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Effects Elecitors on Prophenonoloxidase and Superoxide Anion Activities of Freshwater Prawn, *Macrobrachium malcolmsonii*

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Abstract

The prophenoloxidase and superoxide anion activity of haemocyte lysate supernatant (HLS) from Indian River Prawn (*Macrobrachium malcolmsonii*) were examined by treating HLS with various factors such as an increase in temperature from 25 to 60˚C and elicitors such as ß-1,3 -1, 6-glucan, zymosan, levamisole, vitamin C, vitamin E and trypsin. The strongest PO activity was induced at 50˚C followed by 45˚C. The glucan, zymosan, vitamin C and vitamin E showed higher HLS prophenolase activity, whereas levamisole and trypsin showed lower HLS prophenolase activity relative to the control. In vitro elicitor zymosan showed the maximum percentage of average relative PO activity (272.0%) followed by glucan (215.0%). Levamisole showed maximum percentage elevation (33.4%) of superoxide anion, followed by glucan (25.98%), vitamin E (16.34%), vitamin C (14.87%), trypsin (9.56%) and zymosan (7.0%) respectively.

Introduction

Macrobrachium malcolmsonii is the second largest freshwater prawn of cultivable importance in India (FAO 1998). Diseases are the most common hindrance to the aquaculture species including freshwater prawns. As a consequence there may occur mass mortality and production losses. Several alternative measures for disease control have been applied successfully in aquaculture. One of these enhancing nonspecific immune responses in prawn is the application of elicitors. Elicitors are the substances consisting of a heterogeneous group that includes vitamins, minerals, animals, bacterial and fungal derived compounds and several synthetic compounds (Robertson et al. 1994). Elicitors enhance the activities of macrophages, phagocytes, lymphocytes and non-specific heterogeneous group cytotoxic cells, thus resulting in resistance and protection against various disease attacks. These heterogeneous groups of immunostimulatory compounds are often grouped based on function or origin (Anderson 1992). Phenoloxidase, which has been detected in a wide range of invertebrates (Smith and Soderhall 1991, Jackson et al. 1993) is activated by several microbial polysaccharides including ß-1,3 glucan from fungal cell walls (Unestam and Soderhall 1977; Soderhall and Unestam 1979) and peptidoglycans (Ashida et al. 1983) or lipopolysaccharides (Soderhall and Hall, 1984; Soderhall et al., 1990) from bacterial cell walls. Additional factors found to activate the proPO system include calcium, sodium dodecyl sulfate (SDS), trypsin and high temperature (Ashida et al. 1983; Ashida and Soderhall 1984; Duloray and Lackie 1985; Leonard et al. 1985; Sugumaran and Nellaiappam 1991). However, no report is available so far on the effect of the different elicitors viz; glucan, zymosan, levamisole, vitamin C, vitamin E, and trypsin on the phenoloxidase activity in *M. malcolmsonii*. Thus, the present study was carried out to ascertain the effect of elicitors on phenoloxidase and superoxide anion activity.

Materials and Methods

Collection of test animals

Fifty adults of M. malcolmsonii were collected from the freshwater prawn units of Central Institute of Freshwater Aquaculture, Bhubaneswar. The animals were brought to the laboratory of Aquatic Animal Health Division and acclimatized in 500 litre fiber reinforced tanks for a period of two weeks.

Preparation of elicitors

The elicitors used in the experiments were: (i) ß-1, 3-1, 6-glucan extracted from *Saccharomyces cerevisiae* (Sigma); (ii) levamisole (Sigma); (iii) zymosan, a ß-1, 3-glucan-protein-lipid compound extracted from the cell walls of *S. cerevisiae* (Sigma); (iv) vitamin C (Roche); (v) vitamin E (Roche); and (vi) trypsin (Hi Media, India). Prior to the experiments, all of these elicitors were prepared independently at a concentration of 1 mg per mililitre of distilled water, except vitamin E, ß-glucan and zymosan. ß-1, 3-1, 6-glucan and zymosan were treated with 70% alcohol and boiled for 30 minute and vitamin E was dissolved in coconut oil, respectively. Finally all solutions of these elicitors were sonicated and stored in a refrigerator.

Experimental Design

Haemolymphs collected from 50 individuals were equally divided into seven groups including a control group. From each group, haemolymphs were exposed to individual elicitors for respective superoxide anion, prophenol oxidase activity and temperature assay. For each immunological assay 30 observations were taken.

Collection of haemolymph

Haemolymph was collected from the heart (Cephalothorax region) of M. malcolmsonii aseptically by using 2 ml syringe (26 g x ½ in.) containing 0.2 ml of anticoagulant with osmolarity of 490 $mOs.kg^{-1}$ (0.114 M trisodium citrate, 0.1 M sodium chloride, pH 7.45). Approximately 1 ml of haemolymph was collected from each test individual and kept in refrigerator for preparation of HLS (haemocyte lysate supernatant).

Preparation of HLS

Haemocytes were collected by centrifuging these extracts at 300 g for 10 min at 4°C; after washing haemocytes with 0.01 M phosphate buffered saline (PBS, pH 7.0), these samples were again centrifuged at 300 g for an additional 10 min. Following the supernatant removal, the haemocyte pellets were resuspended in 0.01 M of PBS. The cell suspension was then homogenized with a sonicator equipped with a micro tip (out put 5, duty cycle 50%) and centrifuged at 1,000 g for 30 minute at 4°C. The resultant HLS, which was used as an enzyme source, was kept at –20°C before the assay for PO activity. The total haemolymph protein was determined following the method of Bradford (1976) using Bovine serum albumin (BSA) (Sung et al. 1998).

In vitro ***superoxide anion assay***

Superoxide anion production by phagocytic cells was determined by slight modification of the method described by Misoky and Smolowitz (1989). Each well of a 96-well microtitre plate was coated with 100 µl of Poly-L-Lysine solution (0.2 %, Sigma) to increase the number of adhesive haemocytes, then 100 µl of haemolymph was collected and added to each of four wells and then cyto-centrifuged at 300 x g for 10 min. After removing the supernatant, haemocytes were washed three times with MCHBSS (Modified Complete Hanks' balanced Salt Solution) then stained with NBT solution (0.03%, 100 µl) for 30 min, at 37ºC. The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After three washings with 70% methanol, the haemocytes were air-dried and coated with a solution of KOH (120 µl, 2 M) and DMSO (140 µl) to dissolve the cytoplasmic formazan; the optical densities of the dissolved cytoplasmic formazan at 630 nm (OD_{630}) were then measured with a precision microplate reader, (Emax).

Assay of PO activity

PO activity was assayed according to the procedures described by Sung et al. (1994) using 1-3, 4-dihydroxyphenyl-alanine (L-Dopa, Sigma) as a substrate. HLS (100 µl) was pre-incubated at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) for 15 minutes, after which 200 µl of L-Dopa (1.6 $mg.ml^{-1}$in Cacodylate Acid Citrate buffer) was added and reacted for 1 minute. Each reaction mixture was further diluted with 200 µl of CAC buffer, and then absorbance was measured at 490 nm. For these experiments, 1 unit of enzyme activity was defined as an increase in absorbance of $0.001.min^{-1}.mg^{-1}$ protein (Soderhall and Unestam 1979).

Effect of elicitors on PO activity

Samples containing 150 µl of HLS plus an equal volume of the elicitor (150 µl) were preincubated for 15 min at 37°C; while CAC buffer substituted for elicitor in control sample. Next, 300 µl of L-Dopa was added, the new mixtures were incubated for 1 min, and the absorbance was subsequently measured at 490 nm.

Data analysis

The variation of superoxide anion, prophenoloxidase and temperature effect on proPO was analysed by one way ANOVA by using MS Excel software and the degree of significance was calculated by Duncan's New Multirange Test (DMRT) using SAS Package version 5.0.

Results

In vitro ***effect of elicitors on superoxide anion assay***

The elicitor, levamisole produced a superoxide anion activity which ranged from 1.92 to 2.15 OD.min^{-1}, with an average of 2.09 OD.min^{-1}.

The control HLS without elicitors showed an average activity of 1.57 ± 0.16 OD.min^{-1}. Levamisole showed the highest superoxide anion activity followed by ß-glucan, vitamin E, trypsin and zymosan (Fig. 1). Levamisole showed maximum percentage of elevation (33.4%) superoxide anion followed by glucan (25.98%), vitamin E (16.34%), vitamin C (14.87%), trypsin (9.56%) and zymosan (7.0%).

The protein content varied from 5.5 to 5.7 mg.dl^{-1}.

Fig. 1

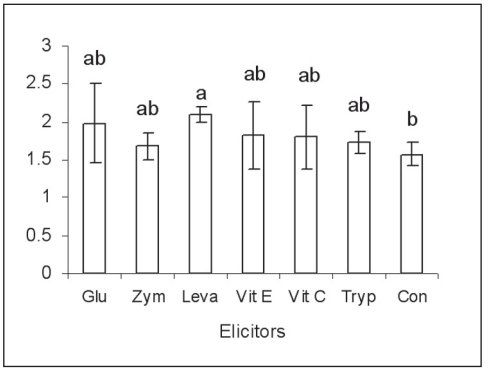

Fig. 1. Effect of elicitors on superoxide anion activity of freshwater prawn *M. malcolmsonii* (Glu- Glucan, Zym- Zymosan, Lev- Levamisole, Vit E- Vitamin E, Vit C- Vitamin C, Tryp- Trypsin, Con- Control) (Bars bearing common superscript are not significant in comparison to each other)

Effect of elicitors on Prophenoloxidase activity of M. malcolmsonii

It was noticed that glucan, zymosan, vitamin C and vitamin E showed higher HLS proPO activity as compared to control, where as levamisole and trypsin showed low HLS proPO activity as compared to control (Fig. 2). Zymosan showed maximum percentage of elevation (172.95%) in HLS proPO activity followed by glucan (115.49%), vitamin E (91.64%), vitamin C (77%), trypsin (-3%) and levamisole (-7.88%). The average relative PO activity (RA) showed varied results. The in vitro elicitor zymosan showed the maximum percentage of average relative PO activity (27.2%) followed by glucan

Fig. 2

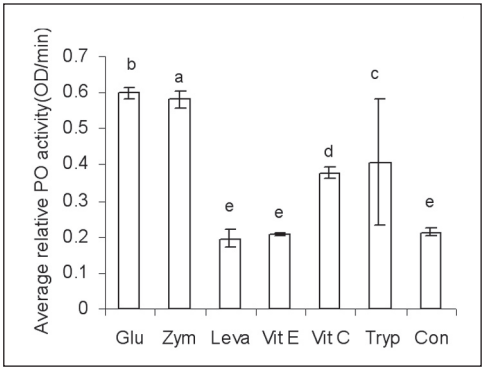

Fig. 2. Effect of elicitors on prophenoloxidase activity of freshwater prawn *M. malcolmsonii* (Glu- Glucan, Zym- Zymosan, Lev- Levamisole, Vit E- Vitamin E, Vit C- Vitamin C, Tryp- Trypsin, Con- Control)
(Bars bearing common superscript are not significant in comparison to each other)

(215%), trypsin (191%), vitamin C (177%), vitamin E (97%) and Levamisole (92%), respectively (Fig. 3).

Fig. 3

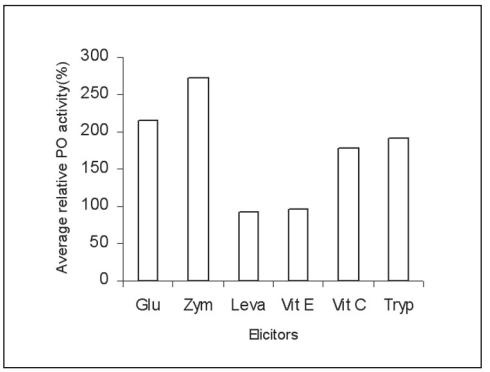

Fig. 3. Average relative activity of prophenoloxidase activity of HLS of freshwater prawn *M. malcolmsonii* after in vitro immunostimulation (Glu- Glucan, Zym- Zymosan, Lev- Levamisole, Vit E- Vitamin E, Vit C- Vitamin C, Tryp- Trypsin, Con- Control)

Fig. 4

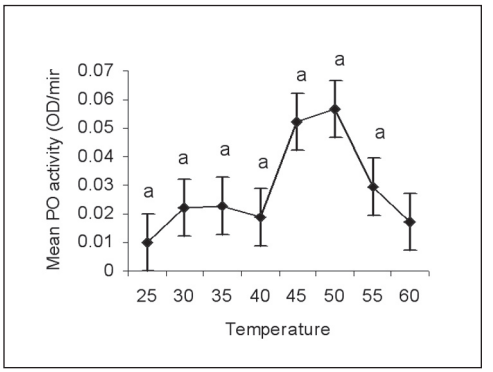

Fig. 4. Effect of temperature on prophenoloxidase activity of freshwater prawn, *M. malcolmsonii* (Mean bearing common superscript are not significant in comparison to each other)

Effect of temperature on HLS Prophenoloxidase activity

The HLS proPO activity was highest at temperature 50°C followed by 45°C. The HLS proPO activity gradually increased from 25°C to 50°C (except 40°C) and thereby reduced up to 60°C (Fig. 4).

Discussion

In the recent year there is an increase in trend for culturing the freshwater prawn in the monoculture and polyculture system. With intensification of culture practices, the disease outbreaks have become evident. Infectious diseases constitute the main barrier to the development and continuation of aquaculture. Crustacean haemocytes play an important role in the host defense responses including self or non-self recognition, cell to cell communication, superoxide anion activity, melanisation, phagocytosis, cytotoxicity and encapsulation. Phagocytosis is the most common of the cellular immune mechanisms and together with natural humoral effectors undoubtedly forms the first line of defense. Many oxygen compounds are toxic to living cell. The most reactive oxygen species can be said to be the superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. The reactive properties of oxygen compounds are not simply harmful, if directed at right target, they can also be used in

the destruction of foreign materials.

Our results demonstrated that HLS of the Indian freshwater prawn, *M. malcolmsonii*, produce superoxide anions when stimulated by various elicitors like ß-glucan, zymsan, levamisole, vitamin C, vitamin E and trypsin. Out of all elicitors, levamisole produce highest activity followed by glucan. The differential effect of the elicitors on the HLS may indicate a repertoire of surface receptors on the phagocyte membrane. It indicates that crustacean haemocytes have the ability to produce a respiratory burst when stimulated by exogenous materials. This phenomenon probably plays a part in the antimicrobial defense of the organism in a similar way to that reported for mammals (Babior et al. 1973). However, the ability of superoxide radicals to act in an antimicrobial manner in crustacean host defense still needs to be elucidated. Analysis of the respiratory burst is a potentially valuable way of investigating phagocytic activation in invertebrates. Evaluation of the magnitude and duration of the response should reveal much about the range of cell surface receptors and about the nature of signal transduction during non-specific recognition.

One of the hallmarks of the crustacean immune system is the prophenoloxidase enzyme cascade. Despite the primitive phylogenetic nature of crustacea, they possess the complex and effective mechanism like proPO system for eliminating pathogens. Recent in vitro researches have shown that the PO activating system and associated factors of proPO system are important activators in crustacean immunity. Prophenoloxidase (proPO) system acts as a major recognition and defense pathway in crustaceans (Jackson et al. 1993). Prophenoloxidase system is activated by several microbial polysaccharides including ß-1, 3-1, 6-glucan (Soderhall and Unestam 1979) and peptidoglycan (Ashida et al. 1983) or lipopolysaccharide (Soderhall et al. 1984) from bacterial cell wall. The enzyme is a part of complex system of proteinases, pattern recognition proteins and protein inhibitors constituting the so called proPO system. The activation of proPO system results in the production of various proteins including PO, which participating in melanisation around the parasite, coagulation, opsonisation of foreign materials and direct microbial killing (Soderhall and Hall 1984).

Both melanin and quinones have also been found to act as inhibitors of bacterial enzymes and to be fungistic (Rowley et al. 1990). It has been reported that phenoloxidase activity is detectable in tiger shrimp haemocytes and can be enhanced by either *in vitro* (Sung et al. 1994) or *in vivo* (Sung et al. 1996) treatment with ß-glucan, the same glucan treatment also acts to strengthen vibriosis resistance in shrimp (Sung et al. 1994). Therefore, proPO system plays an important role in prawn mechanisms, which are similar to these that occur in crayfish.

The present research studied the effect of various elicitors on proPO activation system in *M. malcolmsonii*. Results showed that PO activity is dependent on temperature and proPO system can be activated by prior treatment with zymosan,

glucan, vitamin E, vitamin C and trypsin. In addition, proPO was not purified for assay in this experiment, it cannot be excluded that DOPA used as a substrate as oxidized by peroxidases or other susbstances such as reactive oxygen metabolites, within HLS. According to previous studies done by many researchers we considered that DOPA oxidation was primarily mediated through the enzyme, phenoloxidase within HLS and the level of DOPA oxidation can be used to represent PO activity in this experiment.

If the activation of proPO system can be used as an indicator of host defense, our results suggest that culture of prawns and shrimps in winter waters will always have the low resistance to disease. Our result also shows that a broad range of elicitors examined for this study to enhance *in vitro* PO activity. Sung et al. (1994) reported that treatment with ß-1, 3-1, 6-glucan at 0.5 and 1 $mg.ml^{-1}$ is sufficient for strengthening tiger shrimp resistance to vibriosis *in vivo*. In our present study out of the six elicitors, four elicitors are found to be enhance the disease resistance by increasing the proPO activity system, the mechanism of this system in both prawn and the resolution of the same species are crucial to understand the functionality of the system by the action of these elicitors. It is therefore, necessary to correlate this humoral and cellular immune effectors systems by the effect of these elicitors so that in a long run, disease management strategies can be forecasted by incorporating these elicitors in the feed. Further, it is necessary to study the mechanism of the activations of this proPO system in this prawn.

It is concluded from the above experiment that superoxide anion assay and prophenoloxidase system are the two important immune effectors, which are the indicators to study for either immunostimulations or bacterial infections in crustaceans.

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