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Enhanced immune response and resistance to white tail disease in chitin-diet fed freshwater prawn, *Macrobrachium rosenbergii*

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ABSTRACT

Chitin is one of the natural biopolymer found abundantly in the shells of crustaceans, insects and in cell walls of fungi. In this study, we determined the effect of dietary administration of 0.5, 0.75 and 1% chitin on the immune response and disease resistance in freshwater prawn, challenged against *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV). We observed a significantly enhanced immune response, indicated as higher prophenoloxidase activity and respiratory burst of hemocytes, in 0.75% chitin-diet fed prawns compared to the chitin-free-diet fed prawns. Importantly, the relative percent survival (RPS) following challenge with white muscle disease (WTD) viruses was found relatively high in *M. rosenbergii* fed with diet containing 0.75% chitin (63.16%), suggesting an increased resistance to disease susceptibility. These results indicate that the incorporation of chitin in prawn diet would be beneficial in stimulating the immune response and thereby developing resistance against diseases.

1. Introduction

Among the crustaceans, *Macrobrachium rosenbergii* has found its importance in terms of its economic value and ease of captive farming. In India, annual production of *M. rosenbergii* has been increased over the years from 7140 t in 1999–2000 to 42820 t in 2005–06, later declined to 6568 t in 2009–2010 (FAO, 2011). The sharp decline in the production of *M. rosenbergii* was mainly attributed to the emergence of a new disease namely, white tail disease (WTD) or white muscle disease (WMD) (Sahul Hameed et al., 2004a,b). The sudden outbreak of diseases was ascribed to the rapid expansion of prawn farming, intensification of culture, and frequent translocation of seed and brood stock (New and Nair, 2012). Hence forth, this disease has become a serious concern to giant freshwater prawn farming in India. The WTD is mainly diagnosed by the opaqueness of the abdominal muscle, degeneration of telson and uropods, and 100% mortality within a span of 4 days (Sahul Hameed et al., 2004a,b; Vijayan et al., 2005). The first case of WTD was reported in Island of Guadeloupe in 1995, then in Martinique (French West Indies) (Arcier et al., 1999), Taiwan (Tung et al., 1999), China (Qian et al., 2003) and India (Sahul Hameed et al., 2004a,b). The etiology of WTD in *M. rosenbergii* was initially reported as virus, later it was identified that *M. rosenbergii* nodavirus (MrNV) and extra small virus (XSV) (Qian et al., 2003; Sahul Hameed et al., 2004a,b; Bonami et al., 2005) were the main causative agents of this disease.

The application of antibiotics in general to control diseases is being commonly practiced in crustacean farming. However, the overuse of antibiotics has made pathogens to develop resistance against them and also the food safety measures adopted for the human consumption have reduced or restricted their use in culture practices. In addition, antiviral agents are hardly available to combat various aquatic viral diseases. Alternatively, immunostimulants and probiotics are generally being suggested to use in feed to control or prevent diseases in farming. Among these, immunostimulant supplementation in diet had shown to develop resistance in fish and shrimp against diseases (Anderson, 1992; Song and Huang, 1999). Several natural compounds, such as glucan, chitosan, and other polysaccharides along with some herbs, have been used as immunostimulants and studied their role in stimulating immune response in various crustaceans, but are poorly studied in freshwater prawns. In addition, our previous studies show that the dietary administration of nucleotide and brewer's yeast enhances the immune response and disease resistance in *M. rosenbergii* (Shankar et al., 2012; Parmar et al., 2012).

In some studies, chitin has been used as an immunostimulant and was known to improve the disease resistance in *Litopenaeus*

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vannamei against *Vibrio alginolyticus* (Wang and Chen, 2005). Chitin is made up of polysaccharides, linear β-1,4-linked polymer of *N*-acetyl-D-glucosamine, one of the most abundant polysaccharide in nature and a general component of insects, exoskeleton of crustacean shells and fungal cell walls. It is commercially manufactured from the shrimp and crab shells. Among the aquatic organisms, most of the research on protective effect of chitin has been reported in fishes against bacterial and viral diseases (Sakai et al., 1992; Kawakami et al., 1998; Gopalakannan and Arul, 2006; Vahedi and Ghodratizadeh, 2011). Additionally, we reported recently that chitin supplementation in the diet had stimulating effect on the growth and survival of *M. rosenbergii* (Naveen Kumar et al., 2013). Further, to determine the effect of chitin on immunomodulation of freshwater prawn, in this study we fed 0.5, 0.75, and 1% chitin incorporated diet to *M. rosenbergii* for 90 days and challenged against MrNV and XSV viruses. We found significantly increased prophenoloxidase (proPO) activity, respiratory burst (RB) of hemocytes, and relative percent survival (RPS) in 0.75% chitin-supplemented diet fed groups. Our data suggest that chitin incorporation in the diet could enhance the immune response and disease resistance in *M. rosenbergii*.

2. Material and methods

2.1. Prawns

The larvae of *M. rosenbergii* produced in the Freshwater Prawn Hatchery at the College of Fisheries, Mangalore, India (Murthy, 1998), were reared to postlarvae (PL) stage and then used in the study. They were acclimatized to closed recirculatory system consisting of fiberglass tanks (66 cm dia., 120 l capacity) and fed with dry pellet feed for a period of two weeks.

2.2. Diet preparation

Four diets containing different levels of chitin at 0% (free), 0.5%, 0.75% and 1% were formulated using square method with marinating protein level at 35%, and prepared according to a diet described for *M. rosenbergii* by Naveen Kumar et al., 2013. The chitin extracted from the shrimp shell was obtained from Central Institute of Fisheries Technology, Cochin, India. Briefly, required quantities of feed ingredients such as fish meal, ground nut oil cake, rice bran, and tapioca flour were mixed together with water and hand kneaded until a smooth dough resulted. It was cooked under steam in a pressure cooker for 20–30 min. Each diet was then cooked and cooled at room temperature (RT) in an enamel tray and required levels of chitin and vitamin–mineral premix were added, mixed and blended to get a homogeneous mixture. Next, this mixture was passed through an extruder with a die, and the resulting pellets (2 mm dia.) were dried in a hot air oven at 60 °C till the moisture levels were reduced to below 10%. After proper drying, the pellets were then packed in an air-tight high density polyethylene bags, labeled and stored at 4 °C until use.

2.3. Design of experiments

After the acclimation period, 12 (four treatment groups with three replicates) fiberglass tanks were stocked each with 30 uniform-sized PL with an average initial weight of 0.20 ± 0.05 g. Tanks received continuous aeration, and water was replaced once in two days to maintain the water quality throughout the treatment period of 90 days. During the experimental period, the water pH ranged from 6.46 to 7.8, temperature from 26 to 28 °C, dissolved oxygen from 6.1 to 8.5 mg/l, free carbon dioxide from 0.15 to 3.73 mg/l, total alkalinity from 34.73 to 78.23 mg/l of $CaCO_3$, and ammonia-nitrogen from 0.01 to 4.93 μg at N/l. Prawns were fed their respective diets at a rate of 10% body weight twice daily for the first month and later reduced to 5% body weight. Prawns were weighed biweekly, and the daily ration was adjusted accordingly. The uneaten food and faeces were siphoned out daily morning before feeding.

2.4. Measurement of immune parameters

At the end of the feeding trial, ten prawns from each treatment were randomly sampled for hemolymph collection. Hemolymph (150 μl) was withdrawn from the ventral sinus of each prawn with a 1 ml sterile insulin syringe (26 gauge) and placed in a pyrogen-free microfuge tubes containing 150 μl anti-coagulant solution (0.03 M trisodium citrate, 0.34 M sodium chloride, and 0.01 M EDTA, pH 7.5) This diluted hemolymph (hemolymph-anticoagulant mix) was subsequently used to determine the proPO activity and RB of hemocytes. The presence of proPO in hemolymph was analyzed in triplicates according to previously described protocol (Hose et al., 1987). Briefly, a drop of diluted hemolymph (50 μl) was placed on a sterile glass slide to make a thin layer of hemolymph smear and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 h at 4 °C. This hemolymph-smear was washed thrice, each for 15 min, in a phosphate buffer, incubated with 0.1% L-DOPA in phosphate buffer for 16–18 h at RT. The black-stained granular cells were examined and counted under a light microscope and calculated the percentage of proPO positive hemocytes.

The RB of hemocytes was analyzed using nitroblue tetrazolium (NBT) reduction assay kit (Sigma–Aldrich, USA) according to the manufacturer descriptions. Briefly, a drop of hemolymph (50 μl) was placed on a sterile glass slide, smeared and incubated for 30 min at RT on a damp paper. After gentle washing in phosphate buffered saline (PBS), pH 7.5, 100 μl of 0.2% NBT in PBS was placed on slides and dried for 30 min. Then stained with Wright's stain for 30 s and washed once with distilled water. Air dried the slides and observed through oil immersion lens under light microscope. The formazan-containing granular cells that are mostly in spherical shape, were considered as NBT-positive cells (activated) were counted and the percentage of positive cells determined.

2.5. Viral inocula and challenge study

WTD viral inocula were prepared according to the previously established protocol (Sudhakaran et al., 2007). Briefly, naturally WTD infected PL of *M. rosenbergii* were collected from the prawn hatcheries around Nellore, AP, India. The PL were washed once in sterile water, placed in a 1.5 ml sterile microfuge tubes, transported on dry ice to the laboratory and stored at −40 °C until use. Five frozen PL (weighing each approximately 0.2 g) were homogenized in 1 ml TN buffer (20 mMTris–HCl, 0.4 M NaCl, pH 7.4) using a sterile microfuge tube homogenizer. The homogenate was centrifuged at 4000 *g*, 4 °C for 20 min, supernatant was transferred to a fresh tube and further centrifuged at 10000 *g*, 4 °C for 20 min. Again, the supernatant was collected, membrane filtered (0.22 μm) and stored in aliquots at −80 °C until use.

After 90 days of feeding trial, the challenge experiment was conducted in triplicates by intramuscular (between second and third abdominal segment) injection of 50 μl WTD inoculum to ten randomly selected prawns from each treatment. The TN buffer (50 μl) injected prawns were served as unchallenged control. These prawns were reared under similar conditions that were followed during the feeding trial. The challenged prawns were checked twice daily to observe the development of WTD clinical signs or any mortalities for a period of 15 days. The mortality of prawns were recorded daily and calculated the relative percent survival (RPS) at the end of the challenge study. The cause for mortality of challenged prawns was confirmed by nested reverse transcrip-