Phenoloxidase Activity of Hemocytes Derived from *Penaeus monodon* and *Macrobrachium rosenbergii*

Hung-Hung Sung,^{*,1} Hung-Jun Chang,^{*} Cheng-Hao Her,^{*} Jen-Chang Chang,^{*} and Yen-Ling Song[†]

^{*}Department of Microbiology, Soochow University, Taipei, Taiwan; and [†]Department of Zoology, National Taiwan University, Taipei, Taiwan, Republic of China

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The phenoloxidase (PO) activity of hemocyte lysate supernatant (HLS) from both tiger shrimp (*Penaeus monodon*) and giant freshwater prawn (*Macrobrachium rosenbergii*) was examined by treating HLS with various factors, such as an increase in temperatures from 25 to 70°C, one of four elicitors (β -1,3-1,6-glucan, zymosan, heat-killed *Vibrio* cells, and lipopolysaccharide), trypsin, one of three protease inhibitors (soybean trypsin inhibitor, *p*-nitrophenyl-*p'*-guanidinobenzoate, and benzamidine), and one of two divalent cations (Mg^{2+} and Ca^{2+}). The strongest PO activity in both animals was induced at 37°C, while enzyme activity varied according to the concentration of the elicitors or cations added to the HLS samples. The following optimum concentrations were recorded: lipopolysaccharides at 0.5 mg/ml, both β -glucan and zymosan at 1 mg/ml, and *Vibrio* cells at 10^6 cells/ml. In addition, for giant freshwater prawn, PO activity increased when HLS was treated with trypsin and decreased when it was separately treated with three protease inhibitors. However, effects of either trypsin or protease inhibitors did not occur in tiger shrimp. Strongest PO activity occurred in HLS treated with 20 mM of either calcium ion or magnesium ion, and the addition of the two cations led to an increase in enzyme activity; a decrease was noted following the treatment with EDTA. Cytochemical analysis revealed that prophenoloxidase system exists in the granulocytes of both tiger shrimp and giant freshwater prawn. © 1998 Academic Press

Key Words: Prophenoloxidase activating system; phenoloxidase activity; elicitor; divalent cation; *Penaeus monodon*; *Macrobrachium rosenbergii*.

INTRODUCTION

Recent arthropod research has produced evidence to support that phenoloxidase is the terminal enzyme in

the prophenoloxidase (proPO) activating system. It has also been suggested that activation and regulation of the proPO system serve recognition and defense functions; in addition, factors produced after the activation have been shown to be directly involved in the communication between blood cells (Söderhäll, 1982, 1992; Smith and Söderhäll, 1983; Söderhäll and Smith, 1986a,b). Several proteins of the proPO system have been isolated and partially characterized in the hemocytes of crustaceans (Söderhäll *et al.*, 1990) and can be released by a regulated exocytosis (Johansson and Söderhäll, 1989). The proPO-system proteins released from semigranular cells of crayfish are in the presence of microbial polysaccharides; in contrast, these released from granular cells are in the presence of a 76-kDa factor or β -1,3-glucan binding protein which has been treated with β -1,3-glucan (Barraco *et al.*, 1991). These proteins recently purified from crayfish hemocytes are subsequently activated outside the cells by elicitors of the proPO system, and at the same time prophenoloxidase is converted to its active form, phenoloxidase, by a serine protease, ppA (Johansson and Söderhäll, 1985; Aspán and Söderhäll, 1991). Outside the hemocytes in the plasma, protease inhibitors such as a trypsin inhibitor (Hergenroth *et al.*, 1987) and an α_2 -macroglobulin are present (Hall *et al.*, 1989), and both these high-molecular-mass protease inhibitors can inhibit ppA and thus the activation of the proPO system (Aspán *et al.*, 1990).

Phenoloxidase, which has been detected in a wide range of invertebrates (Smith and Söderhäll, 1991; Jackson *et al.*, 1993), is activated by several microbial polysaccharides, including β -1,3-glucan from fungal cell walls (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979) and peptidoglycans (Ashida *et al.*, 1983) or lipopolysaccharides (Söderhäll and Hall, 1984; Söderhäll *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 Söderhäll, 1984; Dularay and Lackie, 1985; Leonard *et*

¹ To whom correspondence and reprint requests should be addressed. Fax: 886-2-8831193. E-mail: hhsung@mbm1.scu.edu.tw.

al., 1985; Sugumaran and Nellaippan, 1991). According to Ashida and Söderhäll (1984), these elicitors activated the proPO system through different mechanisms. Phenoloxidase is a redox enzyme which is capable of oxidizing phenols to quinones, which leads to spontaneous formation of melanin as an end product. In invertebrates, the pigment is produced in the cuticle after wounding (Ratcliffe *et al.*, 1985), in nodules following bacterial infection, and during encapsulation process following parasite infection (Ratcliffe *et al.*, 1985; Gotz, 1986). Both melanin and quinones have also been found to act as inhibitors of bacterial enzymes (Kuo and Alexander, 1967) and to be fungistatic (Söderhäll and Ajaxon, 1982; St. Leger *et al.*, 1988; Rowley *et al.*, 1990).

According to the results of our earlier research, phenoloxidase activity is detectable in tiger shrimp hemocytes and can be enhanced by either *in vitro* (Sung *et al.*, 1994) or *in vivo* (Sung *et al.*, 1996) treatment with β -glucan; the same β -glucan treatment also acts to strengthen vibriosis resistance in shrimp (Sung *et al.*, 1994). We therefore believe that the proPO system plays an important role in shrimp defense mechanisms which are similar to those that occur in crayfish. For the present research the influence of temperature, elicitors, and divalent cations on the phenoloxidase activity in two important Southeast Asian food species, *Penaeus monodon* and *Macrobranchium rosenbergii*, was studied. Both species are susceptible to several diseases when raised in aquacultural environments; the purpose of this study is to find reference indicators to improve the quality of pond water in order to increase disease resistance, and consequently reduce the economic loss many shrimp farmers face.

MATERIALS AND METHODS

Experimental Animals

Tiger shrimp (*P. monodon*), weighing approximately 30 g each, and giant freshwater prawn (*M. rosenbergii*), weighing approximately 40 g each, were purchased from a fish market in Pingtung, Taiwan. Tiger shrimp and giant freshwater prawn were respectively acclimated in 2.5% saline pond water and fresh pond water at 30°C in 120-liter plastic container for 3 days prior to use in these experiments. The stocking density was four individuals per liter for tiger shrimp and two individuals per liter for giant freshwater prawn.

Elicitors

The elicitors used in our experiments were: (a) heat-killed *Vibrio vulnificus* TG 617 cells (a gift from Dr. Y. L. Song, Department of Zoology, National Taiwan University in Taiwan), which was isolated from diseased tiger shrimp cultured in Taiwan (Song *et al.*, 1990) with the cell wall containing the lipopolysaccharides (LPS) on

the surface; (b) β -1,3-1,6-glucan extracted from *Saccharomyces cerevisiae* (Biotec Mackzymal, Tromsø), which had been examined, can enhance the defense reactions of tiger shrimp (Sung *et al.*, 1994, 1996); (c) zymosan, a β -1,3-glucan-protein-lipid compound also extracted from the cell walls of *S. cerevisiae* (Sigma); and (d) LPS extracted from the cell walls of *Escherichia coli* 0111:B4 (Sigma). Prior to our experiments, the concentrations of *Vibrio* cells suspensions were adjusted to 10^5 , 10^6 , 10^7 , 5×10^7 , and 10^8 cells per milliliter of cacodylate (CAC) buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 10 mM CaCl_2 , 26 mM MgCl_2 , pH 7.0). The other elicitors were prepared separately at concentrations of 0.1, 0.5, 1.0, 2.5, and 5.0 mg per milliliter of CAC buffer. To ascertain that these elicitors were responsible for the enhancement of phenoloxidase (PO) activity, prior to the assay for induction of PO activity, the β -1,3-1,6-glucan and zymosan were treated with 70% alcohol and boiling for 30 min, respectively; the LPS were treated with polymyxin B at a final concentration of 1000 units per milliliter for 15 min as a control.

Hemocyte Lysate Supernatant (HLS) Preparation

Using a 2-ml syringe (26 G $\times$ $\frac{1}{2}$ in.) containing 0.2 ml of anticoagulant with an osmolarity of 780 mOs/kg (0.01 M Tris-HCl, 0.25 M sucrose, 0.1 M trisodium citrate, pH 7.6) for tiger shrimp or an osmolarity of 490 mOs/kg (0.114 M trisodium citrate, 0.1 M sodium chloride, pH 7.45) for giant freshwater prawn, approximately 1 ml of hemolymph was drawn from each shrimp. Hemocytes were collected by centrifuging these extracts at 300g for 10 min at 4°C; after washing hemocytes with 0.01 M phosphate-buffered saline (PBS, pH 7.0), these samples were again centrifuged at 300g for an additional 10 min. Following supernatant removal, hemocyte pellets were resuspended in 0.01 M of PBS. The cell suspension was then homogenized with a sonicator (Vibra cell, AC-600) equipped with a microtip (output 5, duty cycle 50%) and centrifuged at 43,000g for 20 min at 4°C. The resultant HLS, which was used as an enzyme source, was kept at -20°C before assaying for PO activity. HLS protein concentration was determined by the Bradford method (1976) using bovine serum albumin (Bio-Rad Protein Assay Kit II) as a standard.

Assay of PO Activity

PO activity was assayed according to procedures described by Sung *et al.* (1994) using L-3,4-dihydroxyphenyl-alanine (L-dopa) (Sigma) as a substrate. HLS (200 μ l) was preincubated at six different temperature (25, 30, 37, 50, 60, and 70°C) for 15 min, after which 400 μ l of L-dopa (1.6 mg/ml in CAC buffer) was added and reacted for 1 min. Each reaction mixture was further diluted with 400 μ l of CAC buffer, and then the

absorbance of optical density at a wavelength of 490 nm was measured. For these experiments, 1 unit of enzyme activity was defined as an increase in absorbance of 0.001/min/mg protein (Soderhall and Unestam, 1979). In this study, PO activity was detected by using trypsin as an activator and three protease inhibitors (soybean trypsin inhibitor, *p*-nitrophenyl-*p*'-guanidinobenzoate, and benzamidine) as inactivators with the standard L-dopa test (Söderhäll, 1981; Ashida and Söderhäll, 1984).

Effect of Elicitors on PO Activity

Samples containing 150 μ l HLS plus an equal volume of the elicitor being examined were preincubated for 15 min at 37°C; while CAC buffer substituted for elicitor in control sample. Next, 300 μ l of L-dopa was added, the new mixtures were incubated for 1 min, and the absorbance of optic density was subsequently measured. Because the PO activity in HLS varies according to batches of shrimp purchased at different time (Sung *et al.*, 1994), the values given in this study are the means of average relative PO activity (RA) $\pm$ the standard error of the mean of five replicates from five individuals. The value of RA was calculated as

$$RA = \frac{\text{PO activity of a HLS treated with an elicitor}}{\text{PO activity of an untreated HLS}}.$$

Effect of Divalent Cations on PO Activity

The effects of either Ca^{2+} or Mg^{2+} were determined by means of 0.01 M PBS (pH 7.0) containing 0–100 mM cations, 20 mM ethylenediaminetetraacetic acid (EDTA), 20 mM EDTA saturated with 20 mM Ca^{2+} , and a combination of 10 mM Ca^{2+} and 26 mM Mg^{2+} .

Cytochemistry

The following procedure was used to determine which of hemocyte contained the prophenoloxidase system: in addition to using Evans staining to count the number of different types of hemocytes presented, a sequential assay was performed according to procedures described by Lanz *et al.* (1993). Briefly, hemocytes were fixed in a neutral formalin solution (10% formaldehyde in 0.02 M phosphate buffer, pH 7), washed three times in PBS (0.01 M, pH 7.0), incubated in 1 ml L-dopa (2 mg/ml) for 10 hr at room temperature, and then observed by light microscope (Leitz Orthoplan). Control incubations were performed in the presence of 2 mM phenylthiourea.

RESULTS

The Effect of Temperature on PO Activity

In either tiger shrimp (*P. monodon*) or giant freshwater prawn (*M. rosenbergii*), the PO activity was tempera-

ture-dependent; strongest activity was detected at 37°C. At 25 and 30°C, enzyme activity in tiger shrimp was respectively measured at 39 and 57% of that recorded at 37°C, while for giant freshwater prawn the measured activity was 42 and 56%, respectively. Besides, PO activity of giant freshwater prawn at optimum temperature (37°C) was 2.7 times higher than that measured in tiger shrimp.

The Effect of Elicitors on PO Activity

PO activity in tiger shrimp was enhanced by pretreatment with β -1,3-1,6-glucan, zymosan, LPS, or *Vibrio* cells, with optimum concentration measured at 1 mg/ml, 1 mg/ml, 0.5 mg/ml and 10^6 cells/ml, respectively. A quantitative analysis of the stimulative effect of the four elicitors on PO activity in tiger shrimp revealed that β -1,3-1,6-glucan and *Vibrio* cells had the strongest effect, with RA measured at 2.6 each, followed by LPS (2.46) and zymosan (1.78). The strongest stimulative effect of the four elicitors on PO activity in giant freshwater prawn were LPS (4.7), zymosan (2.8), *Vibrio* cells (2.6), and β -1,3-1,6-glucan (2.0) (Fig. 1). Using trypsin as an activator with the standard L-dopa test, PO activity increased in giant freshwater prawn, but did not increase in tiger shrimp. Furthermore, a decrease activity was detectable in HLS of giant freshwater prawn treated with three protease inhibitors, but no decrease was detected in tiger shrimp.

The Effect of Divalent Cations on PO Activity

As shown in Figure 2, PO activities in both shrimp and prawn were enhanced by treatment with either calcium ions or magnesium ions; these effects were concentration-dependent with the optimum concentrations measured at 20 mM for both. No significant differences in PO activity for animals treated with PBS containing 0 and 100 mM calcium ion were reported. The measured RA for HLS treated with 100 mM calcium was 1.1 for either tiger shrimp or giant freshwater prawn. The stimulative effect of magnesium ion on PO activity decreased in the two animals at concentrations higher than 20 mM. However, at 100 mM RA was measured at 2.1 for tiger shrimp and 4.0 for giant freshwater prawn—in both cases still higher than those measured at 0 mM magnesium. For both animals, PO activity was reduced to zero in cation-free HLS treated with 20 mM EDTA; it was noted that, following the addition of 20 mM calcium or magnesium, PO activity returned to the same level measured in cation-free HLS without EDTA. An additive effect of the two cations in HLS collected from either animal and treated with a combination of 10 mM Ca^{2+} and 26 mM Mg^{2+} was also detected.

Location of proPO Activating System

As shown in Fig. 3, the hemocytes of both animals contained dark black granules located within granulocytes, although this was not true within hyaline hemocytes. In addition, a quantitative analysis of different hemocyte types revealed a dominance of granulocytes in both animals; however, the amount of granulocytes as a percentage of all hemocytes was higher in freshwater prawn ($87.7 \pm 2.9\%$) than in tiger prawn ($64.9 \pm 3.8\%$).

DISCUSSION

Söderhäll *et al.* (1994) have reported that the proPO system of invertebrate hemocyte lysates are activated specifically by either β -1,3-glucan from fungal cell wall or lipopolysaccharides and peptidoglycans from bacterial cells. Other researchers have found that such factors as temperature and calcium concentration also effect proPO system activation (Ashida *et al.*, 1983; Ashida and Söderhäll, 1984; Dularay and Lackie, 1985). The present paper is the first study on the effects of various elicitors on proPO system activation in two crustacean species, *P. monodon* and *M. rosenbergii*. Results show that: (1) PO activity in these two species is dependent on temperature; (2) the proPO system can be activated by prior treatment with β -1,3-1,6-glucan, zymosan (β -1,3-glucan), LPS, or *Vibrio* cells; and (3) divalent cations (Ca^{2+} and Mg^{2+}) are both necessary for the activation of proPO system. In addition, proPO was not purified for assay in this experiment, it cannot be excluded that dopa used as substrate was oxidized by peroxidases or other substances, such as reactive oxygen metabolites, within HLS. According to previous studies done by many researchers, we consider 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 shrimp culture in the winter waters of Taiwan will always have low resistance to disease, because of the relatively low average temperature of 25°C . Yeast (*Debaryomyces hansenii* and *Metschnikowia bicuspidata*) is a primary pathogen in giant freshwater prawn during winter months in Taiwan. While the lower

FIG. 1. Effects of four elicitors on PO activity of hemocyte lysates from tiger shrimp (*Peneaus monodon*) and giant freshwater prawn (*Macrobrachium rosenbergii*). The elicitor treatment and concentration for each bar: none, HLS prepared in CAC buffer but not treated with elicitors; β -glucan, 1 mg/ml; zymosan, 1 mg/ml; LPS, 0.5 mg/ml; *Vibrio* cell, 10^6 cells/ml; trypsin, 0.1 mg/ml; trypsin inhibitor, 0.5 mg/ml; *p*-nitrophenyl-*p'*-guanidinobenzoate (p-NPGB), 1 mM; and benzamide, 10 mM.

FIG. 2. Stimulative effects of divalent cations on PO activity in the HLS of tiger shrimp (*Peneaus monodon*) and giant freshwater prawn (*Macrobrachium rosenbergii*). The treatment and concentration of each bar: the first to the sixth bar, HLS prepared in CAC buffer containing various concentration of either calcium or magnesium at 0, 10, 20, 25, 50, and 100 mM; 7th bar, the cation-free HLS was treated with 20 mM EDTA; the last bar, the cation-free HLS was treated with EDTA and 20 mM calcium or EDTA and 20 mM magnesium.

water temperature serves to weaken prawn resistance to disease, it is warm enough for yeast to flourish (I-Chiu Liao, personal communications, 1994).

Our results also show that a broad concentration range of elicitors examined for this study enhance *in vitro* PO activity in both species. Previous studies have shown that treatment with β -1,3-1,6-glucan at 0.5 and 1 mg/ml is sufficient for strengthening tiger shrimp resistance to vibriosis *in vivo* (Sung *et al.*, 1994); when immersion is used to treat shrimp with β -1,3-1,6-glucan, only one-tenth of this dosage enters the hemolymph of shrimp and is required to produce similar results (Sung *et al.*, 1996). While safety issues regarding treatment procedures and concentrations still require further study, we suggest that the four elicitors used on the present study be considered as candidates for enhancing disease resistance in shrimp in the following dosages: 1 mg/ml for two β -glucan and LPS, and 10^7 cells/ml for *Vibrio* cells.

Similar to other invertebrates studied, the proPO of giant freshwater prawn could be activated and it was illustrated by its conversion to the active form by trypsin; however, on the other hand, the proPO of tiger shrimp cannot be activated by trypsin. The possible reasons include: (1) proenzyme form may not exist in tiger shrimp; (2) the proPO can be cut by trypsin, but the active site of PO may be partially or incompletely exposed; (3) the proPO may be not cut by trypsin; (4) most of the proPO may be converted to PO during preparation and handling; and others. Since the results of PO activity were enhanced when HLS was treated with elicitors, the influence of preparation and handling could be limited. These possibilities must be studied further.

The present results also show that both calcium and magnesium are required to enhance PO activity in tiger shrimp and giant freshwater prawn. Similar calcium-mediated enhancement of PO activity has been reported for several types of insects (Ashida *et al.*, 1983; Dunphy, 1991) and crustacean species (Söderhäll, 1981; Ashida and Söderhäll, 1984; Kondo *et al.*, 1992), but similar reactions have not been reported for magnesium. Optimum Ca²⁺ concentration varied according to species: 50 mM for *Lymantria dispar*, 10–20 mM for *Galleria mellonella* (Dunphy, 1991), 10 mM for *Astacus astacus* (Söderhäll, 1981), and *Pacifastacus leniusculus* (Söderhäll, 1981); for the present study, we found the optimum concentration to be 20 mM for both Ca²⁺ and Mg²⁺. In addition, Chung (1994) has reported that ion concentrations in the hemolymph of kuruma shrimp (*P. japonicus*) vary with environmental conditions, such as temperature and dissolved oxygen. Combined, these results suggest that the quality of pond water is critical to maintaining proper concentration of Ca²⁺ or Mg²⁺ in order to enhance the proPO system, and consequently

FIG. 3. The cytologic location of proPO activating system detected by cytochemical analysis and observed with a bright field microscope. A, hemocytes from giant freshwater prawn; B, hemocytes from tiger shrimp; 1, untreated hemocytes as control; and 2, hemocytes incubated with L-dopa as a substrate. Bar, 10 μ m.

strengthen the defense mechanisms of shrimp; however, this suggestion requires further study.

Using L-dopa as substrate, other researchers have reported the presence of proPO enzymes in semigranular and granular cells, for example, localized within the vesicles of hemocytes of *A. astacus* and distributed through the cytosol of hemocytes of *Procambarus clarki* (Smith and Söderhäll, 1983; Johansson and Söderhäll, 1985; Lanz *et al.*, 1993). We found that the proPO system primarily exists in granular hemocytes. Since the differentiation between granular or semigranular cells of either tiger shrimp or giant freshwater prawn by Evan's staining, we cannot determine whether the proPO system exists in semigranular cells. Differentia-

tion of hemocytes will be able to perform by using the monoclonal antibodies against either granular or semigranular cells. The work is being carried out in our laboratory. In addition, our data show that PO activity in giant freshwater prawn is significantly greater than that in tiger shrimp. Possible reasons for this difference include either a large number of or a higher level of granular hemocytes in the former, or a combination of both factors. While evidence supporting the first suggestion from the cell count has been described in an earlier section, further study is necessary for the other two suggestions.

Finally, the study, inspired by Soderhall and his colleagues' research on crayfish, provided important

evidence on the effect of various agents examined in strengthening the innate immune protection of the two aquacultural species. However, the role of these agents in the proPO activating system, the mechanism of the system in both species, and the resolution of some questions derived from this study will be important topics for future study in improving the resistance to disease of Taiwan valuable aquacultural products.

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