Effect of saponin on hematological and immunological parameters of the giant freshwater prawn, *Macrobrachium rosenbergii*

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Received 7 June 2006; received in revised form 31 August 2006; accepted 31 August 2006

Abstract

Giant freshwater prawns, *Macrobrachium rosenbergii* (17.9±2.7 g), exposed to different concentrations of saponin at 0, 0.3, 0.6, 0.9 and 1.2 mg l⁻¹ for 168 h were examined for osmolality, electrolyte levels, oxyhemocyanin, protein levels, acid-base balance status, total hemocyte count (THC), phenoloxidase activity, and respiratory bursts. Hemolymph oxyhemocyanin, protein, and pO₂ were inversely related to the saponin concentration. Hemolymph oxyhemocyanin, protein, pO₂, pCO₂, and pH of prawns exposed to 1.2 mg l⁻¹ saponin were significantly lower than those of prawns exposed to 0.3 mg l⁻¹ and control solutions. However, no significant difference was observed in osmolality or electrolyte levels of prawns exposed to different concentrations of saponin for 168 h. The THC of prawns following 168 h of exposure to 0.9 and 1.2 mg l⁻¹ saponin increased, but the phenoloxidase activity decreased suggesting that the decrease in phenoloxidase activity under saponin stress was not a consequence of the increase in THC. We concluded that saponin at as low as 0.9 mg l⁻¹ decreases the respiratory protein level and acid-base balance, and modulates the immune system of *M. rosenbergii*.

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Keywords: *Macrobrachium rosenbergii*; Hemolymph; Oxyhemocyanin; Acid-base balance; Hemocyte count; Phenoloxidase activity; Respiratory burst

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0444-8486/ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2006.08.051
1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is commercially important in the world as a primary inland cultured species. Disease outbreaks caused by yeast infections in the cool season and bacteria in the hot season have resulted in declining production of farmed prawns in Taiwan (Hsu, 1993; Cheng and Chen, 1998; Chen et al., 2001).

Several studies demonstrated the disruption of normal osmotic and ionic balance after exposure to pollutants (Caldwell, 1974; Inman and Lockwood, 1977; Neufeld and Pritchard, 1979), and osmoregulatory capacity has been proposed as a potential indicator of the physiological condition and a stress indicator among crustaceans (Boitel and Truchot, 1989; Charmantier et al., 1989; Young-Lai et al., 1991; Lin et al., 1993; Bambang et al., 1995a,b). Hemolymph hemocyanin protein levels, and the acid-base balance of crustaceans are altered by changes in ambient salinity, temperature, dissolved oxygen (Truchot, 1983; Ferraris et al., 1986), ammonia-N (Chen and Cheng, 1993a), and saponin (Chen and Chen, 1996). Previous studies indicated that both sublethal and lethal concentrations of trichlorfon affect the hemolymph acid-base balance, osmolality, and ion concentrations in *M. rosenbergii* kept in freshwater systems (Yeh et al., 2005; Chang et al., 2006).

In decapod crustaceans, hemocytes are involved in phagocytosis, which eliminates microbes or foreign particles (Bachère et al., 1995; Johansson, 1995). Hemocytes are associated with proteins like prophenoloxidase (proPO) which is involved in encapsulation, melanization, and cytotoxicity as a non-self recognition system (Johansson and Söderhäll, 1989). Phenoloxidase is the terminal enzyme in the proPO activation system and is activated by several microbial polysaccharides, including β-1,3-glucan from fungal cell walls (Smith et al., 1984). A high pH and low dissolved oxygen (DO) have been reported to cause reductions in hemocyte counts in *M. rosenbergii* (Cheng and Chen, 2000; Cheng et al., 2002b) and in blue shrimp *Litopenaeus stylirostris* (Le Moullac et al., 1998). Primary physicochemical changes like high temperature, high pH, and low dissolved oxygen, and environmental toxicants like ammonia, nitrite, and copper sulfate have been reported to decrease the phenoloxidase activity of *M. rosenbergii* (Cheng and Chen, 2000, 2002; Cheng and Wang, 2001, Cheng et al., 2002a,b).

Reactive oxygen species (ROS) like superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^-$) are produced during phagocytosis. This phenomenon, known as a respiratory burst, plays an important role in microbicidal activity (Song and Hsieh, 1994). The generation of O$_2^-$ has been reported in hemocytes of tiger shrimp, *Penaeus monodon* (Song and Hsieh, 1994), *L. stylirostris* (Bachère et al., 1995), and white shrimp, *Litopenaeus vannamei* (Muñoz et al., 2000). Environmental toxicants like ammonia, nitrite, copper sulfate, and trichlorfon have been reported to increase the release of superoxide anions in *M. rosenbergii* (Cheng and Wang, 2001; Cheng and Chen, 2002; Cheng et al., 2002a; Chang et al., 2006).

Tea seed cake, the residue of *Camellia* sp. seeds after oil extraction contains 5.2%–7.2% saponin (Minsalan and Chiu, 1986). Tea seed cake is widely used as a piscicide. The application of tea seed cake is very effective in eradicating predatory fish in prawn ponds (Terazaki et al., 1980). An application rate of tea seed cake of 15 mg l$^{-1}$ is considered suitable (Minsalan and Chiu, 1986). Since prawn farmers often apply excess amounts of tea seed cake in pond management, the concentration of saponin in the water and its effect on the physiological and immune systems of cultured prawn are of primary concern. The 24- and 96-h LC$_{50}$ values (median lethal concentration) of saponin to Kuruma shrimp, *Marsupenaeus japonicus*, juveniles have been reported to be 27.08 and 18.14 mg l$^{-1}$, respectively (Chen et al., 1996). Saponin has been reported to decrease the hemocyanin level and affect the acid-base balance of *M. japonicus* (Chen and Chen, 1996). However, little is known about the effect of saponin on lethality, physiological responses, and immune responses of *M. rosenbergii*.

In the present study, we attempted to examine physiological and immune responses of *M. rosenbergii* to
saponin stress. Hemolymph osmolality, and electrolyte, oxyhemocyanin, and protein levels, and the acid-base balance including hemolymph $pO_2$, $pCO_2$, pH, $HCO_3^-$, and $TCO_2$ were determined in order to characterize the physiological response. The total hemocyte count (THC), phenoloxidase activity, and respiratory bursts (production of superoxide anion) were examined in order to characterize the immune response.

2. Materials and methods

2.1. M. rosenbergii

*M. rosenbergii* individuals were obtained from a commercial farm in Pingtung, southern Taiwan, and acclimated in the laboratory for 2 wk before experimentation. In order to minimize intrinsic variations, only prawns in the intermolt stage (stage C) were used in this study. The molt stage was determined by examination of the uropoda in which partial retraction of the epidermis could be distinguished (Peebles, 1977). Prawns weighed 15–21 g with an average of 17.9±2.7 g (mean±SD), and had total lengths of 10.6–14.8 cm with an average of 12.1±0.3 cm (mean±SD). No significant difference in weight was observed among the treatments. For the physiological study and immune activity assays, tests were carried out on 10 replicates which were observed for 168 h. During the acclimation and experimental periods, the water temperature was maintained at 28±1 °C, pH at 7.1–7.5, total hardness at 100 mg l$^{-1}$, osmolality at 2 mOsm kg$^{-1}$, $Na^+$ at 0.5 mmol l$^{-1}$, $Ca^{2+}$ at 0.09 mmol l$^{-1}$, and $Mg^{2+}$ at 0.34 mmol l$^{-1}$. However, the concentrations of $K^+$ and $Cl^-$ were too low to be determined. Prawns were fed twice daily with a formulated prawn diet (Shinta Feed Company, Pingtung, Taiwan).

2.2. Test solution

Saponin test solutions were first prepared by dissolving 4.505 g of saponin (Sigma, St. Louis, MO, USA, product no. S7900, from Quillaja bark containing 11.1% saponin) with 500 ml distilled water to make 1000 mg l$^{-1}$ saponin as a stock solution. Saponin test solutions were prepared by diluting appropriate amounts of the stock solution with water to make concentrations of 0, 0.3, 0.6, 0.9, and 1.2 mg l$^{-1}$ saponin.

2.3. Effects of saponin on the physiological response

Prawns were sampled from the holding tank and individually transferred to 20-l circular plastic tanks containing 10 l of test solution. There were 5 treatments, and 10 replicates for each treatment with 1 prawn in each replicate. The test solution was renewed daily, and the experiment lasted for 168 h.

At the end of the experiment, individual hemolymph samples were taken using a 1-ml sterile syringe (25-gauge) from the ventral sinus of each prawn. Hemolymph osmolality and medium osmolality were measured by injecting 20 μl of a sample into a micro-osmometer (Model 3MO plus, Advanced Instruments, Norwood, MA, USA). To determine $Na^+$, $K^+$, and $Cl^-$, 100 μl of a hemolymph sample was immediately injected into an Ion Selective Electrode Analyzer (Medica EasyLyte PLUS, Bedford, MA, USA).

Hemolymph protein was determined with Bio-Rad Protein Assay Kit no. 500-0006 (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin (66 kDa) as the standard, through a method derived from Bradford (1976). Hemolymph oxyhemocyanin was measured using the method described by Hagerman (1986) and Chen and Cheng (1993b). The ratio of oxyhemocyanin to protein was calculated by dividing the concentration of oxyhemocyanin (mmol l$^{-1}$) by that of protein (mmol l$^{-1}$) which was converted from mg ml$^{-1}$ to mmol l$^{-1}$ by dividing by 66 (Chen and Cheng, 1993b).

Hemolymph pH, $pCO_2$, and $pO_2$ levels were determined by immediately injecting the hemolymph (40 μl) into a pH/blood-gas analyzer (Stat Profile pHOx Analyzer, NoVa Biomedical, Waltham, MA, USA) with the thermostat set to 37 °C, and corrected automatically to a value at 28 °C (NOVA, 1998). Hemolymph $HCO_3^-$ and $TCO_2$ were calculated based on the formulae from the data for pH and $pCO_2$ (NOVA, 1998).

2.4. Effects of saponin on the immune parameters of M. rosenbergii

Individuals of *M. rosenbergii* were obtained, fed, and treated as described above. Hemolymph (100 μl) was withdrawn from the ventral sinus of each prawn into a 1-ml sterile syringe (25-gauge) containing 0.9 ml of an anticoagulant solution (0.114 M trisodium citrate and 0.1 M sodium chloride, at pH 7.45 and with an osmolality of 490 mOsm kg$^{-1}$). A drop of the hemolymph suspension was placed on a hemocytometer, and the total hemocyte count (THC) was assessed using a microscope. The remaining portion of the hemolymph mixture was used for subsequent (phenoloxidase activity and respiratory burst) tests.

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced following the procedures of Herández-López...
et al. (1996). The diluted hemolymph was centrifuged at 300 ×g at 4 °C for 10 min, the supernatant fluid was discarded, and the pellet was rinsed, re-suspended gently in 1 ml cacodylate–citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate; pH 7.0), and then centrifuged again. The pellet was then re-suspended in 200 μl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.26 M magnesium chloride; pH 7.0). One hundred microliters of the cell suspension was incubated with 50 μl of trypsin solution (1 mg trypsin dissolved in 1 ml cacodylate buffer), which served as an elicitor, for 10 min at 25 °C (1 mg trypsin dissolved in 1 ml cacodylate buffer), followed by the addition of 800 μl of cacodylate buffer 5 min later. The optical density at 490 nm was measured using a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The control solution which consisted of 100 μl of the cell suspension, 50 μl of cacodylate buffer (to replace the trypsin solution), and 50 μl of L-DOPA was used for the background phenoloxidase activity in all test conditions. The optical density values of the background phenoloxidase activity were in the range of 0.03–0.05. The optical density of phenoloxidase activity was expressed as dopachrome formation per 50 μl of hemolymph.

Respiratory bursts of hemocytes were quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O2−) production (Bell and Smith, 1993; Song and Hsieh, 1994; Le Moullac et al., 1998). One hundred microliters of hemolymph in an anticoagulant solution was deposited in microliter plate wells previously coated with 100 μl of a poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 700 ×g for 15 min. Plasma was removed, and then 100 μl zymosan (0.1% in Hank’s solution minus phenol red) was added and allowed to react for 30 min at room temperature. The zymosan was discarded, and the hemocytes were washed 3 times with 100 μl of Hank’s solution, and then stained with 100 μl of an NBT solution (0.3%) for 30 min at room temperature. The NBT solution was removed and the hemocytes were fixed, washed 3 times with 100 μl of 70% methanol, and air-dried. The formazan was dissolved by adding 120 μl of 2 M KOH and 140 μl of DMSO. The optical density at 630 nm was measured in triplicate using an ELISA reader (Dynex Mrx II, Chantilly, VA, USA). Respiratory bursts were expressed as NBT reduction per 10 μl of hemolymph.

2.5. Statistical analysis

A multiple comparisons (Tukey’s) test was conducted to compare significant differences among treatments using the SAS computer software-ANOVA procedure (SAS Institute, Cary, NC, USA). Before analysis, the percent data (oxyhemocyanin/protein ratio) were normalized using an arc-sine transformation before analysis. Statistically significant differences required that p<0.05.

3. Results

3.1. Effect of saponin on physiological response of Macrobrachium rosenbergii

No significant differences in osmolality, or Cl−, Na+, or K+ concentrations were observed among the prawns exposed to 0, 0.3, 0.6, 0.9, and 1.2 mg l−1 saponin after 168 h. Hemolymph osmolality varied from 423.3±11.2 to 440.6±7.0 mOsm l−1; Cl− varied from 211.9±2.7 to 229.3±9.2 mmol l−1, Na+ varied from 204.0±7.3 to 212.3±2.9 mmol l−1, and K+ varied from 3.8±0.3 to 4.4±0.1 mmol l−1.

Hemolymph oxyhemocyanin decreased with increasing concentrations of saponin below 0.9 mg l−1. Hemolymph oxyhemocyanin of prawns exposed to 0.6, 0.9, and 1.2 mg l−1 saponin were significantly lower than those of prawns exposed to 0.3 mg l−1 saponin and the control solutions (Fig. 1A). Hemolymph pH, pCO2, PO2, HCO3−, and TCO2 levels decreased with increasing concentrations of saponin (Table 1). Hemolymph pH and pCO2 of prawns exposed to 1.2 mg l−1 saponin were significantly lower than those of prawns exposed to ≤0.6 mg l−1 saponin. Hemolymph pO2 values of prawns exposed to 0.9 and 1.2 mg l−1 saponin were significantly lower than those of prawns exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control. Hemolymph HCO3− and TCO2 of prawns exposed to 0.9 and 1.2 mg l−1 were significantly lower than those exposed to 0.3 mg l−1 and the control.

![Graph](image) Fig. 1. Mean (±SE) hemolymph oxyhemocyanin of Macrobrachium rosenbergii exposed to different concentrations of saponin. Each bar represents the mean value from 10 determinations with the standard error. Data with different letters significantly differ (p<0.05) among treatments.
saponin were significantly lower than those of prawns exposed to 0.3 mg l$^{-1}$ saponin and the control.

3.2. Effects of saponin on the immune parameters of *M. rosenbergii*

No significant differences in THCs were observed among the prawns following 168 h of exposure to 0, 0.3, 0.6, and 0.9 mg l$^{-1}$ saponin. However, the THC of prawns following 168 h of exposure to 1.2 mg l$^{-1}$ saponin was significantly higher ($p<0.05$) than those of prawns exposed to $\leq 0.6$ mg l$^{-1}$. The THCs of prawns following 168 h of exposure to 0.9 and 1.2 mg l$^{-1}$ saponin increased by 16% and 43%, respectively, as compared to control prawns (Fig. 2A).

No significant differences in phenoloxidase activity were observed among prawns following 168 h of exposure to $\leq 0.9$ mg l$^{-1}$ saponin. However, phenoloxidase activity of prawns following 168 h of exposure to 1.2 mg l$^{-1}$ was significantly lower than those exposed to $\leq 0.6$ mg l$^{-1}$ saponin. Phenoloxidase activities of prawns following 168 h of exposure to 0.9 and 1.2 mg l$^{-1}$ saponin decreased by 18% and 30%, respectively, compared to control prawns (Fig. 2B).

No significant differences in respiratory bursts (the release of superoxide anions) were observed among prawns following 168 h of exposure to 0, 0.3, 0.6, 0.9, and 1.2 mg l$^{-1}$ saponin.

4. Discussion

In the present study, hemolymph osmolality, and Cl$^-$, Na$^+$, and K$^+$ concentrations of prawns following exposure to 0–1.2 mg l$^{-1}$ saponin showed insignificant changes suggesting that osmollalic and electrolytic regulation by *M. rosenbergii* was not affected or that adaptive regulation was maintained following 168 h of exposure to 0.3–1.2 mg l$^{-1}$ saponin.

The respiratory pigment, hemocyanin, may account for 80%–95% of hemolymph proteins in arthropods (Jeuniaux, 1971). Chen and Chen (1996) indicated that when *M. japonicus* was exposed to 0–20 mg l$^{-1}$ saponin, hemolymph oxyhemocyanin, and the oxyhemocyanin/protein ratio decreased with an increasing concentration of saponin, and no significant difference in hemolymph proteins was observed with saponin exposure. They also indicated that the physiological response was more evident in oxyhemocyanin levels than in protein levels. In the present study, when *M. rosenbergii* was exposed to 0–1.2 mg l$^{-1}$ saponin for
168 h, hemolymph protein and oxyhemocyanin levels decreased, and the oxyhemocyanin/protein ratio slightly increased with an increasing concentration of saponin. This physiological response suggests that hemolymph proteins decrease mainly as a result of the decrease in oxyhemocyanin in *M. rosenbergii*.

Chen and Chen (1996) indicated that *M. japonicus*, following exposure to increased concentrations of saponin, showed decreased hemolymph pCO₂, HCO₃⁻, and ammonia-N indicating that the animal may utilize NH₄⁺ and HCO₃⁻ to form urea via the ornithine–urea cycle. In the present study, increases in hemolymph pCO₂, HCO₃⁻, and TCO₂ were found upon exposure of *M. rosenbergii* to >0.9 mg l⁻¹ saponin, which suggests that HCO₃⁻ may react with ammonia to form urea under saponin exposure in *M. rosenbergii*. Further research is needed to determine the hemolymph ammonia-N and urea levels of *M. rosenbergii* under exposure to saponin.

Low salinity has been reported to cause elevated hemolymph pH of the shore crab, *Carcinus maenas*, and blue crab, *Callinectes sapidus* (Truchot, 1973; Weiland and Mangum, 1975), and lead to an increase in oxygen affinity of hemocyanin with no change in pO₂. Research exposing *M. japonicus* to elevated saponin at 10 and 20 mg l⁻¹ for 24 h was conducted by Chen and Chen (1996) who reported that the hemolymph pH, pCO₂, HCO₃⁻, and TCO₂ of *M. japonicus* significantly decreased, while its hemolymph pO₂ increased. They also reported that the hemolymph oxyhemocyanin and protein of *M. japonicus* decreased, leading to a decrease in the oxygen affinity of hemocyanin. However, the present study indicated that reductions in oxyhemocyanin and protein levels were not accompanied by an increase in hemolymph pO₂. These contradictory results are thought possibly to be due to differences in exposure time, concentrations, and species.

It is well known that the life cycle, food intake, disease outbreak, pollutants, and environmental stresses affect both the quantity and quality of circulating hemocyte counts of crustaceans (Persson et al., 1987; Söderhäll et al., 1988; Smith and Johnston, 1992; Le Moullac and Haffner, 2000; Cheng and Chen, 2001). Circulating hemocytes are also affected by extrinsic factors such as temperature, pH, salinity, and dissolved oxygen, which have been reported to affect THCs in several species of decapod crustaceans. *M. rosenbergii* reared at temperatures of 27–28 °C and 30–31 °C had significantly higher THCs than those reared at 20–21 °C and 33–34 °C, and prawns reared at pH 7.5–7.7 had significantly higher THCs than those reared at pH 4.6–5.0 and 9.0–9.5 (Cheng and Chen, 2000). *M. rosenbergii* following 120 h exposed to dissolved oxygen of as low as 2.75 mg l⁻¹ DO showed decreases in THCs and hyaline cell numbers (Cheng et al., 2002b). In contrast, no significant differences in the THCs of *M. rosenbergii* were observed among prawn groups exposed to 0.07–3.18 mg l⁻¹ ammonia-N for 168 h (Cheng and Chen, 2002), 0.01–1.68 mg l⁻¹ nitrite-N for 168 h (Cheng et al., 2002a), 0–0.4 mg l⁻¹ copper sulfate for 96 h (Cheng and Wang, 2001), or 0–0.4 mg l⁻¹ trichlorfon for 168 h (Yeh et al., 2005). However, the present study indicated that the THCs of *M. rosenbergii* were significantly higher in prawns exposed to 1.2 mg l⁻¹ saponin after 168 h.

Le Moullac et al. (1998) reported that the THC and respiratory bursts of *L. stylirostris* following 24 h of hypoxia exposure at 1 mg l⁻¹ DO decreased, but its phenoloxidase activity and susceptibility to Vibrio infection increased. They indicated that the increase in phenoloxidase activity was related to a lower amount of plasma inhibitors regulating the prophenoloxidase system. Cheng et al. (2002b) indicated that the THC, hyaline cells, phenoloxidase activity, and superoxide anions of *M. rosenbergii* decreased following 120 h of exposure to low DO (2.75 mg l⁻¹), accompanied by an increase the susceptibility to *Lactococcus garvieae* infection. They also indicated that the resistance of prawn to *L. garvieae* was correlated with phenoloxidase activity and clearance efficiency, which were more relevant than THC, DHC, phagocytosis, or NBT reduction. Cheng and Chen (2002) indicated that the increased susceptibility of *M. rosenbergii* following exposure to ammonia-N was considered to be related to the decrease in phenoloxidase activity, which was more relevant than the hemocyte count or respiratory burst products to the resistance of prawns infected by *L. garvieae*. In the present study, THC increased, whereas phenoloxidase activity decreased for prawns after 168 h of exposure to 0.9 and 1.2 mg l⁻¹ saponin. This fact indicates that the decrease in phenoloxidase activity under saponin stress was not a consequence of the increase in THC, but may have possibly increased the susceptibility of prawns to the pathogen and increased hemolymph inhibitors regulating the prophenoloxidase system.

In crustaceans, an increase in the THC is considered to enhance the immune capability during periods of stress (Truscott and White, 1990) leading to disease resistance (Le Moullac et al., 1998). In addition, hemocytes not only play an important role in immune defense but also are involved in physiological functions including carbohydrate metabolism, and transport and storage of proteins and amino acids (Ratcliffe et al., 1985; Martin et al., 1991). In the present study, the circulating hemocytes of *M. rosenbergii* significantly increased, but its phenoloxidase activity significantly decreased under saponin stress. These facts suggest
saponin’s induction of the mobilization of the reserve pool of sessile hemocytes and/or an increase of hematopoiesis towards adaptive physiological functions and/or modulation of immunological functions in *M. rosenbergii*. Further research is necessary to determine the other immune parameters and the susceptibility of prawns to pathogens while under saponin stress.

Nitroblue tetrazolium (NBT) staining has been used for both qualitative and quantitative analyses of O$_2$ generated by hemocytes which is the first product of respiratory bursts (Holmblad and Söderhäll, 1999). Le Moullac et al. (1998) indicated that the production of superoxide radicals which decreased in hypoxic *L. stylirostris* was due to a decrease in the THC, suggesting that NADPH oxidase which is responsible for the production of superoxide was not affected by hypoxic conditions. In the present study, the fact that no significant differences in respiratory bursts or increases in THC were observed among the prawns exposed to 1.2 mg l$^{-1}$ saponin indicates that the activity of NADPH oxidase might have been depressed under saponin stress.

In conclusion, the present study documents that as low as 0.9 and 1.2 mg l$^{-1}$ saponin affects respiratory proteins and the acid-base balance, and decreases the immune competence of *M. rosenbergii* by decreasing its phenoloxidase activity. This might lead to increased susceptibility of *M. rosenbergii* to pathogens.

**Acknowledgements**

The paper was supported by the National Science Council (NSC91-2313-B-020-013) of the R.O.C. We appreciate Ms. C. S. Wang for her assistance with the experiments.

**References**


