ImmuPlus (AquaImmu)’ in giant freshwater prawn, *Macrobrachium rosenbergii* (De Man)

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ImmuPlus, a polyherbal commercial formulation was used to modulate the immune system of commercially important giant freshwater prawn *M. rosenbergii*. The prawns were fed with basal diet supplemented with ImmuPlus @ 1 g/kg feed for 4 weeks. Results showed that the phenoloxidase activity (PO), haemagglutination and lysozyme activities were significantly elevated in ImmuPlus-fed prawn up to 3 weeks of feeding and declined after 4 weeks of feeding. The total protein level in ImmuPlus-fed prawn raised up to 2"nd week of feeding. Incorporation of ImmuPlus at the rate of 1 g/kg feed in the diet of prawn for 3 weeks may be beneficial in raising the immune status of prawn.

**Keywords:** *Macrobrachium rosenbergii*, Immunomodulation, ImmuPlus

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man), is commercially important worldwide as a primary inland cultured species. However, the disease outbreaks caused by the pathogens and environmental stress result in declined production. A major constraint in the commercial scale aquaculture production of this species is the inadequate availability of seed. The serious effect of infections in wild stock are most probably underestimated as the diseased animals rapidly vanish in nature, and only in aquacultural farms mortality are reported causing economic loss. Although the freshwater prawns are comparatively resistant than brackishwater shrimps, they are not free from diseases. Shell diseases such as Brown and Black spot caused by bacteria and vibrosis are commonly found in adult prawns. Further, high stocking density in culture system aggravates the infections. Studies towards a better understanding of prawn defense mechanisms constitute one approach towards overcoming disease problems. Many workers have tried to study how shrimp defense factors, including possible induction mechanisms, influence shrimp health and resistance to disease, but the information gained has not yet led to any general conclusions. One problem may be the lack of reliable and effective indicators for measuring defence status. To this end, many cellular and humoral factors of shrimp and a few factors in prawns have been studied with the hope that they may be developed as indicators or as measures for the effectiveness of potential immunostimulants. In this study, phenoloxidase (PO) enzyme activity, agglutinin, lysozyme and protein levels have been examined to determine whether these could be used as indicators for the stimulatory activity for one of the commercial polyherbal immunopotentiators, viz., ImmuPlus.

Few of the immunostimulants viz., β-1, 3 glucan, peptidoglycan, lipopolysaccharide (LPS) and commercial stimulants, PENSTIM, BRM-01 and BRM-02 have been successfully tried to modulate non-specific immunity in shrimps and to enhance resistance against various bacterial, viral and fungal infections. Similarly, benzalkonium chloride, having immunostimulant property has been evaluated in freshwater prawn.

Medicinal plants, used in various traditional systems, have immune potential against various diseases. More than 13,000 plants have been studied during the last five years for various pharmacological properties in animals and human beings. ImmuPlus (AquaImmu), a polyherbal formulation (Indian Herbs Supply and Research Company Limited, Saharanpur, India) contains the extracts of selected Indian medicinal plants viz., *Ocimum sanctum* (Tulsi), *Withania somnifera* (Ashwagandha), *Tinospora..."
cordifolia (Guduchi) and Emblica officinalis (Amalaki) as major constituents in optimum concentration. Antioxidant and immunomodulatory activities of these plants in human being and animals have been well documented. Therefore ImmuPlus was used in this investigation to modulate the immune system of freshwater prawn.

Materials and Methods

Two diets were compared in the present study. The control diet was a basal diet (Table 1) containing 36.75% crude protein, 6% crude lipid and 22.0% ash. In the experimental diet, the basal diet was supplemented with ImmuPlus (Aquaimmu, 1 g/kg). Trial studies with postlarvae of M. rosenbergii showed that 1 g/kg supplementation of ImmuPlus (Aquaimmu) increased growth and resistance to stress. The feeding ingredients were thoroughly mixed, manually pelletized, air-dried and stored at room temperature.

The experimental prawns (M. rosenbergii, 35-40 g) were artificially propagated and raised from a single stock at the Institute for six months prior to the experiment. The inter-moult prawns were selected and divided into two groups of 72 each. Each group was further divided into four subgroups (for each week of 4 weeks study). Each subgroup had 3 replicates of six prawns. Each replicate was held in a 300 l plastic tank with continuous aeration. The prawns were acclimated to laboratory condition for 2 weeks before starting the experiment. The prawns were fed twice a day at the rate of 5% of their body weight at 1000 and 1700 hrs. Unconsumed food and excreta were removed every morning before feeding with replacement of 15% freshwater. During the experiment the mean physico-chemical parameters of water were as follows: water temperature 27±1.0°C, pH 7.2±0.4, dissolved oxygen 8.2±0.2 mg/l, hardness as CaCO3 92±0.5 mg/l, NO3-N 0.007±0.003 mg/l and NH3 0.021±0.005 mg/l. The prawns were fed with the basal/experimental diet for 4 weeks.

Haemolymph was collected at the end of each week from the ventral-sinus cavity of three prawns from each tank using a 26-gauge needle and 1 ml syringe containing anticoagulant solution (1:9) (tri-sodium citrate: 0.114 M, sodium chloride: 0.10 M, pH 7.45) to measure phenoloxidase (PO) activity.

From the collected haemolymph, haemocyte lysate supernatant (HLS) was prepared. Briefly, PBS-washed haemocyte suspension was homogenized with a sonicator (Artek) equipped with a microtip (output 50, duty cycle 50%) and centrifuged (12,000 g) to collect HLS. PO activity was assayed spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine by using L-3, 4 dihydroxyphenylalanine (L-DOPA, Hi-Media, Mumbai) as substrate in U-bottom microwell plates. HLS (50 μl) was preincubated for 15 min at 37°C, after which 100 μl of L-DOPA (1.6 mg/ml in tris buffer saline, TBS: 50 mM Tris, 100 mM NaCl, pH 7.3) was added and allowed to react for 1 min. Each reaction mixture was further diluted with 100 μl TBS, and then the absorbance at 490 nm was measured. The control solution, which consisted of 50 μl PBS (to replace HLS), 100 μl L-DOPA, and 100 μl TBS, was used for the background PO activity in all test conditions. The background PO activity (optical density) values were in the range of 0.01-0.02. The PO activity in terms of optical density was expressed as dopachrome formation/50 μl HLS.

From the remaining three prawns in each replicate, haemolymph was collected as described above without anticoagulant, allowed to clot at room temperature for 30 min and left at 4°C for 1 hr. The tubes were then centrifuged at 4524 g at 4°C for 30 min to collect the supernatant serum. The serum samples were stored at −30°C till further analysis.

The haemagglutinin assay was carried out in U-bottom microwell plates using 1.5% sheep red blood cells (SRBC). Two-fold serial dilutions of serum (50 μl) samples were made in TBS. An equal volume of 1.5% (v/v) SRBC was added to each dilution of serum. The plates were incubated at 25°C

<table>
<thead>
<tr>
<th>Table 1—Composition of the basal diet of M. rosenbergii</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Ingredient</td>
<td>Percentage</td>
</tr>
<tr>
<td>Groundnut cake</td>
<td>50</td>
</tr>
<tr>
<td>Fish meal</td>
<td>25</td>
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<tr>
<td>Rice bran</td>
<td>13</td>
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<tr>
<td>Soybean meal</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin and mineral mixture*</td>
<td>3</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>3</td>
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<tr>
<td>Carboxymethyl cellulose</td>
<td>1</td>
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*Each 1000 g vitamin and mineral mixture provides vitamin A-2,000,000 i.u., Vitamin B1-100,000 i.u., vitamin B2-0.8 g, vitamin E-100 mg, vitamin C-0.3 mg, vitamin K-0.4 g, calcium pantothenate-1.00 g, nicotinamide-0.4 g, vitamin B12-2.4 mg, choline chloride-60 g, calcium-300 g, manganese-11 g, iodine-0.4 g, iron-3.00 g, zinc-6.00 g, copper-0.8 g and cobalt-0.18 g.
for 45 min. Haemagglutinin titre was recorded as the reciprocal of the last dilution, resulting in agglutination after 45 min incubation. Negative controls comprised mixed equal volumes of RBC and TBS.

Lysozyme levels in serum were measured using the turbidimetric assay with slight modification. Briefly, a solution of 20 mg of Micrococcus lysodeikticus suspension in 100 ml acetate buffer (0.02 M, pH 5.5) was used. To 125 μl of serum, 125 μl of acetate buffer was added and then 1.25 ml of substrate suspension added to each tube. The reaction mixture was incubated for 1 hr at 25 °C. Difference between the initial and final turbidity (ΔOD) was taken as the measure of enzyme activity. The difference of 0.001 in OD observed at one hour is taken as one unit of enzyme activity. The protein content of serum was measured following Bradford method, using bovine serum albumin as a standard protein. The means±SE for each parameter was calculated for each group. Data were analysed using two-way analysis of variance. Means were compared using Duncan's multiple range test. Difference was considered significant when P<0.05.

Results and Discussion

The ImmunoPlus-fed prawns showed a significant (P<0.05) rise in PO activity than the control group throughout the experimental period and enhanced activity was observed till 3 weeks of ImmunoPlus feeding which declined after 4 weeks of feeding (Fig. 1a). A fluctuation in PO activity was noticed in control group prawns throughout the four weeks of experiment. A similar trend of rise in haemagglutinin levels in immunostimulant-fed prawns in comparison to control group was observed for 3 weeks of feeding which became comparable with the control value after 4 weeks (Fig. 1b). Lysozyme activity differed considerably amongst individual animals and a fluctuation in lysozyme level in control prawns was noticed during different periods. The ImmunoPlus-fed prawns showed significantly (P<0.05) enhanced serum lysozyme level till 3 weeks of feeding (Fig. 1c). A rise in serum total protein concentration was marked in ImmunoPlus-fed prawns until 2 weeks of feeding compared to control which subsequently declined and became on par with level of control group (Fig. 1d).

In any culture system special care must be taken to control opportunistic pathogens such as vibrios, chitinoclastic bacteria, filamentous bacteria, fungi and protozonas. Although the immunostimulants do not eradicate all pathogens, they can increase non-specific disease resistance, leading to reduction in mortality caused by opportunistic pathogens or stressors. The present results showed that oral administration of one of the commercial herbal immunomodulators (ImmunoPlus) could enhance non-specific immunity of...
the adult prawns. Some of the plant extracts/products have been evaluated for their immunostimulatory and antioxidant properties in fishes.\textsuperscript{20,21} ImmuPlus is one of the above herbal products which has also been evaluated for its efficacy to stimulate the non-specific immunity in vertebrates.\textsuperscript{22} The herbs included in ‘ImmuPlus’ have been studied to stimulate immune system of human beings and animals through enhanced phagocytosis, production of reactive oxygen and nitrogen species.\textsuperscript{31} The herbal product contains Amlaki (Emblica officinalis), as one of its ingredients, is rich in vitamin C which is a potent immunostimulant and antioxidant.\textsuperscript{22-25} The ascorbic acid present in this fruit is conjugated to gallic acid and reducing sugars, forming a tannoid complex, which is more stable in nature and enhances the bioavailability of ascorbic acid.\textsuperscript{31} Similarly, another constituent, Guduchi (Tinospora cordifolia), is well known to augment phagocytic cell functions and enhance protection against infections in animals and human beings.\textsuperscript{26} The other constituents, Aswagandha (Withania somnifera) and Tulsi (Ocimum sanctum) are also well known for their immunomodulatory roles.\textsuperscript{11,27} Thus, it is expected that similar enhancing effects may be reflected on the immune parameters in treated prawns.

In the present study, elevated PO activity, haemagglutinin, lysozyme and total protein levels were recorded in ImmuPlus-fed M. rosenbergii. The preliminary assay of lysozyme activity in prawns as an index of immunostimulation was made and the activity was well correlated with the other immune indices viz., PO activity and haemagglutinin titre. The enhanced immunity indices were noticed to be there up to three weeks of feeding, which declined after 4 weeks of feeding. That is why it is essential to know the optimum dose and period of feeding of each of the substances in any species.

Much more to be studied to understand the mechanisms behind these immunomodulatory effects as well as to employ these herbal substances as possible therapeutants. This concept of using herbal products for health, also gains a little more credibility, when we realize that herbal antioxidants concurrently exhibit significant immunomodulatory activities.

Acknowledgement
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