Phenoloxidase Activity of *Macrobrachium rosenbergii* after Challenge with Two Kinds of Pathogens: *Lactococcus garvieae* and *Aeromonas veronii*

Hung-Hung Sung*, Yen-Tung Huang and Li-Ting Hsiao

Department of Microbiology, Soochow University, Taipei, Taiwan 111, ROC

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**ABSTRACT**—Prawns *Macrobrachium rosenbergii* injected with Gram-positive *Lactococcus garvieae* and Gram-negative *Aeromonas veronii* were monitored for changes in phenoloxidase (PO) activity. Challenge-stimulated PO activity (Pos) was measured by adding trypsin inhibitor to hemocyte lysate to prevent proPO activation. Challenge with either *L. garvieae* at $5 \times 10^5$ cells/g of prawn or *A. veronii* at $2 \times 10^2$ cells/g of prawn (1/10 LD_{50}) gave a maximum increase in POs at 18 h post-injection, and the response was higher with *L. garvieae* than with *A. veronii*. On the other hand, when prawns were separately injected with two kinds of formaldehyde-inactivated bacteria at the same dose ($5 \times 10^5$ cells/g), the POS was similar for both. These results suggest that POs response was positively related to bacterial dosage but not to bacterial species. Furthermore, the total PO activity (POT) resulting from all intrahemocytic proPO was also examined after bacterial injection. POT increased after challenge with either viable or inactivated *L. garvieae* and *A. veronii*. However, POT expression with viable bacteria was greater and appeared more quickly than with inactivated bacteria. Regardless of dosage, inactivated bacteria enhanced a milder increase in POS or POT. These results suggest that inactivated bacteria have potential for use as a bacterin to induce a mild defense response in cultured prawns.

**Key words:** phenoloxidase, *Macrobrachium rosenbergii*, prawn, *Lactococcus garvieae*, *Aeromonas veronii*, bacterin

During 1987 to 1991, Taiwan aquaculturists increased their production of the giant freshwater prawn *Macrobrachium rosenbergii* from 1,400 tons to over 16,000 tons (FAO, 1994), thereby reaching the top in the world in terms of yearly-expansion (overtaking a declining Thai fishery, in particular). However, Taiwan's prawn production fell steeply in 1992 and 1993 due to a major outbreak of yeast infections during the winter months, along with an overall reduction in growth, which was thought to have been caused by inbreeding (New, 1995). Bacterial diseases associated with *Aeromonas, Pseudomonas, Vibrio, Beneckea* and *Leuconthrix* have been reported in Brazilian prawn hatcheries (Lombardi and Labao, 1991a, b), where they led to iblack-spoti bacterial necrosis and gill obstruction. Brady and Lasso (1992) have also reported a predominance of *Aeromonas* spp., *Bacillus* spp., and *Pseudomonas* spp. among bacteria isolated from the hemolymph of diseased prawns. More significantly, both *Aeromonas* and *Pseudomonas* species have been isolated from the hepatopancreas of healthy-looking prawns (Sung and Hong, 1997), so that *Aeromonas* species may function as opportunistic pathogens (Sung *et al.*, 2000). Muscle necrosis of *M. rosenbergii* has been found to occur in Taiwan only during the summer, and especially during phytoplankton blooms (Cheng and Chen, 1998a). The causative bacterial pathogen has been identified as *Lactococcus garvieae* (Chen *et al.*, 2001). Therefore, in order to clarify the pathogenicity of *Aeromonas* and *Lactococcus* and prawn defense mechanism for effective disease control, we examined the immune responses of prawns to infection with much attention of phenoloxidase activity.

The primary immune response in crustaceans is a nonspecific cellular response (Anderson, 1992). According to previous studies (Söderhäll, 1982; Ratcliffe *et al.*, 1985; Smith and Söderhäll 1991), several proteins associated with the hemocyte prophenoloxidase-activating system (PAS) play an important role in non-self rec-
ognition and host defense for elimination of foreign particles in the body cavity of crayfish and other crustaceans (Söderhäll et al., 1994). Therefore, the PAS may be used as a defense indicator in crustaceans, including cultured prawns (Rodriguez and Moullac, 2000). It can be activated by microbial polysaccharides such as β-1,3-glucan from fungal cell wall (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Sung et al., 1998), lipopolysaccharides (LPS) from Gram-negative (G-) bacteria (Söderhäll and Hall 1984; Söderhäll et al., 1990; Sung et al., 1998) and also peptidoglycan from Gram-positive (G+) bacteria (Ashida et al., 1983). In addition, the PAS is also easily activated by several environmental and biological and even experimental manipulations, including calcium, sodium dodecyl sulfate (SDS), trypsin, high temperature (Ashida et al., 1983; Ashida and Söderhäll, 1984; Dularay and Lackie 1985; Leonard et al., 1985; Sugumaran and Nellaiappan, 1991; Sung et al., 1998). However, it is inhibited by infection (Sung et al., 2000) and environmental contaminants such as phthalate esters (Sung et al., 2003). Therefore, PO activity may not be adequately used as a defense indicator.

Hsiao and Sung* also showed that PO formation from intrahemocytic proPO was inhibited when hemocyte lysate supernatant (HLS) was treated with trypsin inhibitor (TI) but activated when HLS was treated with trypsin. In this study, the concentrations of TI and trypsin required to inhibit and activate PO activity were determined, respectively. The results confirmed that the use of TI in HLS preparation can block PO activity triggered via manipulation, and that trypsin added to HLS can activate all intrahemocytic proPO to form PO. Therefore, in the following experiments, PO activity in the HLS of prawns challenged with bacterial cells was measured by two different methods: For method 1, trypsin was used to activate all intrahemocytic proPO so that total PO activity (POT) could be measured. For method 2, trypsin inhibitor was used to block the activation of intrahemocytic proPO so that challenge-stimulated PO activity (POs) could be measured.

Materials and Methods

Experimental prawns

A batch of apparently healthy giant freshwater prawns Macrobrachium rosenbergii ranging from 25 to 32 g in weight, were purchased from a commercial shrimp farm. They were acclimated in aerated freshwater in 120 L plastic containers held at 25°C for at least three days prior to each experiment. Stocking densities were generally maintained at 20 prawns per container and artificial feed was given twice a day.

Challenge experiment

For the challenge experiment with viable bacterial cells, each batch of prawns was divided into three groups with 24 prawns in each group. The ventral muscle of each prawn in the first two groups was injected with 100 mL of one of the bacterial suspensions to give a sublethal dose (1/10 LD50) respectively, 2 × 10^2 cells/g of prawn for A. veronii (Sung et al., 2000) and 5 × 10^5 cells/g of prawn for L. garvieae (Sung and Sun, 2002), while the third group was injected with an equal volume of sterile PBS. In the treatment with formaldehyde-inactivated bacterial cells, which was used to measure the phenoloxidase activity, only prawns from group 1 and 2 were used as test groups, which were injected with the same dose (10^5 cells/g of prawn) of A. veronii and L. garvieae, respectively. After injection, prawns from each group were held separately in 120 L plastic containers at 25°C with aeration.

Preparation of bacterial suspensions

Two kinds of bacteria, Lactococcus garvieae, a gift from Dr. Winton Cheng (Department of Aquaculture, National Pingtung University of Science and Technology), and Aeromonas veronii, were separately cultured overnight at 37°C in brain heart infusion broth (BHB; Sigma, USA) (Sung and Sun, 2002) and trypticase soy broth (TSB; Sigma) (Sung et al., 2000), respectively. Subsequently, 1 mL of each of the bacteria-inoculated solutions was subcultured separately into 100 mL BHB and TSB, and these mixtures were cultured at 37°C until bacterial growth reached the late log phase: about 5 h for L. garvieae and 3 h for A. veronii. Before using in challenge experiments, a portion of the bacterial cells was washed once and suspended in sterile phosphate buffered solution (PBS, 0.01 M, pH 7.0); the rest of the bacterial cells was fixed and inactivated with 4% formaldehyde at 4°C for 24 h. These inactivated bacterial cells were then washed twice and also resuspended in sterile PBS. The concentrations of the either viable or inactivated bacterial cells were then adjusted to required levels for different experiments by dilution with PBS via a cell counting method (Petroff Hausser Counting Chamber, Hauser Scientific Co., USA) and a microscope (Eclipse E800, Nikon, Japan).

Preparation and observation of hemocytes

In this experiment, three groups, including uninjected, PBS-injected and A. veronii-injected prawns, were used to examine the change of the total number of hemocytes. At the intervals of 6, 18, and 24 h after injection, 1.8 mL of hemolymph was drawn from each prawn with 0.2 mL of anticoagulant with an osmolarity of 490 mOsm/kg (0.114 M trisodium citrate, 0.1 M sodium chloride, 0.1 mg/mL trypsin inhibitor; pH 7.45). The hemocyte sample was prepared from 50 mL of hemolymph according to procedures described by Sung

et al. (1998). The remaining hemolymph was used for the assay of phenoloxidase activity. Hemolymph (50 μL) was mixed with an equal volume of trypan blue, and cell counting and observation were then carried out using a hemacytometer (Bright-Line hemacytometer, Hauser Scientific Horham USA).

Preparation of hemocyte-lysate supernatant (HLS)

In order to determine PO activity, hemocyte-lysate supernatant (HLS) was prepared from hemocytes of prechallenge prawns as the control value, and of bacteria-challenged prawns at the intervals of 6, 18 and 24 h after injection. Each HLS sample was prepared by mixing hemocytes of six prawns randomly collected from one test group. Then, HLS was prepared according to procedures described by Sung et al. (1998). Briefly, hemocytes were collected by centrifuging these extracts at 300 × g for 10 min at 4°C; after washing the hemocytes with 0.01 M cacodylate buffer (CAC, pH 7.0; Sigma, USA), these samples were centrifuged at 300 × g for an additional 10 min. Following the removal of the supernatant, hemocyte pellets were resuspended in 0.01 M CAC. The cell suspension was then homogenized with a sonicator (Vibra-cell AC-600, Sonics, USA) equipped with a microtip (output 5, duty cycle 50%) and centrifuged at 12,000 × g for 30 min at 4°C. The resultant HLS was used as an enzyme source. HLS protein concentration was determined by the Bradford method (1976) using bovine serum albumin (Protein Assay Kit II Bio-Rad, USA) as a standard. In this study, the same experiment process was repeated three times with three different batches of prawns, because the PO activity may vary in batches of prawns purchased at different times (Sung et al., 1994) or as a result of the injection procedure (Sung et al., 2000).

In order to determine the optimal concentration of trypsin inhibitor (TI) which can be used to inhibit spontaneous PO activity elicited either when hemolymph is collected or when hemocytes are broken with a sonicator, both anticoagulant and CAC buffer, used in preparing the hemocyte suspension before homogenization, were prepared with different concentrations of TI (0, 0.05, 0.1, 0.25 and 0.3 mg/mL of final concentration). Furthermore, to determine whether trypsin can be used not only in neutralizing the inhibition effect of TI on PO activity but also in catalyzing all intracellular proPO to form PO, all HLS samples prepared with either TI-containing anticoagulant or TI-containing CAC buffer were treated with different concentrations of trypsin (0, 0.5, 1, 2, 3 and 5 mg/mL of final concentration) at 37°C for 15 min before detection of PO activity.

Assay of intrahemocytic phenoloxidase (PO) activity

The PO activity of the resultant HLS was determined with a spectrophotometer set by measuring the absorbance at 490 nm, using L-dihydroxyphenylalanine (L-dopa; Sigma) as a substrate: 100 μL of each HLS was kept at 37°C for 15 min, after which 200 μL of L-dopa (0.01 μM in 0.01 M CAC buffer) was added and allowed to react for 1 min. The optical absorbance at 490 nm was measured.

In this experiment, each HLS sample was divided into two portions and the measurement of two PO activities of prawns after injection with bacterial cells were detectable: (1) challenge-stimulated PO activity (POs), which resulted from the intrahemocytic proPO activated to form PO via challenge induction, which was still detected when the HLS was prepared with TI-containing CAC buffer; and (2) total intrahemocytic PO activity (POt), which resulted from all the intrahemocytic proPO being completely catalyzed to form PO when HLS prepared with TI-containing CAC buffer was treated with trypsin.

The experiments for PO activity were repeated three times. Therefore, the data show the average of relative PO activity (RA) ± standard error from three different HLS samples. The value of RA was calculated as follows:

RA = PO activity of HLS mixture at X time after prawns injected with bacterial cells
PO activity of HLS mixture of prechallenge prawns

Statistics

All data from the experiments described above, including measurements of two types of PO activity (POs and POT), were statistically analyzed using ANOVA and Duncan’s multiple range tests with a significance level of p<0.05.

Results

To avoid the spontaneous elicitation of PO activity via experimental manipulations, the effect of trypsin inhibitor (TI) on PO activity was first examined. By comparison with HLS without TI, PO activity of HLS treated with TI was significantly reduced and was dose-dependent (Fig. 1B). To determine whether or not trypsin can neutralize the inhibition effect of TI on PO activity, PO activity was measured after treatment of HLS with trypsin. As shown in Fig. 1A, 0.5 mg/mL of trypsin added to HLS was enough to elicit a maximum PO activity. A maximum relative PO activity of HLS, which was prepared with either anticoagulant containing TI or CAC buffer containing TI, could be detected and was not different from control HLS prepared without TI (Figs. 1C and 1D). The results indicate that trypsin can not only neutralize the effect of TI but also catalyze proPO to form PO. Furthermore, we found that PO activity of HLS prepared with TI-containing CAC buffer was not different from that with Ca2+-free CAC buffer (data not shown). Therefore, in the following experiments, HLS was prepared with TI-containing anticoagulant (final concentration of 0.3 mg/mL) and Ca2+-free CAC buffer.
addition, challenge-stimulated PO activity (PO<sub>S</sub>) and total intrahemocytic PO activity (PO<sub>T</sub>) were measured in HLS without or with trypsin (final concentration of 0.5 mg/mL).

The total hemocyte counts (THCs) were shown to decrease in prawns that were injected with either PBS or A. veronii at 1/10 of the LD<sub>50</sub>, but remained stable in uninjected prawns (Table 1). A significant increase in the percentage of granulocytes in the hemolymph was detected after challenge with bacterial cells (p<0.05); however, in PBS-injected prawns, the proportion of granulocyte did not vary significantly within 24 h post-injection (Table 1).

After challenge with 1/10 of the LD<sub>50</sub> of A. veronii, the relative PO<sub>T</sub> of prawns was found to increase significantly from 6 h to 24 h post-injection, with the highest activity at 18 h post-injection (Fig. 2). Whereas, after challenge with 1/10 of the LD<sub>50</sub> L. garvieae, the PO<sub>T</sub> was found to increase significantly at 6 h post-injection, although reduced to its pre-challenge level at 24 h (Fig. 2). A comparison of two types of PO activities after challenge with two bacterial species showed that PO<sub>S</sub> values of prawns challenged with L. garvieae were higher than those of prawns challenged with A. veronii from 6 h to 18 h post-injection, and that a higher PO<sub>T</sub> was detected at both 18 h and 24 h post-injection with A.
Phenoloxidase activity of challenged prawn

Table 1. Changes in the total hemocyte counts and the percentage of hemocytes in the hemolymph of prawns injected with a sublethal dose (2×10^2 cells/g of prawn) of Aeromonas veronii

<table>
<thead>
<tr>
<th>Time after injection (h)</th>
<th>Number of total hemocytes (× 10^6 cells/mL)(^a) / % of granulocytes(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (N=6)</td>
</tr>
<tr>
<td>preinjection</td>
<td>65.2 ± 2.5 / 93.2 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>63.4 ± 4.1 / 93.1 ± 0.1</td>
</tr>
<tr>
<td>18</td>
<td>66.3 ± 3.6 / 92.9 ± 0.3</td>
</tr>
<tr>
<td>24</td>
<td>65.4 ± 3.1 / 93.1 ± 0.2</td>
</tr>
</tbody>
</table>

N, the number of prawns.

\(a\), Total hemocyte count.

\(b\), % of granulocytes = the number of granulocytes/the number of total hemocytes; granulocytes containing granular and semigranular cells.

\(c\), No injection.

\(d\), Prawn injected with sterile PBS or bacterial cells.

\(e\), Significant difference (p<0.05) from the preinjection group. The data were analyzed using the ANOVA and Duncan's multiple range tests.

veronii than with L. garvieae.

As shown in Fig. 3, an increase in the relative PO\(_T\) was detected from 6 h to 24 h post-injection with inactivated A. veronii and also at 18 h and 24 h post-injection with inactivated L. garvieae. However, the PO\(_T\) at 6 h after injection of prawns with A. veronii was found to be
higher than that 6 h after injection with *L. garvieae*. A maximum in PO$_5$ was detected at 18 h and a significant increase was still detected at 24 h after challenge with either *L. garvieae* or *A. veronii*. No difference was found between the PO$_5$ in the two test groups (Fig. 3).

The results showed that the relative PO increased after challenge with either viable or inactivated *L. garvieae* or *A. veronii* (Fig. 4). A maximum in PO$_T$ of prawns injected with either viable or inactivated *A. veronii* at a dose of $10^5$ cells/g of prawn was detected at 18 h, but all PO$_T$ values detected after injection with viable cells were found to be higher than those after injection with inactivated cells. Furthermore, the appearance of a maximum PO$_T$ after injection with a high dose of bacterial cells ($10^5$ cells/g) was detected at 6 h after injection (Fig. 4A). As for injection with *L. garvieae*, a maximum in PO$_T$ was observed at 6 h and 18 h after challenge with viable cells and inactivated cells, respectively. Also, the PO$_T$ stimulated by viable cells was found to be significantly higher than that stimulated by inactivated cells (Fig. 4B).

**Discussion**

The prophenoloxidase-activating system (PAS) is easily elicited by several environmental and biological factors, and even by experimental manipulations. In this study, we found that PO formation from intrahemocytic proPO can be inhibited when hemocytes are collected from each prawn by using anticoagulant containing trypsin inhibitor (TI) (Fig. 1C). In future experiments, we suggest that TI should be used to avoid spontaneous elicitation of proPO. Furthermore, total intrahemocytic proPO can be activated to form PO when HLS is treated with trypsin (Fig. 1D). Therefore, challenge-stimulated PO activity (PO$_5$) can be directly detected when HLS is prepared with TI-anticoagulant but not treated with trypsin; whereas, the total intrahemocytic PO activity (PO$_T$), which can be representative of the total intrahemocytic proPO content of prawns, can be detected when HLS is treated with trypsin.

Smith (1990) indicated that fast-growing pathogens are able to overwhelm the initial and non-specific defense responses of crustaceans and cause diseases before more powerful immune defense begins to operate fully. Both types of defense are likely to succeed in functioning against slow-growing pathogens. Previous studies have also shown that prawns are able to clear invading bacteria from their hemolymph within 4–6 h (Adams, 1991; Martin et al., 1993; Sung et al., 1996); as in other decapods, a large number of bacteria may be removed directly by phagocytosis. The PAS, which functions in non-self recognition and host defense (Söderhäll, 1982; Ratcliffe et al., 1985; Smith and Söderhäll, 1991) has been shown to be directly involved in the communication between hemocytes and also in the elimination of foreign particles from the body cavity of crayfish (Söderhäll et al., 1994). Previous studies have suggested that both the *A. veronii* strain and *L. garvieae* strain function as opportunistic pathogens and only cause disease in prawns that have already weakened due to environmental stresses (Sung et al., 2000; Chen and Cheng, 1998b and 1999). Results from challenge experiments have shown that the growth of *A. veronii* strain was faster than that of *L. garvieae* strain, and that the virulence of *A. veronii* strain was greater than that of *L. garvieae* strain (Sung et al., 2000; Sung and Sun, 2002). In this study, in contrast to the changes in PO$_5$ activities measured after prawns were challenged with *L. garvieae*, the PO activity was not significantly enhanced immediately after prawns were challenged with either viable *A. veronii* at a low dose (Fig. 2) or inactivated *A. veronii* at a high dose (Fig. 3). The results suggest that, due to the fact that the virulence of *A. veronii* is greater than that of *L. garvieae*, the fast-growing *A. veronii* may delay the activation of the proPO system. Since PO activity is not immediately induced after infection, the ini-
ential defense reactions, such as cytotoxicity, phagocytosis, nodule formation or encapsulation, have not enhanced in order to kill or remove the invading bacteria quickly.

According to the results of both the previous study (Sung et al., 2000) and present study on changes in total hemocyte count (THC), the researchers found that though THC decreased rapidly in both bacteria-treated and PBS-treated prawns, the granulocyte percentage of prawns injected with PBS remained unchanged after injection. Therefore, in this study, the prawns injected with PBS were used as the control group to evaluate the expression of the PAS in the early phase of infection. By comparison with pre-injection values, the percentage of granulocytes significantly increased at 6 h after challenge with bacterial cells; however, there was no difference in data collected from 6 to 24 h post-challenge. The changes in both granulocyte percentage and PO activity suggest that the expressions of both POs and PO enzymatic activity could be primarily related to the percentage of granulocytes at 6 h after infection; however, after 6 h, it seems that the change of those expressions is not related to the granulocyte percentage and may be influenced by pathogen species, bacterial dosage, or other reactions such as intrahemocytic proPO production or gene expression, which needs further investigation.

Previous studies have shown that PO can be activated in prawns either in vitro or in vivo by lipopolysaccharide (LPS) from Escherichia coli cell walls, β-1,3-glucan and zymosan from yeast cell walls, Vibrio bacterin (Sung et al., 1996; Sung et al., 1998), and peptidoglycan from G+ bacterial cell walls (Sritunyalucksana et al., 1999), and that the PAS in crayfish is triggered by microbial products such as LPS and β-1,3-glucan through different activating pathways (Söderhäll and Hall, 1984). In this study, by challenge with the same virulence (1/10 the LD₅₀ dose of A. veronii and L. garvieae), the effects of two pathogens on the activation of the PAS showed that POs activity was dependent on the bacterial dosage, and that the appearance of the greatest POT occurred earlier post-injection and was greater than that after treatment with L. garvieae (Fig. 3). Therefore, the results from this study indicate that although the activation of the PAS can be rapidly enhanced in the early stages of infection, the enhancement effect of G+ L. garvieae may be stronger than that of G+ A. veronii.

After examining the effect of viable and inactivated cells on the activation of the PAS, it is also found that viable bacteria induce a stronger activation than inactivated bacteria (Fig. 4). A stronger response induced by the pathogen is beneficial to the prawns in preventing disease or death in the early stages of infection. However, regardless of the dosage, the inactivated bacteria also provoke a mild activation of the PAS (Fig. 4). Since the inactivated bacteria were easier and cheaper to prepare than purified compounds such as LPS, glucan, and peptidoglycan, the inactivated bacteria may be used as a bacterin to induce a mild defense response without an adverse effect on the host, and can be used in the reduction of disease outbreak or in the prevention of disease in prawn populations during cultivation.

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References


