Peroxinectin gene transcription of the giant freshwater prawn
*Macrobrachium rosenbergii* under intrinsic, immunostimulant,
and chemotherapeutant influences

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Received 15 November 2005; revised 30 May 2006; accepted 18 June 2006
Available online 25 July 2006

**Abstract**

Peroxinectin (PE) gene expressions were determined using real-time PCR in the giant freshwater prawn *Macrobrachium rosenbergii* based on moulting; prawns were fed diets containing different concentrations of sodium alginate, and were exposed to different concentrations of copper sulphate, benzalkonium chloride (BKC), and trichlorfon. Results showed that PE mRNA expression of prawns was the highest in stage A, significantly decreased in stage B, and reached the lowest level in stages D0/D1. The PE transcript was significantly higher in prawns fed the 1.0 g kg⁻¹ sodium alginate-containing diet than those fed the 2.0 g kg⁻¹ sodium alginate-containing diet and those fed the control diet. PE transcripts significantly decreased in prawns exposed to 0.1—0.4 mg L⁻¹ copper sulphate after 96 h, 0.3—1.0 mg L⁻¹ BKC after 96 h, and 0.2—0.4 mg L⁻¹ trichlorfon after 48 h. It was concluded that the status of PE gene expression was seriously affected by the moult cycle, immunostimulant, and chemotherapeutants.

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**Keywords:** *Macrobrachium rosenbergii*; Peroxinectin; Transcription; Moult stage; Immunostimulant; Copper sulfate; Benzalkonium chloride; Trichlorfon

1. **Introduction**

The giant freshwater prawn *Macrobrachium rosenbergii* is the most important aquaculture species of decapod crustaceans used for freshwater culture in Taiwan. During the past few years, commercial prawn farming has been severely adversely impacted by epidemics associated with yeasts in the cool season [1] and bacteria in the hot season [2], which have caused serious economic losses.

Disease outbreaks result from interactions among the environment, hosts, and pathogens. Several strategies have been used to prevent and control disease outbreaks in prawn culture, such as improving the management of cultivation conditions, administering immunostimulants to increase the immune ability of hosts, and using different chemicals and probiotics to respectively kill and inhibit the pathogens or to maintain a stable quality of pond water. Environmental factors not only affect pathogen aggression but also the weakened defence ability of hosts. Therefore, it is...
necessary to determine which environmental changes are related to immune ability in order to improve culture techniques for the prevention and control of disease outbreaks.

Invertebrates, including crustaceans, do not have acquired immunity; instead they have an innate immune system, which includes melanisation by activation of the prophenoloxidase-activating system (proPO system), a clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action, and cell agglutination [3]. Several components or associated factors of the proPO system have been found in crustaceans including the recognition protein [4,5], cell adhesive protein [6–8], protease inhibitor [9], and proPO-activating proteinase [10,11].

Peroxinectin, a cell adhesion protein, is synthesised and stored in semi-granular and granular haemocytes in an inactive form, is released in response to a stimulus, and is activated outside of cells to mediate haemocyte attachment and spread [12–14]. It is generated concomitantly with activation of the proPO system [12]. Peroxinectin has multiple functions of degranulation [15], encapsulation enhancement [16], opsonification [17], and activation of peroxidase [6]. When a foreign particle enters the haemolymph of a host, the haemocytes recognise the foreign intruder as non-self and change from non-adhesive cells to adhesive cells, and strongly adhere to the foreign target. Haemocytes (both semi-granular and granular ones) attach and spread across the surface of the foreign intruder, and form a multilayered sheath of cells during encapsulation [18].

As peroxinectin has peroxidase activity and has been shown to be an opsonin, the binding of peroxinectin to an extracellular superoxide dismutase (EC-SOD) could be of biological significance. SOD may catalyse the production of H₂O₂ from superoxide anions which peroxinectin may then use to produce microbicidal substances (such as HOCl) on the surface of invading micro-organisms or parasites [19]. Therefore, peroxinectin is essential in crustacean cellular defence reactions for enhancement of encapsulation and phagocytosis.

Variations in physiological factors and chemotherapeutants that induce changes in the immune status of *M. rosenbergii*, and sodium alginate administration to enhance the immune ability of *Litopenaeus vannamei* were reported in previous studies [20–25]. Peroxinectin of *M. rosenbergii* was also cloned in a previous study [26]. The aim of the present study was to evaluate peroxinectin cDNA expression of the giant freshwater prawn *M. rosenbergii* based on moulting; prawns were fed diets containing different concentrations of sodium alginate, and were exposed to different concentrations of copper sulphate, benzalkonium chloride (BKC), and trichlorfon.

### 2. Materials and methods

#### 2.1. Prawns

Giant freshwater prawns *M. rosenbergii* obtained from a commercial farm in Pingtung, Taiwan, were acclimated in recirculating water tanks and fed a commercial prawn diet (which did not contain sodium alginate; Shinta Feed, Pingtung, Taiwan) daily for 2 weeks. Only prawns in the intermoult stage were used for the study except for the moult cycle test. Prawns weighing 14.8–19.6 g were used for the study of peroxinectin expression, and no significant size differences among the different moult stages or among treatments were detected. During the experiment, water temperature was maintained at 27 ± 1 °C.

#### 2.2. Peroxinectin expression of prawns based on different moult stages

There are three moult stages—postmoult, intermoult, and premoult—which can be distinguished by the degree of hardness of the exoskeleton. Each moult stage is divided into several substages according to the retraction of the epithelium within the setae of the antennal scale [27]. They are (1) A and B for the postmoult; (2) C for the intermoult; and (3) D₀, D₁, D₂, and D₃ for the premoult. Five moult stages (A, B, C, D₀/D₁, and D₂/D₃) were used, and six prawns from each stage were sampled to examine PE expression in prawns in this study.

#### 2.3. Peroxinectin expression of prawns administrated a sodium alginate-containing diet

Three diets containing different levels of sodium alginate were prepared by the Shinta Feed Company (Pingtung, Taiwan). The basal diet contained 0.2% cellulose. Sodium alginate (Kimitsu Algina I-1, Kimitsu Chemical Industries, Chiba, Japan) was added to the test diets at the levels of 1.0 and 2.0 g (kg diet)⁻¹ with a corresponding decrease in the amount of cellulose. The finished pellets were stored in a cool room at 4 °C until used.
At the beginning of the trial, 18 glass aquaria (60 L) containing 40 L aerated water were each stocked with five prawns. Each diet was fed to six groups of prawns (six replicates) for 14 days. The aquaria were continuously aerated, and 30% of the water was exchanged daily to maintain the water quality. The haemolymph was individually collected, and PE expression was examined at the beginning and after 7 and 14 days of feeding.

2.4. Peroxinectin expression of prawns under copper sulphate, BKC, and trichlorfon stress

Five prawns were kept in each 60-L glass aquarium containing 40 L of test solutions with different concentrations of copper sulphate (0, 0.1, 0.2, 0.3, and 0.4 mg L\(^{-1}\)) and at different concentrations of BKC (0, 0.3, 0.6, and 1.0 mg L\(^{-1}\)), and ten prawns were kept in each 60-L glass aquarium containing 40 l of test solutions with different concentrations of trichlorfon (0, 0.2, and 0.4 mg L\(^{-1}\)). Prawns were exposed to the test solution for 96 h. Six replicates of each test were run. For measurement of PE expression, haemolymph was sampled individually at the beginning and after 48 and 96 h of exposure. Prawns were fed as described above.

2.5. Peroxinectin expression of prawns

2.5.1. Haemocyte collection

Haemolymph (0.50 ml) was individually withdrawn from the ventral sinus cavity of each shrimp into a 1-ml sterile syringe (25 gauge) containing 0.5 ml of a pre-cooled (4 °C) anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, and 10 mM EDTA, at pH 7.5 and with an osmolality of 780 mOsm kg\(^{-1}\)). The diluted haemolymph was centrifuged at 500 \(g\) and 4 °C for 20 min, and the haemocyte pellet was washed once with cacodylate buffer (10 mM sodium cacodylate, 0.45 M sodium chloride, and 20 mM calcium chloride; pH 7.0). The resulting haemocyte pellet was then used for the total RNA isolation.

2.5.2. Total RNA isolation and reverse transcription (RT)

Total RNA was extracted and purified using the guanidinium thiocyanate method described by Chomczynski and Sacchi [28]. First-strand cDNA synthesis by reverse transcription (RT) was as described previously [29].

2.5.3. Quantification of peroxinectin gene expression by real-time RT–PCR

In all tests, the mRNA expression of peroxinectin was measured by real-time RT–PCR. The cDNA was used for the assay of real-time RT–PCR. The SYBR green I real-time RT–PCR assay was carried out in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The amplifications were performed in a 96-well plate in a 25-μl reaction volume containing 12.5 μl of 2 × SYBR Green Master Mix (Perkin-Elmer Applied Biosystems), 2.5 μl each of the forward (5′-CAACGTCTTGTGATGATCGAT-3′) and reverse (5′-CGCCACTGCACTCACAGATG-3′) primers (10 μM), 1 μl of template (1 μg cDNA), and 9 μl of DEPC-water. The thermal profile for the SYBR green real-time RT–PCR was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was run in duplicate. DEPC-water replaced the template as the negative control. The same method was applied to detect the β-actin gene of prawns using specific primers (forward: 5′-CATCACCAACCTGGGAGACATGGA-3′; reverse: 5′-GAGCAACACCGAGATTCTGTTGT-3′) at different moult stages, and controls and treatments as a housekeeping gene.

Data analysis of the RT–PCR was performed with SDS software version 2.0 (PE Applied Biosystems). Relative quantitation of gene expression was performed according to the manufacturer’s instructions. Briefly, the threshold PCR cycle (Ct) is defined as the cycle number at which a statistically significant increase in the fluorescence of SYBR green against the internal passive dye, ROX (ΔRn), is first detected. The copy number of the target gene and Ct values are inversely related; thus, a sample containing a higher number of copies of the target gene has a lower Ct value than that of a sample with a lower number of copies of the same target. Differences in the Ct values of PE genes and the corresponding internal control β-actin gene, called ΔCt, were calculated to normalise for any difference in the amount of total RNA added to the cDNA reaction mixture and the efficiency of the reverse-transcription reaction. The value of ΔCt for the treated sample was subtracted from the value of ΔCt of the control sample. The difference was expressed as the ΔΔCt value that allowed measurement of the change in expression of PE genes in the treatment sample relative to the control sample. The test of PE expression of prawns was based on different moult
stages, the stage C was used as a control sample. A 3.3-fold change in the Ct value was considered to be equivalent to a 10-fold change in expression level.

2.6. **Statistical analysis**

A multiple comparison (Tukey) test was conducted to compare the significant differences of peroxinectin gene expression of prawns based on moult ing; prawns were fed diets containing different concentrations of sodium alginate, and were exposed to different concentrations of copper sulphate, BKC, and trichlorfon using the SAS computer software (SAS Institute, Cary, NC, USA). A significance level of $p = 0.05$ was chosen.

3. **Results**

PE mRNA transcripts were measured in haemocytes of prawns from different moult stages using real-time RT–PCR. There were no significant differences in PE mRNA transcription for the prawns between stages $D_{0/1}$ and $D_{2/3}$, or between stages C and $D_{2/3}$. PE mRNA transcription of prawns at stage A was significantly higher than that of prawns at stage B; the PE mRNA transcription of prawns at stage B was significantly higher than that of prawns at stage C; and the PE mRNA transcription of prawns at stage C was significantly higher than those of prawns at stage B.

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**Fig. 1.** Analysis of haemocyte peroxinectin (PE) mRNA expression in *Macrobrachium rosenbergii* based on different moult stages by SYBR green RT–PCR. Each bar represents the $\Delta Ct$ of PE (the Ct value of the PE gene minus the Ct value of the $\beta$-actin gene) as measured by SYBR green RT–PCR. Numbers above the bars indicate the $\Delta\Delta Ct$ value (the $\Delta Ct$ value of PE in each treatment shrimp minus the $\Delta Ct$ value of PE in shrimp at stage C) (A). A change in the Ct value ($\Delta\Delta Ct$) of 3.3 is equivalent to a 10-fold difference between different moult stages and shrimp at stage C (B). Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among moult stages.
The PE gene expression was 17.9-fold ($\Delta \Delta C_t = -5.9$) and 10.4-fold ($\Delta \Delta C_t = -3.4$) higher in prawns at stages A and B, respectively, and was 7.9-fold ($\Delta \Delta C_t = 2.6$) and 5.5-fold ($\Delta \Delta C_t = 1.8$) lower in prawns at stages D0/D1 and D2/D3, respectively, than in prawns at stage C (Fig. 1).

The mRNA expressions of PE in haemocytes of prawns fed the 0, 1.0, and 2.0 g kg$^{-1}$ sodium alginate-containing diets for 14 days were measured using real-time RT–PCR. After 14 days, PE gene expression was significantly higher in prawns fed the 1.0 g kg$^{-1}$ sodium alginate-containing diet than those fed the 2.0 g kg$^{-1}$ sodium alginate-containing diet and the control diet (0 g kg$^{-1}$). However, no significant difference in PE gene expression was observed between the 2.0 g kg$^{-1}$ sodium alginate-containing diet and the control diet. The PE gene expression was 5.3-fold ($\Delta \Delta C_t = -1.7$) higher in prawns fed the 1.0 g kg$^{-1}$ sodium alginate-containing diet than those fed the control diet (Fig. 2).

PE mRNA transcripts were measured in haemocytes of prawns exposed to different concentrations of copper sulphate, BKC, and trichlorfon using real-time RT–PCR at the beginning and after 48 and 96 h of exposure. There were no significant differences in PE mRNA transcription in prawns following 48 h exposure to 0–0.4 mg L$^{-1}$ copper sulphate. After 96 h, the PE mRNA transcription of prawns directly decreased with increasing concentrations of copper sulphate in the range 0.1–0.4 mg L$^{-1}$, and the PE mRNA transcription of prawns placed in 0.4 mg L$^{-1}$ copper sulphate was significantly lower than that of prawns placed in the control solution. The PE gene expression was...
11.8- ($\Delta \Delta Ct = 3.9$), 9.3- ($\Delta \Delta Ct = 3.1$), 6.9- ($\Delta \Delta Ct = 2.3$) and 3.1-fold ($\Delta \Delta Ct = 1.0$) lower in prawns exposed to 0.4, 0.3, 0.2 and 0.1 mg L$^{-1}$ copper sulphate, respectively than those exposed to control solution (Fig. 3).

The PE mRNA transcription levels of prawns following 48 h of exposure to 0.6 and 1.0 mg L$^{-1}$ BKC were significantly lower than those of prawns exposed to the 0.3 mg L$^{-1}$ BKC and control solutions. After 96 h, PE mRNA transcription levels of prawns exposed to 1.0 mg L$^{-1}$ BKC were significantly lower than that of prawns exposed to 0.3 and 0.6 mg L$^{-1}$ BKC, and the PE mRNA transcription levels of prawns exposed to 0.3 and 0.6 mg L$^{-1}$ BKC were significantly lower than that of prawns exposed to control solution. The PE gene expression was 24.5- ($\Delta \Delta Ct = 8.1$), 8.7- ($\Delta \Delta Ct = 2.9$) and 10.1-fold ($\Delta \Delta Ct = 3.3$) lower in prawns exposed to 1.0, 0.6 and 0.3 mg L$^{-1}$ BKC, respectively than those exposed to the control solution (Fig. 4).

PE mRNA transcription levels in prawns exposed to 0.2 and 0.4 mg L$^{-1}$ trichlorfon were significantly lower than those of prawns exposed to the control solution from 48 to 96 h. PE mRNA transcription levels of prawns following 48 h of exposure to 0.2 and 0.4 mg L$^{-1}$ trichlorfon were significantly lower than those following 96 h of exposure. The PE gene expression was 20.2- ($\Delta \Delta Ct = 6.6$) and 16.4-fold ($\Delta \Delta Ct = 5.4$) lower in prawns exposed to 1.0, 0.6 and 0.3 mg L$^{-1}$ BKC, respectively than those exposed to the control solution after 96 h (Fig. 5).

Fig. 3. Analysis of haemocyte peroxinectin (PE) mRNA expression by SYBR green RT–PCR in *Macrobrachium rosenbergii* exposed to 0, 0.1, 0.2, 0.3, and 0.4 mg L$^{-1}$ copper sulphate after 48 and 96 h. Each bar represents the $\Delta Ct$ of PE (the Ct value of the PE gene minus the Ct value of the $\beta$-actin gene) as measured by SYBR green RT–PCR. The $\Delta \Delta Ct$ (the $\Delta Ct$ value of PE in each treatment shrimp minus the $\Delta Ct$ value of PE in control shrimp) represents the relative expression of PE between two treatments (A). A change in the Ct value ($\Delta \Delta Ct$) of 3.3 is equivalent to a 10-fold difference between treatment and control shrimp (B). See Fig. 2 for statistical data.
4. Discussion

The immune competence activities in relation with the moult cycle have been reported in M. rosenbergii [22,30]. Among stages A, B, C, D0/D1, and D3 of M. rosenbergii, the total haemocyte count (THC) is lowest in stage D3 and then increases gradually from stage A to C and reaches the highest in stage C [30], while PO activity is significantly higher in stage C and lower in stages A, B, D0/D1, and D3 [22]. There are fewer respiratory bursts in stage C [22]. Phagocytic activity and clearance efficiency of prawns to the pathogen Lactococcus garvieae significantly decrease in stages A, D0/D1, and D3 compared to those in stage C [22]. Among stages A, B, C, D0/D1, and D2/D3 of M. rosenbergii, the proPO mRNA expression of prawns is significantly increased in stage A, and achieves the highest level in stage B, and then sharply decreases in stage C and reaches the lowest in stage D2/D3 in our previous study [31], and the PE mRNA expression of prawns was the highest in stage A, significantly decreased in stage B, and reached the nadir in stage D0/D1 in the present study.

For patterns of haemocyte production and release throughout the moult cycle in the penaeid shrimp Sicyonia ingentis, Hose et al. [32] indicated that large numbers of maturing haemocytes including hyaline and granular haemocytes were channelled into the vessel lumens immediately after moult. Recently, the mRNA of proPO of
Pacifastacus leniusculus was detected by in situ hybridisation with a digoxigenin-labelled cDNA probe, and it was found that proPO was not expressed in haematopoietic tissue, but haemocytes released into the circulation expressed proPO\[33\]. These facts suggest that PE mRNA expression of Macrobrachium rosenbergii occurs earlier than proPO mRNA transcription in newly synthesised haemocytes released from haematopoietic tissue, and accumulation of PE protein may reach the highest level in stage C which has the highest phagocytic activity\[22\]. However, the mechanism of higher PE mRNA expression in stage A and lower expression in stages C, D0/D1, and D2/D3 is still undetermined.

The immune stimulatory effects of immunostimulants like glucan, chitosan, and other polysaccharides have been widely studied in crustaceans\[34\]. Our previous study showed that dietary administration of sodium alginate at 2.0 g kg\(^{-1}\) or less increases phenoloxidase activity, respiratory bursts, SOD activity, phagocytic activity, and clearance efficiency against Vibrio alginolyticus of white shrimp Litopenaeus vannamei\[24\]. In the present study, the PE gene expression was significantly higher in prawns fed the 1.0 g kg\(^{-1}\) sodium alginate-containing diet than those fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet and control diet after 14 days. However, no significant difference was observed between prawns fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet and the control diet. After 7 days, prawns fed the diet containing sodium alginate at 2.0 g kg\(^{-1}\) showed slightly decreased PE mRNA transcription, as compared to those fed the control diet. These facts suggest that sodium alginate added to the diet can enhance immune ability, but in excessive amounts may cause suppression or have no affect in prawns.
In decapod crustaceans, it is known that environmental changes may affect the immune ability leading to susceptibility against pathogenic infections. These environmental variations are often stressful for crustaceans, resulting in a reduction of immune vigour as measured by THC, proPO activation, release of free oxygen radicals, phagocytic activity, and clearance efficiency. Environmental stress can deeply alter the defence since it has been shown that proPO and PE gene expressions are seriously affected by ammonia [35].

In *M. rosenbergii* following exposure to $0.1-0.4 \text{ mg L}^{-1}$ copper sulphate, $0.3-1.0 \text{ mg L}^{-1}$ BKC, and $0.2-0.4 \text{ mg L}^{-1}$ trichlorfon after 96 h, PO activity was significantly decreased, but respiratory bursts significantly increased. However, no significant differences in haemocyte density were observed among treatments [20,21,25]. Prawns exposed to 0.2 and $0.4 \text{ mg L}^{-1}$ trichlorfon showed decreased SOD activity from 48 to 144 h. Phagocytic activity and clearance efficiency towards *L. garvieae* significantly decreased after prawns were exposed to 0.2 and $0.4 \text{ mg L}^{-1}$ trichlorfon for 48 h [25]. *Macrobrachium rosenbergii* exposed to $0.1-0.4 \text{ mg L}^{-1}$ copper sulphate, $0.3-1.0 \text{ mg L}^{-1}$ BKC, and $0.2-0.4 \text{ mg L}^{-1}$ trichlorfon also showed decreased PE gene expression from 48 to 96 h in the present study.

Similar to PO activity and antibiotic peptides, anti-oxidative enzymes are important components necessary for a fully functioning crustacean immune system [19]. PE plays an important role in the cellular responses of phagocytosis, encapsulation, and nodule formation in crustaceans [19]. The PE mRNA transcription of *M. rosenbergii* significantly decreased under copper sulphate, BKC, and trichlorfon stress suggesting that copper sulphate, BKC, and trichlorfon in water weaken the cellular immunity of prawns.

$O_2^*$ is implicated as a toxic species in free radical-mediated cytotoxicity when SOD limits cell injury, and is considered to be beneficial with respect to increased immunity. However, excess generation is a key mediator of cell injury [20,36]. Therefore, the concentration of ROIs has to be tightly regulated in living systems, which include antioxidant enzymes and small antioxidant molecules. PE is one of the anti-oxidative enzymes that scavenger hydrogen peroxide ($H_2O_2$) in crustaceans [19]. From our previous and present studies, we suggest that copper sulphate, BKC, and trichlorfon induce excessive accumulation of ROIs [20,21,25] possibly resulting from decreases in transcription and translation of anti-oxidative enzymes gene.

In conclusion, the present study documents that the PE mRNA expression of *M. rosenbergii* varies with moult stages, and is the highest in stage A, decreases significantly in stage B, and reaches the nadir in stages D0/D1. PE mRNA expression significantly increased in prawns fed a diet containing 1.0 g kg$^{-1}$ sodium alginate, but significantly decreased under copper sulphate, BKC, and trichlorfon stress. To add sodium alginate in the diet, and to decrease frequency or forbidden usage of chemotherapeutants may help immune ability and avoid immune suppression with benefits for prevention and control of disease outbreaks in prawn farming.

Acknowledgements

This paper was supported by a grant (NSC93-2317-B-001-007) from the National Science Council of the R.O.C.

References


