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Effect of Dopamine Injection on the Hemocyte Count and Prophenoloxidase System of the White Shrimp *Litopenaeus vannamei*

PAN Luqing*, HU Fawen, and ZHENG Debin

Department of Aquaculture, the Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, *P. R. China*

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Abstract Effects of dopamine injection on the hemocyte count, phenoloxidase activity, serine proteinase activity, proteinase inhibitor activity and α_2-macroglobulin-like activity in *L. vannamei* were studied. Results showed that dopamine injection resulted in a significant effect on the parameters measured ($P<0.05$), while no significant difference was observed in the control group (0.85% NaCl). In the experimental groups, the hemocyte count reached the minimum in 3 h; granular and semi-granular cells became stable after 12 h and hyaline cells and the total hemocyte count became stable after 18 h. Phenoloxidase activity reached the minimum in 6 h, and then became stable after 9 h. Serine protease activity and proteinase inhibitor activity reached the minimum in 3 h, and α_2-macroglobulin-like activity reached the maximum in 3 h, and all the three parameters became stable after 12 h. The results suggest that the activating mechanisms of the proPO system triggered by dopamine are different from those triggered by invasive agents or spontaneously activated under a normal physical condition.

Key words *Litopenaeus vannamei*; dopamine injection; hemocyte count; prophenoloxidase system

1 Introduction

Crustaceans have a non-specific immune system including circulating hemocytes and various active factors existing in hemocytes or released to the hemolymph from the hemocytes upon activation. The prophenoloxidase (proPO) system plays a key role in immune recognition and defense (Söderhäll and Unestam, 1979; Söderhäll, K., 1982; Söderhäll and Smith, 1983; Söderhäll and Häll, 1984; Söderhäll and Cerenius, 1998; Lin, *et al*., 2007; Shrestha and Kim, 2008). It's a complex enzymatic cascade system localized inside hemocytes (granular and semi-granular cells) in an inactive form, which consists of serine proteases and other associated factors (Ashida and Söderhäll, 1984; Hernández-López *et al*., 1996; Perazzolo and Barracco, 1997). The activating mechanisms of the proPO system in crustaceans triggered by invasive agents have been defined (Söderhäll and Cerenius, 1998); several papers involved in the gene expression of proteins like peroxinectin and serine proteinas have been published (Chiu *et al*., 2007; Dong *et al*., 2009; Lin *et al*., 2010). However, no study has been reported about the activating mechanisms of the crustacean proPO system under environmental stress.

In crustaceans, biogenic amines (BA) are widely distributed in the central nervous system and peripheral organs, which transduce signals as neuroregulators (neurotransmitters and neuromodulators) (Tierney *et al*., 2003), including dopamine (DA), histamine, 5-hydroxytryptamine (5-HT), norepinephrine (NE), octopamine and so on. It has been shown that environmental stress can lead to fluctuation of DA, NE and serotonin concentrations in crustacean hemolymph (Zatta, 1987; Péqueux *et al*., 2002). Environmental stressors have been reported to suppress the immune system of shrimps (Le Moullac and Haffner, 2000; Cheng and Chen, 2000; Liu and Chen, 2004). Cheng *et al*. (2005, 2006) found that after injection of BA, immune ability of the white shrimp *Litopenaeus vannamei* was decreased significantly. It is assumed that BA modulate the immunity of crustaceans (Cheng *et al*., 2005). At present, there have been studies focusing on the effect of dopamine on some immune parameters; however, no research has been published about the effect of BA injection on the hemocyte count and proPO system.

The white shrimp, *L. vannamei*, has high commercial value and excellent property of breeding, and in recent years, *L. vannamei* has become the main aquatic animals cultured in coastal regions of China. The present study was conducted to examine the effect of DA injection on

* Corresponding author. Tel: 0086-532-82032963
E-mail: panlq@ouc.edu.cn

the proPO system of *L. vannamei*, explore the moduculation of BA to *L. vannamei* and understand the physiological mechanisms of immunological regulation of shrimp.

2 Materials and Methods

2.1 Source of Experimental Shrimp and Rearing Conditions

Adults of white shrimp *L. vannamei* (12±0.5 g) were obtained from a shrimp farm in Yinghai, Qingdao, China. Prior to the experiment, the shrimps were acclimated in tanks (70 cm × 60 cm × 50 cm) containing aerated water (salinity 31, pH 8.1) with air-lift at (20±0.5℃) for 15 d. Half of the water in each tank was renewed twice daily. During the acclimation period, the shrimp were fed a formulated shrimp diet daily. Before the experiment the shrimp were starved for 48 h. Only healthy animals at the intermoult stage were used for experiment. The molt stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished (Robsertson *et al*., 1987).

2.2 Experimental Design

DA (Sigma, USA) was dissolved in sterile saline (0.85% NaCl) to concentrations of 2×10^{-3} mol L^{-1} and 2×10^{-2} mol L^{-1}. *L. vannamei* were injected with 50 μL 2×10^{-3} mol L^{-1} and 2×10^{-2} mol L^{-1} DA solutions individually into the ventral sinus of the cephalothoraxes and used as the challenge groups in which DA doses were $10^{-7}$and 10^{-6} mol per shrimp, respectively, while shrimps in the control group were injected with the same volume sterile saline. For each treatment, there were three replicate groups, and each group contained 55 shrimps. The experimental conditions were identical to those of the acclimation period. No shrimp died during the experiment. Six shrimps were sampled randomly from each group at 0, 3, 6, 9, 12, 18, 24, and 48 h.

2.3 Hemolymph Collection

After injection in each treatment, 200 μL hemolymph from each shrimp was withdrawn from the ventral sinus using a 5-gauge needle fitted to a 1.0 mL syringe containing an equal volume of sterile anticoagulant solution (450 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 10 mmol L^{-1} EDTA-Na_2, 10mmolL^{-1} HEPES, pH7.45, osmolality 1930 KPa) (Vargas-Albores *et al*., 1993). Samples of the hemolymph from six shrimps were mixed gently in an Eppendorf tube and processed or analyzed immediately.

2.4 Plasma and Hemocyte Lysate Supernatant (HLS) Preparation

HLS was prepared using methods modified from Smith and Söderhäll (1991). The diluted hemolymph (0.4 mL) was centrifuged at 700×*g* for 10 min at 4℃, and the supernatant fluid was stored at –80℃ as plasma sample. The pellet was rinsed, re-suspended gently in 0.6 mL ice cold cacodylate-citrate buffer (10 mmol L^{-1} sodium cacodylate, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} trisodium citrate, pH 7.0), and centrifuged again. The pellet was then re-suspended with 0.6 mL ice cold cacodylate (CAC) buffer (10 mmol L^{-1} sodium cacodylate, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} calcium chloride, 26 mmol L^{-1} MgCl, pH 7.0). This suspension was homogenized with a sonicator equipped with a microtip (output 20 W, duty cycle 30%) for 1 min, and centrifuged at 15 000×*g* for 20 min at 4℃. The supernatant fluid was stored at –80℃ as HLS.

2.5 Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC)

For the measurements of THC and DHC, 100 μL diluted hemolymph was fixed with an equal volume of 10% formaldehyde for 30 min at 4℃. A drop of the hemolymph suspension was placed on a hemocytometer, and THC and DHC were determined using an inverted phase contrast microscope (Olympus, Japan).

2.6 Phenoloxidase Activity Assay

Phenoloxidase activity in HLS was measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA; Sigma, USA) as a substrate, and trypsin (Sigma) as an elicitor following the method described by Söderhäll and Unestam (1979), Smith and Söderhäll (1991). 200 μL of HLS were incubated with 200 μL of 0.1% trypsin in CAC buffer at room temperature for 30 min, and then 200 μL of L-DOPA 0.3% in CAC buffer were added. Each reaction mixture was further diluted with 600 μL of CAC buffer and mixed. Optic density was measured at 490 nm. Absorbance measurements were made against a blank, consisting of CAC buffer, L-DOPA and elicitor, to control for spontaneous oxidation of the substrate alone. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1}. Protein content in HLS was measured via the method described by Bradford (1976), using bovine serum albumin as a standard protein.

2.7 Serine Proteinase Activity Assay

Serine proteases activity was investigated in HLS using a synthetic chromogenic substrate BAPNA (Na-Bz-Arg-r-Nitroanilide, Sigma, USA) (Perazzolo and Barracco, 1997). A sample of 100 μL of HLS was incubated with LPS (1 mg mL^{-1}) for 15 min at room temperature (25℃). The sample then received 500 μL of Tris-HCl buffer, pH 8.0 and 50 μL of chromogenic peptide. The mixture was incubated at 30℃ for 30 min and the enzyme reaction was ended by the addition of 200 μL of 50% (v:v) acetic acid. In the control, the HLS was replaced by TBS. The release of para-Nitroanilide from the chromogenic peptide was determined spectrophotometrically at 405 nm.

2.8 Plasma Proteinase Inhibitor Activity Assay

Bovine pancreatic trypsin (Sigma, USA) 100 μL (20 μg) in 0.1 mol L^{-1} Tris buffer pH 8.0 was incubated with 200 μL plasma for 10 min at room temperature. Controls

used Tris buffer to substitute shrimp plasma. Protease activity was measured by the hydrolysis of the low molecular weight substrate, N-benzoyl-DL-argininep-nitroanilide (BAPNA) (Sigma, USA). After 5 min, the released p-nitroanilide was measured at 405 nm for 2 min (Le Moullac *et al*., 1998).

The same procedure as above was used to detect an α_2-macroglobulin (α_2M) activity in plasma by adding 2 μL (40 μg) soybean trypsin inhibitor (SBTI, Sigma, USA) to the mixture of enzyme and plasma (Armstrong *et al*., 1990).

2.9 Statistical Analysis

All data were subjected to one-way ANOVA. If significant differences were indicated at the 0.05 level, then the Duncan Multiple Range test was used to identify significant differences among the treatments.

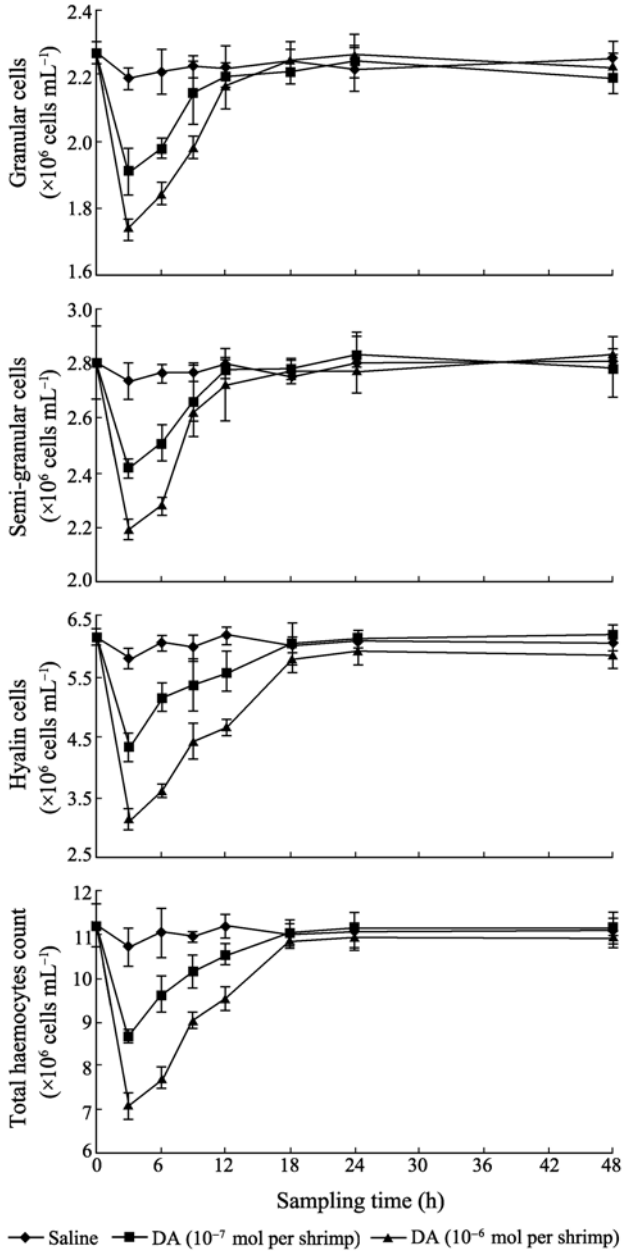

Fig.1 Effect of dopamine injection on hemocyte count of *Litopenaeus vannamei*.

3 Results

3.1 Effect of DA on Hemocyte Count of *L. vannamei*

Injection of DA had a significant effect on hemocyte count of *L. vannamei* ($P<0.05$), while no remarkable

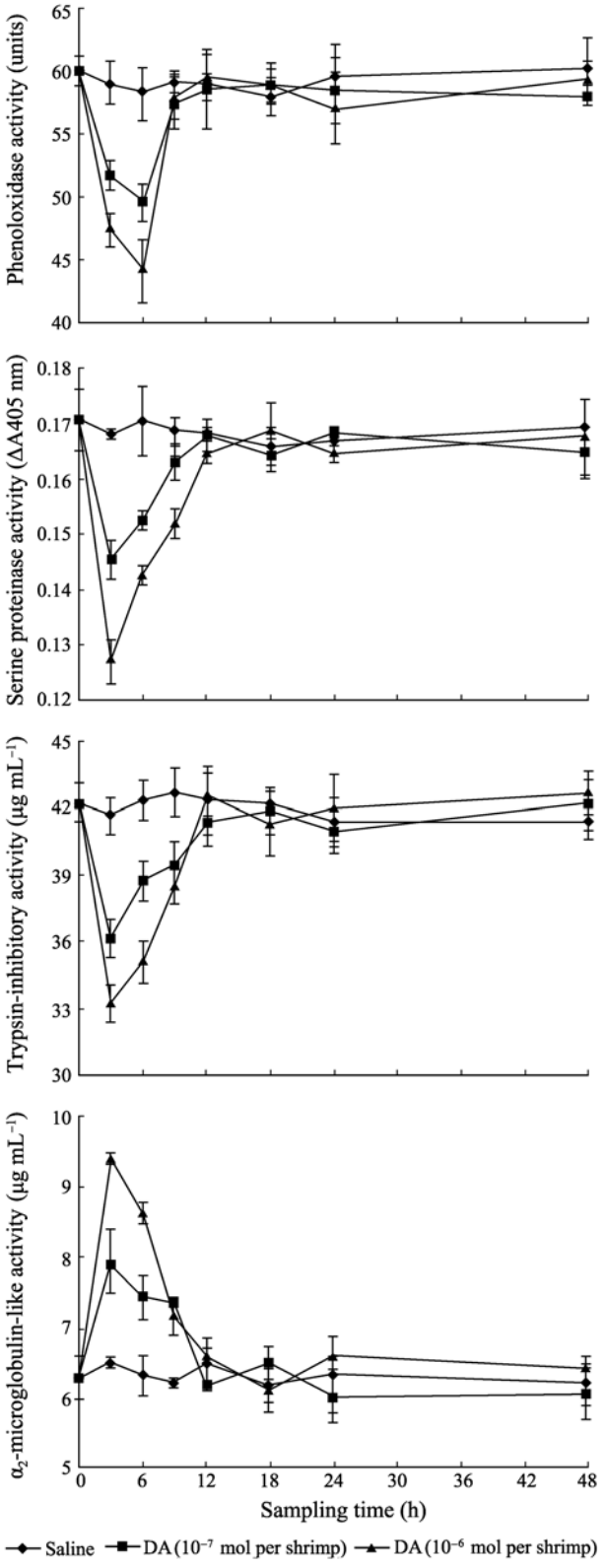

Fig.2 Effect of dopamine injection on the proPO system of *Litopenaeus vannamei* and associated factors.

difference was observed in the control group. Both THC and DHC in the different experimental groups reached the lowest at 3 h, and the peak values were negatively impacted by the dosage of DA injected for each parameter measured. THC and hyaline cells in the different experimental groups recovered to the control level after 18 h, while granular and semi-granular cells became stable after 12 h (Fig.1).

3.2 Effect of DA on the proPO System of *L. vannamei*

DA injection had a significant effect on phenoloxidase, serine proteases, proteinase inhibitor activity and α_2-M activity of *L. vannamei* ($P<0.05$), while no remarkable difference was observed in the control group. Serine proteases and proteinase inhibitor activity reached the minimum at 3 h and phenoloxidase activity at 6 h, while α_2-M activity reached the maximum at 3 h. The peak values for the former three parameters were negatively and that of the latter was positively, related to the dosage of DA injected. Phenoloxidase activity recovered to the control level after 9 h, while serine proteases and proteinase inhibitor activity and α_2-M activity after 12 h (Fig.2).

4 Discussion

It is commonly agreed that crustaceans, in general, do not possess immunoglobulins. Moreover, with an open circulatory system, they must have immediate, non-inducible defense and coagulation mechanisms to entrap parasites and prevent blood loss upon wounding. These reactions are carried out primarily by hemocytes. In decapod crustaceans, circulating hemocytes are generally classified into three types, *i.e.* hyaline, semi- granular and granular cells (Tsing *et al*., 1989). Hemocytes are involved not only in coagulation but also in the production of melanin via the proPO system, which has a role in recognition and defense (Johansson and Söderhäll, 1989; Söderhäll *et al*., 1996). The proPO system consists of serine proteases, phenoloxidase, β-1, 3-glucan binding protein (BGBP), peroxinectin, proteinase inhibitor and so on. Phenoloxidase is the terminal enzyme in the proPO system (Smith *et al*., 1984). The proPO system can be specifically activated by β-1, 3-glucans (Ashida *et al*., 1983; Leonard *et al*., 1985; Vargas-Albores *et al*., 1993), bacteria cell walls (Ashida *et al*., 1983; Rowley and Rahmet-Alla, 1990) and lipopolysaccharide (Söderhäll and Häll, 1984). Semi-granular cells are very sensitive, which can release components of the proPO system in response to environmental stressors, and once outside the cells proPO (76 KD) can be split into two active form phenoloxidases (60 and 62 KD). When in the active forms, the components of the proPO system such as BGBP and peroxinectin cause degranulation of semi-granular and granular cells through cell-cell communication, and then more components of the proPO system are released to the hemolymph and subsequently eliminate foreign particles (Smith *et al*., 1984; Aspán *et al*., 1990; Hernánhdez-López, 1996; Ashida and Brey, 1998; Jiang *et al*., 2004). In addition, proteinase inhibitors like pacifastin and α_2-M play an important role in controlling and regulating the activation of the proPO system (Häll and Söderhäll, 1982; Cerenius and Söderhäll, 2004).

Söderhäll and Unestam (1979) found that mechanisms of the proPO system of *Astacus astacus*, triggered by invasive agents (bacterium, fungus, *etc*.) and spontaneously activated under a normal physical condition, were completely different. Some authors have noted that Ca^{2+} is necessary in the activating process of the proPO system in crustaceans (Ashida and Söderhäll, 1984) and insects (Leonard *et al*., 1985; Brehélin *et al*., 1989). The ion Ca^{2+} activates proPO activating enzyme (PPAE) and then PPAE transforms proPO to an active form when both proteins are released from the cells after stimulus (Gollas-Galván *et al*., 1997). Environmental changes have been reported to start-up the proPO system of crustaceans as extrinsic stimulators or stressors, resulting in decreases of hemocyte count, phenoloxidase activity and other enzyme activities associated with immunity, and an increase in susceptibility to pathogens (Vagas-Albores *et al*., 1998; Perazzolo *et al*., 2002; Cheng *et al*., 2002; Pan *et al*., 2005). However, a decrease in phenoloxidase activity and an increase in immune ability were seen when the crustaceans were challenged by invasive agents (Bachère, 2000; Malham *et al*., 2003). Therefore we assume that, as an important recognition factor, there are probably several different activating mechanisms of the proPO system in shrimp, leading to different immune responses.

Mammal and teleostean are reported to modulate the immune reaction via DA receptors in the cell membrane (Yang, 1997; Meyniel *et al*., 1997; Terasmaa *et al*., 2000; Jordan *et al*., 2007). Lacoste *et al*. (2001a, 2001b) found that when the Pacific oyster *Crassostrea gigas* were subjected to a 15 min mechanical disturbance, NE and DA concentrations in hemolymph increased, and immune parameters such as hemocyte count and phagocytic capacity of the hemocytes decreased; those researchers also suggested that NE could modulate oyster hemocyte phagocytosis via a β-adrenergic receptor/cAMP/protein kinase, a signaling pathway (Lacoste *et al*., 2001c). In the present study, both DHC and THC of *L. vannamei* decreased significantly after injected with DA and reached the lowest in 3 h. Granular and semi-granular cells in the experimental groups stabilized after 12 h, while THC and hyaline cells reached a stable level after 18 h. Then hemocyte count in the experimental groups recovered to normal levels observed in the control group. Biogenic amines are reported to have a negative effect on cell viability, and the activation of the proPO system trigger cell degranulation and excessive degranulation ever causes cell death because of lysis. So, the decrease of hemocyte count can be due to the two reasons: one is cell death and the other is cell rupture, and this may explain the decrease of THC and DHC in the experimental groups. Granular and semi-granular cells, serine proteases activity and proteinase inhibitor activity became stable after 12 h, and hyaline cells and THC became stable after 18 h. We as-

sume that the restored THC after 18 h maybe because of release of new cells from hematopoietic tissues. It is suggested that DA may modulate the immunity of crustaceans and show significant time- and dose-dependent relationships. It is also suggested that shrimp have DA receptors in the cell membrane. DA injection results in a sudden change of DA concentration in hemolymph. With the help of transmembrane proteins in the cell membrane, shrimp may activate the proPO system, decrease the hemocyte count and lead to a series of immune responses, which is different from the activating mechanisms of the proPO system triggered by invasive agents or spontaneously activated under a normal physical condition. Further studies are required on the acti- vating mechanisms of the proPO system of *L. vannamei* under varying DA concentrations in hemolymph.

At present, our study on the activating process of the proPO system of crustaceans is at its preliminary stage. Although studies of the proPO cascade have been reported focusing on serine proteases, no research has been reported on the upstream protease and signal transduction of the proPO system. Aspán and Söderhäll (1991) reported that endogenous serine proteases could split proPO (76 KD) into two active forms of phenoloxidases (60 and 62 KD) through proteolytic activation, whereas only one phenoloxidase (60 KD) was created after being split by trypsinase. In the present study, we found that the phenoloxidase activity, serine protease activity and proteinase inhibitor activity decreased significantly for *L. vannamei* receiving DA injection. The phenoloxidase activity, serine proteases activity and proteinase inhibitor activity reached the minimum at 6, 3, and 3 h, respectively, and recovered to normal levels observed in the control group at 9, 12, and 12 h, respectively. These results suggest that changes of serine proteases activity, proteinase inhibitor activity and phenoloxidase activity are significantly time- dependent after DA injection. Granular and semi-granular cells, serine protease activity and proteinase inhibitor activity became stable after 12 h. Hyaline cells and THC became stable after 18 h. Phenoloxidase activity became stable after 9 h. All parameters of the experimental groups had no marked differences from those of the control group after they were stable. We speculate that there are probably several different activating mechanisms of the proPO system in shrimp under different stressors. Though it is reported that the activating processes of the proPO system are basically alike, the different productions and molecular structures of the proPO system bring about different mechanisms and effects in immunity response.

Hemolymph from the ancient invertebrate *Limulus polyphemus* contains both complement-like and proteinase-inhibitory activities, supporting the hypothesis that *L. polyphemus* α_2-M is both a proteinase inhibitor and part of a lytic system and plays a significant role in host resistance to infection (Enghild *et al*., 1990). In comparison with other inhibitors, α_2-M has a different inhibitory mechanism. It forms a cage and physically entraps the proteinase, avoiding proteolysis of large but not small substrates. Each α_2-M subunit contains an exposed bait region that is susceptible to proteolytic cleavage and an intramolecular β-cysteinyl-g-glutamyl thioester that is buried in a pocket protected from solvent. Cleavage of the bait region by a proteinase from any mechanistic class (not only SP) leads to a conformational change that traps the proteinase in a cavity formed by the α_2-M tetramer (in vertebrates) or dimer (in invertebrates). The change in conformation also leads to formation of covalent crosslinks between the thiolester region of α_2-M and lysine sidechains of the proteinase, resulting in irreversible inhibition of the proteinase, even though its active site is not acted (Starkey *et al*., 1982; Laskowski and Kato, 1980; Sottrup-Jensen *et al*., 1986; Melchior *et al*., 1995; Gollas-Galván *et al*., 2003). In the present study, α_2-M activity increased significantly after injection with DA, reached the maximum in 3 h and reached a stable level after 12 h, and there were no significant differences between the control and experimental groups thereafter. Considering the multiplicative functions and unique mechanism of the α_2-M in hemolymph, the immunoregulation of the α_2-M of *L. vannamei* remains to be investigated.

BA is widely distributed in the central nervous system and peripheral organs, which transduce signals as neuroregulators. The present study documented that DA seemed to play a role in hemocytes and the proPO system, not only enriching studies on the immune physiology of shrimp, but also exploiting new ideas for future studies on the activating mechanisms of the proPO system in crustaceans.

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