

# Prophenoloxidase and Immune Indices of Indian White Shrimp *Fenneropenaeus Indicus*

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## Abstract

Economical importance of shrimp in aquaculture and to the increase of diseases that are threatening shrimp cultivation, leads to the necessity of studies on immune system of shrimp and are receiving increased attention. The Prophenoloxidase (ProPO) system is the origin of melanin production and considered to be an innate defence mechanism in invertebrates. In crustaceans, prophenoloxidase (ProPO) is present in the haemolymph as an inactive form of phenoloxidase. The present study focuses on the phenoloxidase activity and other innate immune responses such as agglutination activity, phagocytic activity and encapsulation, were studied from the haemocytes of Indian white shrimp *Fenneropenaeus indicus*. The PO activity of *F. indicus* showed highest titre value ( $0.022 \pm 0.001$ ) in plasma, with increased concentration of laminarin. *F. indicus* haemocytes showed the strongest agglutination titre against human erythrocytes A ( $45 \pm 5.5$ ) and yeast *Saccharomyces cereviceace*. The results of phagocytic activity showed ingestion of yeast *S. cereviceace* by haemocytes of *F. indicus* and the encapsulation showed highest response against the sepharose 6CLB beads than other DEAE and CM sepharose beads. The present study supplements the knowledge on crustacean immunity, and understands the host pathogen interactions. An advance made through immunological research is an important criteria to increase the yield, food production and also helps for the development of aqua farming.

**Keywords:** Agglutination; Encapsulation; *F. indicus*; Prophenoloxidase

## Introduction

*Fenneropenaeus indicus* is one of the commercially important species in aquaculture along the Indian Ocean especially in east coast of India. Though, farming of the species faces lot of problems due to the occurrence of opportunistic microbial pathogens including bacteria and viruses [1-5]. Nowadays understanding the immune ability of shrimp and their defence mechanisms have become a primary concern in shrimp culture for better production. Invertebrate immune mechanism has the ability to recognize the invading organisms as foreign substance [6-8], these substances were recognized by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan and glucan, present on the surface of microorganisms, which trigger the cellular and humoral responses in crustaceans [9]. Cellular mediated responses include encapsulation, phagocytosis and nodule formation [10], whereas the humoral responses include the clotting cascade pathway, antimicrobial peptides synthesis, proPO cascade [10-12].

Crustaceans haemocytes comprises of important immune recognition components such as PO, lipopolysaccharide  $\beta$ -1,3 glucan binding protein (LGBP) and agglutinins etc. Prophenoloxidase proenzyme activity has been demonstrated in plasma haemocytes or in both [6,13-16]. Activation of ProPO system is triggered by LGBP through a complex cascade of native serine proteases and other factors [16,17]. Number of haemocytes mediated immune responses were studied, among that proPO system, when triggered, generates immunologically active substances such as opsonins, cytotoxic molecules [16,18]. Activation of haemocytes in arthropods is enhanced by microbial cell wall components, which also increases the phagocytic activity of haemocytes against bacteria or erythrocytes [19,20]. However, ProPo activating immune system has been well studied against foreign particles entry, there is no information at the cellular level regarding the *F. indicus* immune responses. The defence mechanism of *F. indicus* is poorly understood, but knowledge of this is an indispensable key for the development of current strategies to control infections in the Indian

white shrimp culture. Hence, the present study reports the functional studies of Prophenoloxidase and the immune indices in Indian white shrimp *Fenneropenaeus indicus*.

## Materials and Methods

### Animals

Indian white shrimp *Fenneropenaeus indicus* used in the study were obtained from a commercial farm in Tamil nadu, India. They were acclimated at  $26 \pm 1^\circ\text{C}$  and a salinity of 20 ppt in tanks with recirculating water in the laboratory. The body weight of the intermoult shrimp ranged from 11.1 to 14.0 g with an average of  $12.5 \pm 1.5$  g (mean  $\pm$  S.D.). During the acclimation period, the shrimp were fed a formulated shrimp diet (Shinta Feed Company, Pingtung, Taiwan) twice daily.

### Total haemocyte count

One hundred microlitres of whole haemolymph were mixed with 100 ml of marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5). This haemocyte suspension was then fixed with 100 ml paraformaldehyde (4% w/v). The number of haemocytes per millilitre was estimated using an Improved Neubauer haemocytometer. The numbers and relative proportions of haemocyte types were calculated using a minimum number of 250 cells/slide.

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### Assay of phenoloxidase (PO) enhancing activity

In PO enhancing activity, haemocyte lysate was serially diluted two-fold with TBS/Ca<sup>2+</sup> (pH 7.4) buffer in flat-bottomed microtitre plates and incubated with 25 µl of laminarin (1 mg/ml in TBS-I, pH 7.4) for 1 h at room temperature [21]. 25 µl of L-β-3, 4-dihydroxyphenylalanine (L-DOPA, 3 mg/ml in distilled water) was added and again incubated for 1 h at room temperature. Controls were made with Tris buffer saline buffer instead of haemocyte lysate, replacing them with TBS/Ca<sup>2+</sup> pH 7.4 buffers. The absorbance at 490 nm was measured at 30 min intervals using a Biophotometer Plus (Eppendorf, Germany) and expressed as units<sup>-1</sup> min<sup>-1</sup> mg protein<sup>-1</sup>.

### Agglutination activity

**Haemo agglutination assay (HA) with erythrocytes (RBC):** Blood samples of Human (A, B and O group) originate from healthy male volunteers (age 25-45 years), mouse and rat were obtained by venous or cardiac puncture and collected in sterile Alsever's solution [22]. Trypsin treatment and subsequent fixation of human A RBC with formalin were performed as described earlier [23]. Prior to HA assays, human RBC were washed three times with 0.9% saline, once with TBS-II (25 mM Tris HCL, 100 mM NaCl, pH 7.5), and finally resuspended in the same buffer as 1.5% (v/v) suspensions. HA assays were performed in V-bottom microtiter plates (Tarsons, Kolkata) by serial dilution of a 25 µl suspension with an equal volume of TBS II. RBC suspension (25 µl) was added to each well, mixed, and incubated for 45 min at 26°C. The HA titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC. Control for all assays consisted of the substitution of the sample by TBS-II. Each experiment was performed in duplicate with at least for test samples and based on the median HA titer values. Necessity of the divalent cations for HA activity and EDTA sensitivity of haemolymph was described [23].

**Yeast agglutination assay:** In agglutination activity, baker's yeast was used as an indicator. Briefly, 25 µl of plasma serially diluted two-fold in TBS-I buffer (50 mM Tris HCL, 100 mM NaCl, 100 mM CaCl<sub>2</sub>, pH 7.5) in the wells of V-bottomed microtitre plates, then an equal volume of yeast solution (1% made up in TBS-I, pH 7.4) was added and incubated at room temperature for 1 to 2 h. Titres were documented as the reciprocal of the highest dilution showing agglutination judged microscopically. Control consisted of TBS I buffer, instead of plasma.

**Agglutination inhibition assays:** The substances to be tested for inhibition were dissolved in TBS-III (10 mM Tris HCL, 70 mM NaCl, 100 mM CaCl<sub>2</sub>, pH 7.5) (carbohydrates, 0.2 M-0.8 M) or in TBS-IV (10 mM Tris HCL, 100 mM KCl, 100 mM CaCl<sub>2</sub>, pH-8.2) (glycoproteins 30 mg/ml). If necessary, the pH was adjusted to 7.8 using concentrated NaOH solution. Plasma to be tested (25 µl) was serially diluted with an equal volume of the effluent sample in microtiter plates and incubated for 30 min at room temperature. Mouse RBC suspension (25 µl) was added to each well and incubated for 30 min at room temperature. The minimal concentration of the test substance was completely inhibited and its HA activity also recorded. Each assay was repeated for 5 times. Agglutinating inhibition activity was tested with the use of 21 carbohydrates (11 monosaccharides, 5 oligosaccharides and 5 polysaccharides).

### Phagocytosis

*In vitro* phagocytosis assays were performed by engulfing foreign substances with haemocytes in suspension following Arumugam et al. [24]. Briefly, 25 µl of haemocyte suspensions were incubated with serially diluted two-fold in TBS-I<sup>+</sup> pH 7.4 buffer or PBS pH 7.8 in flat-

bottomed microtitre plates (Nunc Roskilde, Denmark), and 25 µl of buffer or serum treated heat inactivated yeast *S. cereviceace* was added, mixed, and incubated for 30 min at 25°C. This suspension was smeared on the glass slide, after drying, fix the smear with ethanol. Phagocytised yeast was viewed at 40X magnification and counted under microscope (Nikon, Japan). Alternatively formalin killed yeast *S. cereviceace* also used for the phagocytosis assay, after 24 hr incubation the pellets were collected and washed with potassium or sodium diphosphate buffer and the suspension was used for the phagocytic assay.

### Encapsulation and cell adhesion

In encapsulation response 25 µl of plasma samples from *F. indicus* were serially diluted two-fold in TBS/Ca<sup>2+</sup> pH 7.4 buffer in flat-bottomed micro titre plates. To each well, 25 µl of sepharose beads (6 mg/ml in TBS-I<sup>+</sup> pH 7.4) and 25 µl of haemocyte lysate supernatant were added, mixed, and incubated for 45 min<sup>-1</sup> h at 25°C. Finally, 25 µl of this mixture was taken for the microscopic studies. Positive control was made without sample plasma or HLS (Haemolystae), sepharose beads (Sephacrose CL6B, CM Sepharose and Sodium alginate beads). Encapsulated beads with haemocytes were seen under the microscope (Nikon, Japan) and calculated.

### Statistical analysis

Each experiment was performed 3 to 10 times using samples from different preparations. Differences between control and experimental values were statistically analysed for significance by the paired-sample Student *t*-test.

## Results

### Total haemocyte count

Total haemocyte counts were varied remarkably among individuals, ranging from ~5.8 to 12×10<sup>5</sup> cells/mm<sup>3</sup> of haemolymph (Figure 1). The resulting mean indicates the circulating haemocytes number around 6.0 (×10<sup>6</sup>) cells/ml of haemolymph (n=6) *F. indicus*.

### ProPO activity

PO activity of plasma, haemocyte lysate and haemolymph of *F. indicus* were determined. The results indicate that the plasma showed highest PO activity. In addition, increase in the concentration of laminarin like PAMPs led to a parallel increase in PO activity in *F. indicus* haemocytes (Figure 2).

### Agglutination and agglutination inhibition assays

Plasma, supernatant and pellet samples of *F. indicus* was found

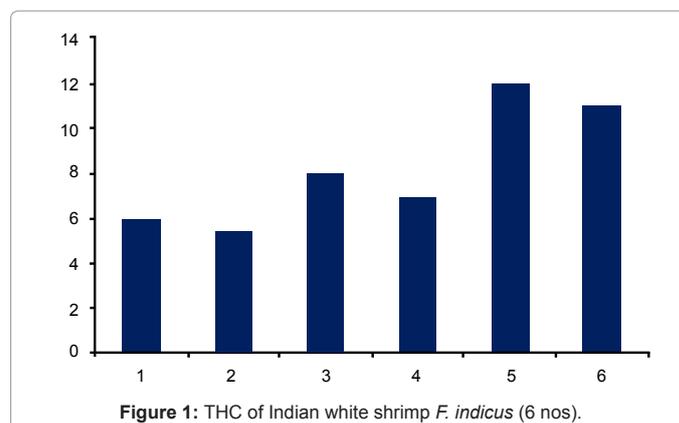
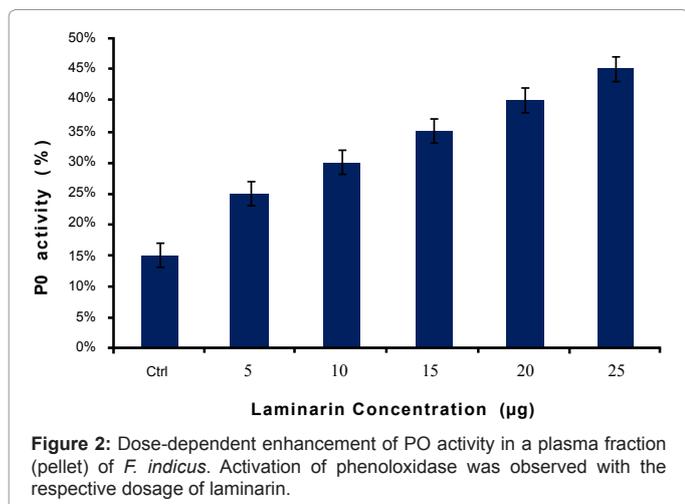


Figure 1: THC of Indian white shrimp *F. indicus* (6 nos).

S.No	RBC types and Yeast	Supernatant	Plasma	Pellet
1	Human A	45	38	15
2	Human B	22	32	14
3	Human O	15	25	10
4	Human AB	23	20	10
5	Mice	15	15	12
6	Rat	15	14	16
7	Yeast (Native)	28	20	15

*F. indicus* plasma was agglutinated with different types of RBCs like human, mouse, rat, rabbit and chicken erythrocytes.

**Table 1:** Agglutinating activity profile of various plasma fractions of *F.indicus* against mammalian RBC types and yeast cells.



**Figure 2:** Dose-dependent enhancement of PO activity in a plasma fraction (pellet) of *F. indicus*. Activation of phenoloxidase was observed with the respective dosage of laminarin.

to agglutinate in all types of the human, mouse and rat erythrocytes. Results indicate that strongest agglutination titre was found in human erythrocytes (Figure 3A, Table 1) and towards yeast *S. cereviceae* (Figure 3B). The binding specificity of plasma was examined by HA inhibition assays using several carbohydrates and glycoproteins. Among the twenty one carbohydrates tested, only ten showed the agglutination inhibition with the plasma of *F. indicus* (Table 2). Monosaccharides and disaccharides had highest binding specificity in inhibition comparing with polysaccharides. Among the monosaccharides, primary sugars showed highest value (100 mM) in agglutination inhibition, and N-acetyl derivatives, showed lower values (32 mM). Tests with amino sugars possess the agglutination inhibition at the low level 50 mM (Table 3) when compared to monosaccharides.

### In vitro phagocytic assays

Haemocyte monolayers (HML) comprising fully haemocytes were rinsed and spread over the surface with iso-osmotic TBS-V ( $Ca^{2+}$ , pH 7.8), all haemocytes tend to get a round configuration within 5 min. The rounded haemocytes were incubated for 10 min with yeast cells suspended in buffer and showed the attachment of few yeast cells to the surface of the haemocyte, free yeast cells were found in monolayer (Figure 4A) (Table 4). Haemocyte monolayer conditions, free and haemocyte attached yeast cells were retractile, surrounded by bright rings and were clearly visible under phase optics. It suggests that *F. indicus* haemocytes involved in the phagocytic activity with yeast cells. Moreover, the haemocytes had the clear cut protecting activity against the foreign particle enter in the host. Upon extension of the incubation time for up to 10 min with yeast cells, it was vividly notable that several haemocytes contained up to 5 yeast cells intracellularly,

CHO tested	Maximum concentration(mM)	Minimum inhibitory concentration(mM)
<b>Monosaccharides</b>		
D-Glucose	200	100
D-Galactose	200	100
D-Mannose	200	100
D-Fructose	200	100
L-Fructose	200	100
De-Oxy sugars		100
2-Deoxy D-Glucose	200	100
2-Deoxy D-Galactose	200	100
<b>Acidic Sugars</b>		
D-Glucouronic acid	200	25
Glucouronic acid	200	28
<b>N-Acetyl sugars</b>		
GluNAC	200	32
GalNAC	200	32
<b>ManNAC</b>		
Amino sugars		
Gal N	200	50
Glu N	200	50
Man N	200	50
<b>Glycosides</b>		
Methyl-β-D		
Glucopyranoside	200	25
p-Nitrophenyl-β-DGlucopyranoside	200	25
<b>Disaccharides</b>		
D-Maltose	200	100
Sucrose	200	100
PAlatinase	200	50
D-Lactose	200	50
D-Melibiose	200	50

The assay was repeated five times for each carbohydrate with identical results

**Table 2:** Inhibition of agglutinating activity (titre= 4) of plasma from the *F. indicus* by various carbohydrates.

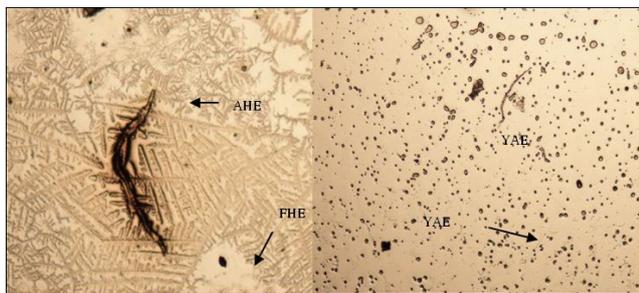
thereby demonstrating phagocytic activity of *F. indicus* haemocytes *in vitro* (Figure 4B). While testing with *F. indicus* haemocytes, the intracellular (Ingested) yeast cells appeared darker and markedly lost their surrounding bright rings as compared with free or extracellularly attached yeast cells. Thus, the apparent differences enabled easy and unambiguous determination of the ingestion (like melanisation) of yeast cells (Figure 4C).

### Encapsulation and cell adhesion

*F. indicus* haemocytes having the capacity to encapsulate chromatography beads with different surface characteristics were tested *in vitro*. Among the different bead tested the haemocytes showed encapsulation response only against sepharose CL6B beads (Figure 5) in test.

### Discussion

In arthropods, phenoloxidase has usually been described as inactive within haemocytes, only being activated with serine proteases, LGBP and other PGRPs once released into the haemolymph Soderhall [25]. In the current study, results indicate that phenoloxidase in *F. indicus* can be activated by laminarin like Pattern Associated Molecular Patterns (PAMP) *in vitro*. In this study normal level of haemocyte count in the



**Figure 3:** Phase contrast and differential interference contrast (DIC) photomicrographs of live hemocytes of *F. indicus* (A, B). Phase contrast images of hemocytes showing agglutination with HB RBC. Note that the Agglutinated erythrocytes & haemocytes (AHE) appeared darker and markedly lost their surrounding bright rings as compared to free (FHE) or attached (YAE) erythrocytes. DIC images of the same area.

Polysaccharides	Max. Concentration (mg/ml)	Minimum Inhibitory Concentration (mg/ml)
Laminarin	1	0.5
Mannan	1	0.58
Dextran	1	0.83
Inulin	2	0.6
Colimonic Acid	5	0.6

The assay was repeated three times for each polysaccharide with identical results using samples from different preparations

**Table 3:** Inhibition of agglutinating activity (titre= 4) of plasma from the *F. indicus* by various polysaccharides.

S.no	RBC types and Yeast	Ctrl	Test
1	Haemolymph	0.022 ± 0.001	0.035 ± 0.002
2	Plasma	0.018 ± 0.002	0.019 ± 0.002
3	Pellet	0.008 ± 0.002	0.038 ± 0.001

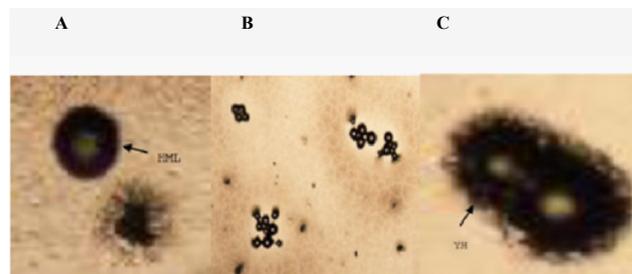
Data represent median values from 10 replicates for each RBC type and yeast cells.

Plasma obtained from the clarified plasma previously incubated with laminarin. Pellet (dissolved and dialysed against TBS-I) was collected after centrifugation (25,000 x g, 15 min, 4°C) of supernatant previously incubated with laminarin.

**Table 4:** Phagocytic activity in different plasma fractions of *F. indicus*.

Indian white shrimp *F. indicus* was found to be  $1.04$  to  $12.21 \times 10^5$  cells/mm<sup>3</sup>. Previous reports showed the high disparity in total haemocyte count (THC) of different crustacean species. For example, in *Penaeus californiensis* THC level was about  $11 \times 10^3$  cells/mm<sup>3</sup> [43], *Penaeus japonicus*  $5-14 \times 10^6$  cells/mm<sup>3</sup> [26], *Penaeus setiferus*  $8.9 \times 10^6$  cells/mm<sup>3</sup> [27]. These findings propose us the difference in the THC counts in crustaceans showing the variation in the cellular immune responses such as phagocytosis and encapsulation. Phenoloxidase activity in both cell-free haemolymph and haemocytes of *F. indicus* was observed. Cellular haemolymph fraction is of specific interest because it appears to be strongly associated with defence against the pathogen. In the present study plasma of *F. indicus* showed reactivity with different RBC types with highest reactivity against human RBC. Similar results were reported in mollusc [28-32]. Plasma from *F. indicus* was found to agglutinate all the RBCs (Human A, Human B, Mice, Human O, Human AB, Rat and Yeast) tested. The strongest agglutination titre was observed in *F. indicus* haemocytes against human RBCs, yeast *S. cereviceace* and it was viewed under light microscope. Similarly insects showed the agglutination activity with human RBCs (1998). It may be due to pattern recognition molecules occurrences in haemolymph.

In the present study, the differential profile of agglutinating activity of plasma, and agglutination inhibition were tested with 21 carbohydrates, only ten inhibited the agglutinating activity of *F. indicus* haemocytes. Monosaccharides and disaccharides had the highest binding specificity in inhibition. In contrast, some N-acetyl derivatives, namely, N-acetyl neuraminic acid (NeuAc), N-acetyl glucosamine (GlcNAc) and N-acetyl galactosamine (GalNAc) inhibited the HA activity of plasma even at very low concentrations (32 mM). Amino sugars and acidic sugars showed the agglutination inhibition at a low concentration level of 50 mM, while testing the six polysaccharides. Phagocytosis does not appear to be solely dependent on these factors, since a few studies with crustacean haemocytes morphotypes such as agranular or hyaline cells have shown that they are capable of responding to bacterial cells in the absence of detectable levels of PO or its precursor [32-34]. Phagocytic activity in *F. indicus* was tested using yeast, yeast cells appeared darker and markedly lost their surrounding bright rings as compared with free or extracellular attached yeast cells.



**Figure 4:** Rounded haemocytes were incubated with yeast cells and the attachment with few yeast cells to haemocyte surface, free yeast cells were found in monolayer (Haemocyte monolayer HML) (A). *F. indicus* haemocyte attached with 5 yeast cells intracellularly that were surrounded by bright rings and visible under phase optics (B). Ingested yeast cells emerge darker and lost their surrounding bright rings obviously as compared with free or extracellularly attached yeast cells (yeast haemocytes –YH) (C).



**Figure 5:** Encapsulation of chromatographic beads DEAE-sepharose was encapsulated with the *F. indicus* haemocytes that encapsulated beads were marked as (IHE) that was shown. Note: All free, attached and ingested erythrocytes are clearly discernible.

In the present study, encapsulation response of *F. indicus* haemocytes with three types of chromatography beads was tested, encapsulation response only noted against sepharose CL6B beads. The interaction of haemocytes with beads based on surface charges has been reported in insects [35-37], crustaceans [38] and Mollusca [39,40]. In conclusion, the present study reports the ProPo activating system, function and the immune indices of *F. indicus* leads to the understanding of the innate immunity. Further molecular biological studies are needed to confirm the intruding mechanism of Propo system.

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