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One recombinant C-type lectin (*LvLec*) from white shrimp *Litopenaeus vannamei* affected the haemocyte immune response *in vitro*

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ABSTRACT

C-type lectin has received widespread attention in animal immunomodulation functions since it was discovered, but it is still limited in crustaceans. The present study is to explore effects of one recombinant C-type lectin (*LvLec* protein) on haemocyte immune response in *Litopenaeus vannamei* (*L. vannamei*). The methods of keeping haemocyte immune activity were optimised by the Key Laboratory of Mariculture. The experiment was divided into four groups: control group, recombinant protein group (*LvLec* protein, 1.0 mg mL^{-1}), Lipopolysaccharide group (LPS, 1.0 mg mL^{-1}), and LPS combine with *LvLec* protein group (LPS + *LvLec* protein, 1.0 mg mL^{-1} + 1.0 mg mL^{-1}), while each group processes 0, 3, 6, 9, 12, and 24 h respectively. The results showed that the haemocyte count reduced, while the exocytosis PO activity, hemagglutinating activity and phagocytic activity promoted, and the concentration of cGMP and PKA increased after *LvLec* protein treatment. However, the levels of antibacterial activity and bacteriolytic activity as well as the concentrations of cAMP and PKG did not change significantly after treating with *LvLec* protein, LPS or LPS + *LvLec* protein. Therefore, these results suggest that *LvLec* protein can stimulate the exocytosis PO activity through cGMP-PKA pathway to affect the phagocytic activity and hemagglutinating activity of *L. vannamei* haemocytes *in vitro*.

1. Introduction

Invertebrates lack adaptive immune system and mainly rely on innate immunity to resist the invasion of pathogenic microorganisms [1–4]. Invertebrates rely on innate immunity system including circulating haemocytes and various active factors existing in haemocytes or being released from heamocytes into the hemolymph upon activation [1,4]. Haemocytes are involved in phagocytosis, which is an important process of eliminating microorganisms or foreign particle, as well as in melanization and encapsulation [5–7]. The immune factors in hemolymph include phenoloxidase (PO), C-type lectin, lysins, peptides, hormones, etc [8–13]. Recently, researchers have paid more attention to the role of agglutination system in immune regulation of invertebrates including *Penaeus monodon*, *Litopenaeus vannamei*, *Macrobrachium nipponense*, *Portunus trituberculatus*, and *Crassostrea gigas* [14–18]. Lectins, as one of the most crucial agglutinating factors, are well known to take an active participate in the defense functions of vertebrates and invertebrates for they play important roles in the recognition of foreign particle [19,20].

C-type lectins are a superfamily of more than 1,000 members that are defined by one or more characteristic of C-type lectin-like domains (CTLDs), which have been subdivided into 17 subgroups on the basis of their phylogeny and domain organization [21,22]. They are known for their ability to agglutinate particles as carbohydrate-binding protein [23,24], which can serve as recognition molecules and mediate the biological activity of cell-surface saccharides [25–27]. Their specificity is usually defined by the carbohydrates that inhibit lectin-induced agglutination [28–30]. In addition, they are also involved in innate immune responses, including the promotion of phagocytosis, nodule formation, encapsulation, and melanization in invertebrates [31–34]. Furthermore, lectins from some invertebrate species may participate in activation of enzymatic cascades, for example, it from tobacco hornworm *Manduca sexta* (Insecta: Lepidoptera) and *Eriocheir sinensis* are able to activate the prophenoloxidase (pro-PO) system [35,36], and that from *Macrobrachium rosenbergii* can regulate haemocytes oxidative processes [37,38]. In some crustacean species, lectins are stored in haemocyte cytoplasmic granules and released to extracellular space upon infectious challenge [38,39]. After recognition, these factors stimulate immune responses by activating proteases present in the hemolymph and using intracellular signaling pathways in immune-responsive tissues [8,40,41]. However, the relationship between C-type lectin and pro-PO system in invertebrate haemocytes remains unclear.

In haemocytes, several protein kinases, such as protein kinase A (PKA), protein kinase C (PKC) and protein kinases G (PKG), have been

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found playing important parts in the regulation of the innate immune system, which were regulated by second messengers including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [42–45]. Many previous studies have indicated that the activation of the pro-PO system of prawn haemocytes, including degranulation and PO activity, is induced by a PKC-activating signaling [46,47]. However, Solon *et al.* demonstrated that lipopolysaccharide (LPS, major constituents of the outer membrane of bacteria)-induced haemocytes exocytosis in the horseshoe crab (*Limulus polyphenmus*) had no correlation with PKC signal pathway [48,49]. Most studies have reported that cAMP-PKA and cGMP-PKG pathway regulated the immune response in crustaceans [50–52]. Nevertheless, biological function of cGMP-PKA pathway rather than cGMP-PKG pathway has also been found in Japanese tree frog and *Schizosaccharomyces pombe* [53,54]. Furthermore, a C-type lectin overexpressed in hepatocellular carcinoma can alter the PKA signal [55]. However, as one of the most important immune factors, it remains unclear how C-type lectin mediated signal pathways of immune defense is integrated form an effective response against invading bacterial pathogens to kill and eliminate bacteria, especially in crustaceans.

Due to the high commercial value and excellent property of breeding, the white shrimp, *Litopenaeus vannamei* (*L. vannamei*), has become the main aquatic animal cultured in coastal regions of China [50,56]. In recent years, however, the massive outbreaks of diseases have seriously affected the shrimp farming [57]. Therefore, more attentions have been paid to the regulation of immune defense in shrimp. To the best of our knowledge, studies on the mechanism of C-type lectin for immune defense are limited. In the present study, we investigated the effect of recombinant protein (*LvLec* protein) and/or LPS on the activation of the pro-PO system and the signaling pathway involved in activating haemocytes of *L. vannamei in vitro*. The exocytosis path of haemocytes under C-type lectin and/or LPS was also explored, for the sake of providing a scientific basis for the regulation mechanism of C-type lectin on pro-PO system in shrimp.

2. Materials and methods

2.1. Animals

Adults of white shrimp *L. vannamei* (8.2 ± 0.5 cm) were obtained from a shrimp farm in Shazikou, Qingdao, China. Prior to the experiment, the shrimps were acclimated in tanks (40 cm × 50 cm × 60 cm) containing aerated water (salinity 30‰, pH 7.8) with air-lift at temperature (22 ± 0.5 °C) for 7 d. Half of the water in each tank was renewed twice daily. During the acclimation period, the shrimps were fed a formulated shrimp diet daily. Before experiment, the shrimps were starved and immersed in penicillin-streptomycin mix solution (1000 u mL^{-1} penicillin and 1000 μg mL^{-1} streptomycin) for 48 h. Only healthy animals at the intermoult stage were used for experiment. The molt stage was determined by examining the uropod in which partial retraction of the epidermis could be distinguished [58]. All the experiments were carried out in accordance with the Animal Care and Use Committee of Ocean University of China, following the guidelines on animal experiments of Ocean University of China.

2.2. Reagents and culture media

LPS from *Escherichia coli* 055: B5 was purchased from Sigma (USA). Recombinant protein (*LvLec* protein) was obtained from the prokaryotic expression of C-type lectin gene of *L. vannamei* (GenBank: EF583939.1) [59], and then recombinant protein was expressed and detected by SDS-PAGE and western-blot. The recombinant protein was purified by His-Band resin chelating chromatography and Amicon Ultra centrifugal filter devices, and agglutination activity was assayed successfully. The evidence of the production and purification of the C-lectin was published in another paper [60].

The following commercially available cell culture media were tested for their ability to maintain viability of *L. vannamei* haemocytes *in vitro*: 60% 2 × L-15 (GiBCOTM), 20% Foetal bovine serum (FBS) (Solarbio), 20% shrimp muscle extract (SME) which treatment process was composed of collecting abdominal muscles, weighing, adding solution containing the same amount (W/V) of Balanced Salt (BSS) as homogenate (18.0 g L^{-1} NaCl, 0.7 g L^{-1} KCl, 0.5 g L^{-1} $NaHCO_3$, 0.54 g L^{-1} KH_2PO_4, pH was adjusted to 7.2), and making up to 3 to 4 times the volume with BBS in turn. After centrifugation at 10 000 g for 30 min, the supernatant was collected and centrifuged at 16 000 g for 30 min, and then was passed through a 0.22 μm filter. The methods of keeping haemocytes immune activity were optimised by own laboratory. The osmolarity of these solutions were 780 ± 30 mOsM kg^{-1}, a value close to the osmolarity of *L.vannamei* hemolymph (780 mOsM kg^{-1}). The commercially available penicillin-streptomycin mix solution (1000 u mL^{-1} penicillin and 1000 μg mL^{-1} streptomycin were added in 0.9% NaCl, final concentration 1.0%) (Sigma) was added to medium to minimise bacterial contamination.

2.3. Experimental design

The experiment was divided into four groups in the present study: (1) Control group (Phosphate Buffered Saline: 23.59 g L^{-1} NaCl, 1.476 g L^{-1} $CaCl_2$•$2H_2O$, 0.1351 g L^{-1} Na_2HPO_4•$12H_2O$, 0.658 g L^{-1} KCl, 4.0852 g L^{-1} $MgCl_2$•$6H_2O$, 4.242 g L^{-1} Tris, pH = 7.45, 780 mOsm Kg^{-1}), (2) Recombinant protein (*LvLec* protein, 1.0 mg mL^{-1}) [35,61,62], (3) Lipopolysaccharide (LPS, 1.0 mg mL^{-1}) [35,62], (4) LPS and *LvLec* protein (LPS + *LvLec* protein, 1.0 mg mL^{-1} + 1.0 mg mL^{-1}). The concentration of the recombinant protein was selected according to preliminary experiment. For each treatment, there were three replicate groups. 200 μL hemolymph from each shrimp was withdrawn from the ventral sinus using a 25-gauge needle and 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, 10 mmol L^{-1} HEPES, pH 7.5, osmolality 780mOsm Kg^{-1}) [63]. Samples of the diluted hemolymph were mixed gently in sterile eppendorf tube, and then centrifuged at 300 *g* for 10 min at 4 °C. When supernatant fluid was discarded, the pellet was rinsed, and re-suspended gently in cell media. Haemocyte concentration was determined using a hemacytometer and adjusted to about 1×10^6 cells mL^{-1} [11,64]. After 0, 3, 6, 9, 12, 24 h, we harvested and assessed parameters respectively. Haemocytes culture was carried out in biosafety cabinets at 25 °C.

2.4. Total haemocyte count (THC) and cell viability

For the measurements of THC and cell viability, 100 μL haemocyte suspension was fixed with an equal volume of 10% formaldehyde (in order to observe better and prevent the rupture of haemocytes during the observation process) for 30 min at 4 °C, then added a drop of 3% Trypan blue [65]. A drop of the suspension was put in a hemacytometer, and THC was determined using an inverted phase contrast microscope (Olympus, Japan).

2.5. Exocytosis PO activity assay

The culture medium of cultured heamocytes was to detect the activity of exocytosis PO in this study. PO activity was measured spectrophotometrically using L-3, 4-dihydroxyphenyalanine (L-DOPA) as substrate and using trypsin as elicitor following the method described previously [66]. 100 μL CAC (cacodylate buffer, 0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, and 0.26 M magnesium chloride; pH 7.0) [67], 100 μL samples of supernatant fluid separated from haemocyte and 100 μL 0.3% L-DOPA in CAC buffer were mixed and optic density was measured at 490 nm at 2 min intervals for 30 min. Absorbance measurements were made against a blank

consisting of CAC buffer and L-DOPA for spontaneous oxidation of the substrate alone. One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1}.

2.6. The hemagglutinating activity

The method of hemagglutinating activity was consulted and modified by Luo [68]. Mice and rabbit erythrocytes were harvested and resuspended in TBS (0.14 mol L^{-1} NaCl, 0.01 mol L^{-1} Tris-HCl, 0.01 mol L^{-1} $CaCl_2$). The rabbit and mice erythrocytes were collected by centrifugation at 800*g*, 4 °C for 5 min and washed 3 times with TBS. The erythrocytes were resuspended in TBS to a concentration of 2% cells. The hemagglutinating activity of *LvLec* protein was assayed by incubating the erythrocytes (25 μL in TBS) and *LvLec* protein (25 μL 1 mg mL^{-1} in TBS) in 96-well plate and following that was observed after 1 h incubation at room temperature under the microscope.

2.7. Phagocytic activity, antibacterial activity and bacteriolytic activity

Phagocytic activity of haemocytes was measured using *Vibrio alginolyticus* [52]. Briefly, 100 μL of haemocytes suspension (1×10^7 cells mL^{-1}) and equal volume of bacterial suspension (1×10^8 cells mL^{-1}) were pipetted into plastic micro plate. Then the mixture was incubated in a moist chamber for 30 min at 25 °C. After incubation, the micro plate was placed at room temperature (25 °C) for 15 min. One drop of the mixture was pipetted onto a glass slide and then dried at room temperature, fixed in methanol, stained with Giemsa stain, decolorized in MiliQ water, air-dried and observed using Olympus light microscope (10 × ocular, 100 × oil immersion objective). The number of phagocytic haemocytes among random 200 haemocytes was counted. Phagocytic activity defined as phagocytic rate (PR) was calculated as:

PR (%) = (number of phagocytic haemocytes/200 haemocytes) × 100%

Antibacterial activity and bacteriolytic activity in plasma were measured using *Vibrio parahaemolyticus* and *Micrococus lysoleikticus* respectively modified from the method of Hultmark [69]. On the one hand, *Vibrio parahaemolyticus* is the pathogen of *L. vannamei*, so this study selected it to test the antimicrobial activity. On the other hand, detection of bacteriolytic activity by *Micrococus lysoleikticus* is a mature method referenced to Hultmark et al. [65]. Briefly, 3 mL bacterial suspension (OD570 nm = 0.3) prepared with sterile KH_2PO_4/K_2HPO_4 buffer (0.1 M, pH = 6.4) was added into a tube and placed in ice bath (0 °C), then 100 μL plasma was added. The absorbance (A0) at 570 nm was measured immediately after vortex. Afterwards, the tube was incubated in water bath at 25 °C for 30 min then returned to ice bath for 10 min to stop the reaction and the optical density at 570 nm (A) was measured again. The antibacterial activity and bacteriolytic activity, defined as Ua^2 and UL respectively, were calculated as follows:

$Ua^2 = (A0\text{-}A)/A$

$UL = (A0\text{-}A)/A$

2.8. The signal transduction factors

The concentrations of second messengers and protein kinases were measured using shrimp cAMP ELISA kit (BPE94058, Shanghai Lengton Bioscience Co., LTD, China), shrimp cGMP ELISA kit (BPE94048, Shanghai Lengton Bioscience Co., LTD, China), shrimp PKA ELISA kit (BPE94611, Shanghai Lengton Bioscience Co., LTD, China), and shrimp PKG ELISA kit (BPE94112, Shanghai Lengton Bioscience Co., LTD, China) according to the manufacturer's instructions. In order to guarantee the success of this experiment, only solution A and B as well as stop solution were allowed as the blank group; Likewise, just 50 μL standard dilution and 50 μL streptavidin-HRP were added in standard group while the test group was conducted with 40 μL sample extract, 10 μL antibody and 50 μL streptavidin-HRP. The process was conducted at 37 °C for 60 min, and then reacted at 37 °C for 10 min with chromogen solution A and B added after washing the plate 5 times. Ultimately, 50 μL stop solution was added and the OD value was measured at 450 nm within 10 min.

2.9. Statistical analyses

All data are presented as the mean values of three independent sets of experiments. Each value was indicated as means ± standard deviation (S.D.). Statistical analysis was performed using SPSS software (Version 21.0) with one-way ANOVA. If significant differences were indicated at the 0.05 level, the Duncan Multiple Range test was used to identify significant differences among the treatments.

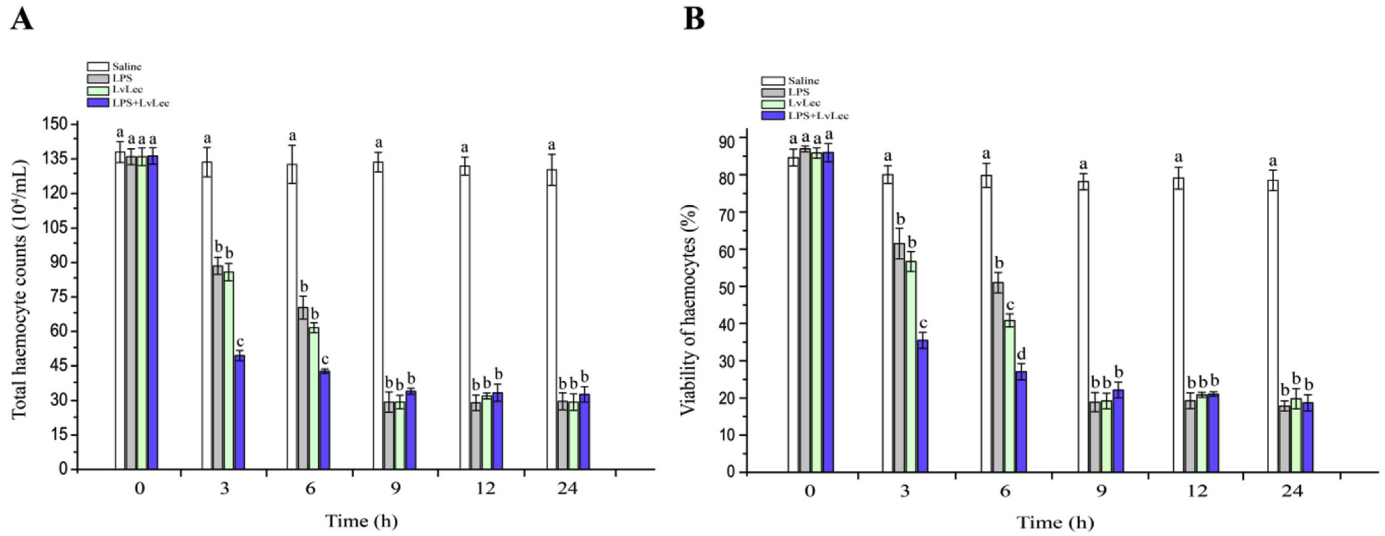

Fig. 1. Independent and simultaneous effect of *LvLec* protein and LPS on the total haemocyte count (THC) and cell viability of *L. Vannamei* heamocytes *in vitro* (A: THC; B: Viability of heamocytes). Each bar represents the mean ± S.D. (n = 3). Data with different letters is significantly different ($P < 0.05$) among treatments.

3. Results

3.1. Effects of LvLec protein on THC and cell viability of L. vannamei haemocytes in vitro

As shown in Fig. 1, the groups of LPS and *LvLec* protein had a significant effect on haemocyte count and cell viability of *L. vannamei* ($P < 0.05$), while no remarkable difference was observed in the control group ($P > 0.05$). Total haemocyte count and cell viability in three treatment groups declined in 3 h, and then kept stable after 9 h. Effects of LPS + *LvLec* on the haemocyte count and cell viability of the shrimp decreased remarkably than those of LPS or *LvLec* independent action group during 3–6 h. The haemocyte count and cell viability of the shrimp in the control group were always higher than those of the other three groups during the experiment.

3.2. Effects of LvLec protein on the exocytosis PO and hemagglutinating activity of L. vannamei haemocytes in vitro

The results showed that LPS and *LvLec* protein groups had significant effect on exocytosis PO activity of *L. vannamei* haemocytes ($P < 0.05$, Fig. 2). The exocytosis PO activity reached the highest peak at 6, 9 and 9 h in LPS group, *LvLec* protein group, and LPS + *LvLec* protein group, respectively. Then the level of exocytosis PO activity in LPS and *LvLec* groups recovered to the level of the control group at 12 h, while it recovered to the control level in LPS + *LvLec* protein group at 24 h. The values of the exocytosis PO activity in LPS + *LvLec* group were higher than that in the *LvLec* protein or LPS groups. The value of exocytosis PO activity was LPS + *LvLec* > *LvLec* > LPS > control group (Fig. 2).

The results in Table 1 indicated that the groups of *LvLec* protein had a significant effect on the hemagglutinating activity ($P < 0.05$, Table 1). While no significant difference was observed in LPS group and control group. Similar with exocytosis PO activity, the level of hemagglutinating activity was LPS + *LvLec* > *LvLec* > LPS > control group.

3.3. Effects of LvLec protein on the phagocytic activity, antibacterial activity and bacteriolytic activity of L. vannamei haemocytes in vitro

The results showed that phagocytic activity in *LvLec* protein group are obviously changed ($P < 0.05$, Fig. 3 A), while it had no significant difference in LPS and control groups ($P > 0.05$, Fig. 3 A). Antibacterial activity and bacteriolytic activity in three treatment groups had no significant difference with the control group ($P > 0.05$, Fig. 3B and C). Phagocytic activity in the *LvLec* protein group increased significantly during 3–24 h compared with LPS and control groups, while there was no significant difference with LPS + *LvLec* group (Fig. 3 A).

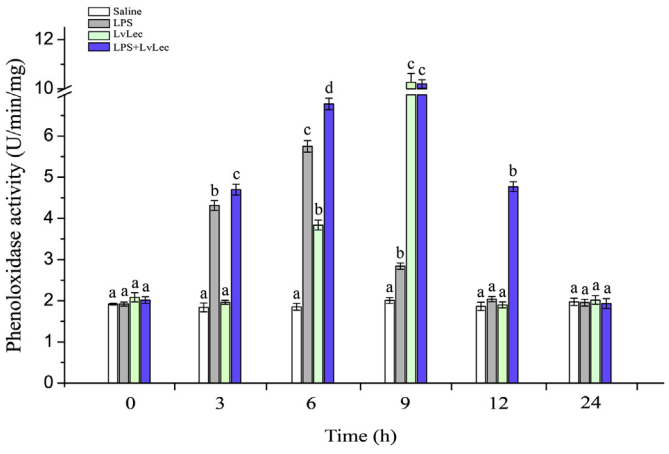

Fig. 2. Independent and simultaneous effect of *LvLec* protein and LPS on the exocytosis PO activity of *L. Vannamei* heamocytes *in vitro*. Each bar represents the mean ± S.D. (n = 3). Data with different letters is significantly different ($P < 0.05$) among treatments.

Table 1
Effect of *LvLec* protein and LPS on the hamagglutinating activity of *L. vannamei*.

Treatment	Time(h)					
	0	3	6	9	12	24
Control	$2^{7a/A}$	$2^{3a/B}$	$2^{3a/B}$	$2^{2a/BC}$	$2^{1a/C}$	$2^{1a/C}$
LPS	$2^{7a/A}$	$2^{4\,ab/B}$	$2^{3a/BC}$	$2^{2a/CD}$	$2^{1a/D}$	$2^{1a/D}$
LvLec protein	$2^{7a/A}$	$2^{5\,b/B}$	$2^{5\,b/B}$	$2^{4b/BC}$	$2^{4b/BC}$	$2^{2ab/D}$
LPS + LvLec protein	$2^{7a/A}$	$2^{6b/AB}$	$2^{6bc/AB}$	$2^{6c/AB}$	$2^{5bc/B}$	$2^{3bc/C}$

Notes: Small letters indicate differences among multiple treatments at the same time-point, and capital letters indicate differences among various time-points in the same treatment.

3.4. Effects of LvLec protein on the signal transduction factors of L. vannamei haemocytes in vitro

To explore the mechanism of C-type lectin of the white shrimp for immune defense, signal transduction factors including cAMP, cGMP, PKA, and PKC were detected. The results showed that LPS and *LvLec* protein groups significantly affected the concentrations of signal transduction factor cGMP and PKA ($P < 0.05$, Fig. 4B and C), while the concentrations of cAMP and PKG didn't change significantly ($P > 0.05$, Fig. 4 A, D). The concentrations of cGMP and PKA showed remarkably increased in LPS group during 3–24 h, while that were significantly increased in *LvLec* group and LPS + *LvLec* group during 6–24 h ($P < 0.05$, Fig. 4B and C). The value of cGMP and PKA reached the maximum at 9 h and decreased at 12 h, then kept stable (Fig. 4B and C).

4. Discussion

C-type lectins play significant roles in both adaptive and innate immunity to defend against pathogens, and they are involved in various immune responses [20,22,70]. Howbeit the immune defense mechanism by C-type lectin remains unclear, especially in crustaceans. To explore the effects of one recombinant *LvLec* on the immune defense of *L. vannamei* haemocytes *in vitro*, this study was performed. As mentioned in the results part, *LvLec* protein and LPS can lead to a decrease in total haemocytes count, cell viability and phagocytic activity but an increase in hemagglutinating activity. Consistent with our results, lectins from the hemolymph in *Mytilus edulis* and *Corbicula fluminea* acted as opsonins to stimulate phagocytosis by haemocytes *in vitro* [71,72]. Moreover, similar results were observed in *Blaberus discoidalis*, *Manduca sexta*, *Scylla paramamosain*, and *Fenneropenaeus chinensis* whose lectins could enhance the cell phagocytosis and have the phenomenon of particle agglutination [35,73–76]. In addition, increasing hemagglutination activity induced by C-type lectin was also reported in *Scylla paramamosain*, *Fenneropenaeus chinensis*, and *L. vannamei* [32,40,59,75,76]. Therefore, the present results indicate recombinant *LvLec* protein of *L. vannamei* inhibiting the haemocyte count, cell viability and phagocytic activity, while promoting the hemagglutinating activity. Moreover, the total haemocyte count reduced significantly in LPS group, probably due to the release of PO from the haemocytes under the LPS stimulation leading to haemocytes death and rupture. Similar results were also presented in *LvLec* treatment, which may be the effects of *LvLec* on some type of haemocytes that was closely related to the release of PO, and then lead to a significant decrease in the cell viability of haemocytes. However, how does recombinant *LvLec* protein regulate these parameters in haemocytes of *L. vannamei in vitro* is still unclear.

To our best knowledge, pro-PO system plays a key role in immune

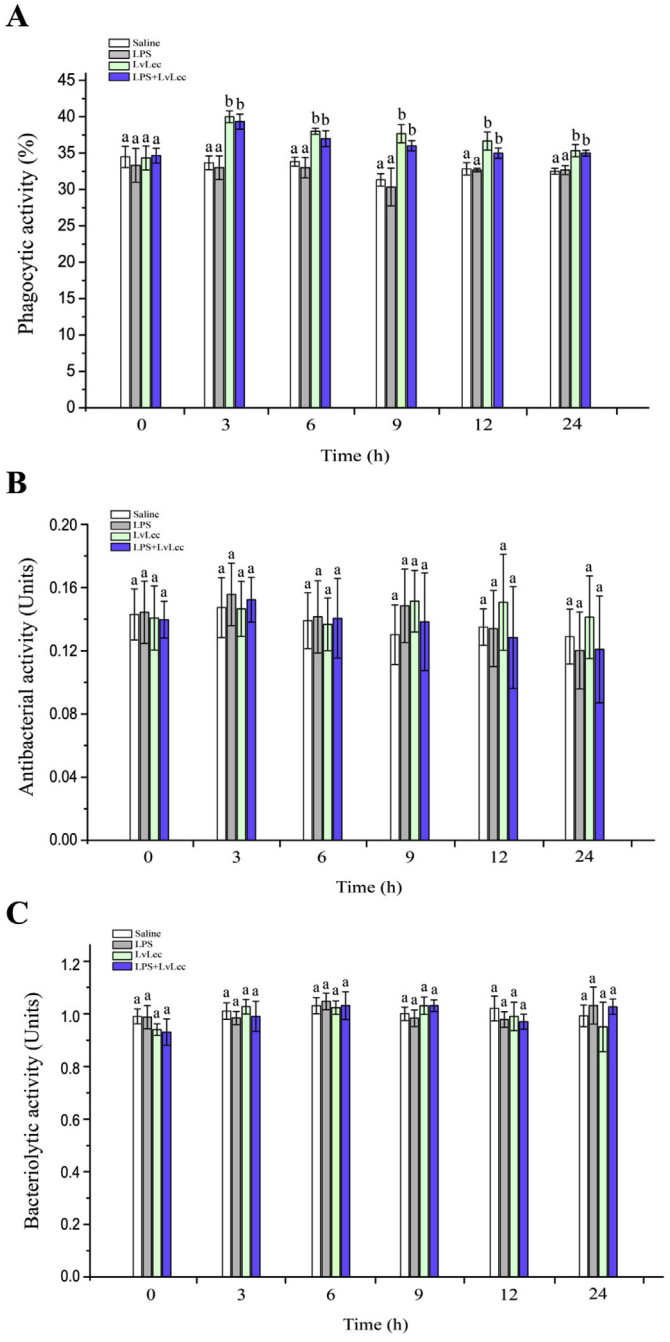

Fig. 3. Independent and simultaneous effect of *LvLec* protein and LPS on the phagocytic activity, antibacterial activity, and bacteriolytic activity of *L. Vannamei* heamocytes *in vitro* (A: Phagocytic activity; B: Antibacterial activity; C: Bacteriolytic activity). Each bar represents the mean ± S.D. (n = 3). Data with different letters is significantly different ($P < 0.05$) among treatments.

response in invertebrate. In order to explore the relationship between *Lvlec* and pro-PO system, the activity of exocytosis PO was detected in this study. Previous studies established that lectin and LPS could activate the pro-PO system which is an important part of invertebrate immune responses [36]. PO is thought to be a key enzyme in invertebrate defense against infection and parasitization, which involves a serine proteinase cascade that can be triggered by microbial polysaccharides and was in some ways similar to the complement system in invertebrate [9,77,78]. Besides, recent studies indicated that pro-PO system could be activated by C-type lectin [35,36,74,79–81]. Moreover, Wang et al. revealed that C-type lectin may function as a sensor, probably be upstream of the pro-PO activating system, that could detect the pathogen invasion [82]. Presently, the exocytosis PO activity peaked at 6 h, 9 h and 9 h, and then recovered to the control level at 12, 12 and 24 h in LPS, *LvLec* protein, and LPS + *LvLec* protein groups, respectively. Nevertheless, cell viability of haemocytes was decreased significantly at 9 h, while PO activity was significant increased at the same time. It can be speculated due to the death of haemocytes at this stage, while the increase of PO activity in culture medium is caused by the rupture of haemocytes. PO activity in *LvLec* + LPS group increased significantly during 3–12 h, but no obvious change has been seen at 3 h while increased clearly during 6–9 h in *LvLec* group. This may be due to the fact that *LvLec* can promote PO activity faster and longer under LPS stimulation than without LPS stimulation. While the PO activity returned to control level in *LvLec* group at 12 h, but it was still significantly higher in LPS + *LvLes* group at the same time, which indicates that the effect of *LvLec* is still persistent in the presence of LPS. Consistent with the results of this study, several reports showed that C-type lectin can activate the extracellular PO activity in invertebrates including *Procambarus clarkii* [82], *Manduca sexta* [34], and *Eriocheir sinensis* [36]. Here, the present study results indicate that *LvLec* protein can activate the pro-PO system and enhance the exocytosis PO activity. Nevertheless, how the *LvLec* protein activates PO activity still remains unclear.

Recently, several studies reported that LPS induce exocytosis in the Limulus GR and all of these compounds are known to activate the IP3/DAG and cAMP signal transduction pathways in both vertebrate and invertebrate cell types [83–86]. Solon et al. showed that G-proteins which are presumably linked to LPS receptors are involved in exocytosis of the Limulus GR [48]. It has been shown that PKA play an important role in recognition protein expression and phagocytosis function in insect haemocytes [87]. In addition, several reports suggested that the activation of the pro-PO system of prawn haemocytes, including degranulation and PO activity, induced by ODN2006 (CpG oligodeoxynucleotide), which is contained in genes of bacterias and viruses, is used as molecular patterns via a PKC-activating signaling pathway [46]. However, Solon et al. demonstrated that LPS-induced haemocytes exocytosis in the horseshoe crab (*Limulus polyphenmus*) had no correlation with PKC signal pathway [48,49]. In order to explore the signal pathways of *LvLec* regulating PO activity to influence the immune defense of haemocytes, the content of second messengers and protein kinases including cAMP, cGMP, PKA, and PKG were examined. In the present study, the concentration of cGMP and PKA in three treatment groups increased significantly, reached the maximum at 9 h, and kept stable after 12 h. Similarly, cGMP through PKA-dependent pathway rather than PKG pathway activates sodium transport in the urinary bladder of the Japanese tree frog [51]. Moreover, Tsukada and Fukuda reported that nitric oxide induced neurite extension through cGMP-PKA pathway [88]. In addition, recent study showed that the *Schizosaccharomyces pombe fbp1* gene is transcriptionally repressed by PKA that is activated by extracellular glucose via a cGMP signaling pathway [54]. Therefore, combined with previous reports and data from the present results, we can find that the signal transduction factors and the immune parameters have a positive correlation. The present study suggests that *LvLec* protein activates exocytosis PO activity through cGMP-PKA pathway rather than PKG pathway, thereby affecting the immune response parameters including haemocyte count, cell viability, phagocytic activity, and hemagglutinating activity of *L. vannamei* haemocytes *in vitro*.

In conclusion, the present study documented that C-type lectin not only modulated the pathogen recognition and hemagglutinating activity, but also activated the pro-PO activating system of *L. vannamei* haemocytes *in vitro*. The C-type lectin correlated with decreases in haemocyte count, changes in exocytosis PO activity, hemagglutinating activity, phagocytic activity and the concentration of cGMP and PKA. In addition, C-type lectin, which exhibits the same performance as LPS, has the same effect on immune defense, and the effect of C-type lectin is

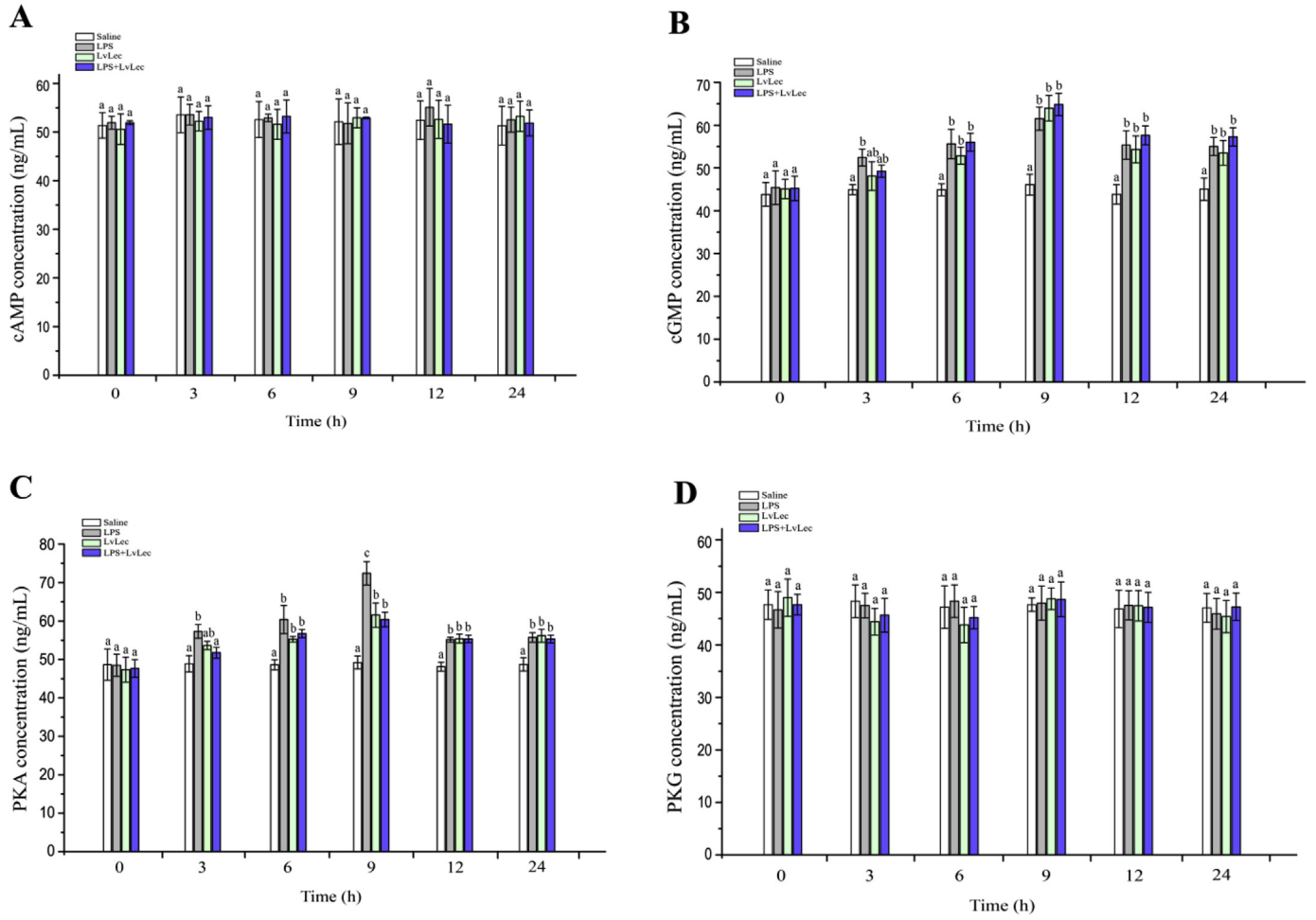

Fig. 4. Independent and simultaneous effect of *LvLec* protein and LPS on the second messenger and protein kinases concentration of *L. Vannamei* heamocytes *in vitro* (A: cAMP; B: cGMP; C: PKA; D: PKG). Each bar represents the mean ± S.D. (n = 3). Data with different letters is significantly different ($P < 0.05$) among treatments.

more remarkable under the induction of LPS. Based on the results, it is speculated that *LvLec* protein activates the exocytosis PO activity by cGMP-PKA pathway, and then affects the phagocytic activity and hemagglutinating activity of *L. vannamei* haemocytes *in vitro*. This study provides a theoretical basis for the investigation of the immune defense mechanism of C-type lectin in invertebrate haemocytes. In future research, we will endeavor to understand how C-type lectin activates the system of proteinases in the pro-PO system cascade of *L. vannamei* haemocytes.

Disclosure summary

The authors have nothing to disclose.

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References

[1] E.S. Loker, C.M. Adema, S.M. Zhang, T.B. Kepler, Invertebrate immune systems-not homogeneous, not simple, not well understood, Immunol. Rev. 198 (1) (2010) 10–24.
[2] J. Alpuche, A. Pereyra, C. Agundis, C. Rosas, C. Pascual, M.C. Slomianny, L. Vázquez, E. Zenteno, Purification and characterization of a lectin from the white shrimp *Litopenaeus setiferus* (Crustacea decapoda) hemolymph, BBA-General Subjects 1724 (1) (2005) 86–93.
[3] Y.C. Liu, F.H. Li, B. Dong, B. Wang, W. Luan, X.J. Zhang, L.S. Zhang, J.H. Xiang, Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*, Mol. Immunol. 44 (4) (2007) 598–607.
[4] N.G. Van, S.M. Hattingh, A.M. Engelbrecht, Invertebrates: why no adaptive immune system? Scand. J. Immunol. 83 (2) (2016) 160–161.
[5] C.J. Bayne, Phagocytosis and Non-Self Recognition in Invertebrates: phagocytosis appears to be an ancient line of defense, Bioscience 40 (10) (1990) 723–731.
[6] R. Medzhitov, C.A. Janeway, Decoding the patterns of self and nonself by the innate immune system, Science 296 (5566) (2002) 298–300.
[7] B. Milutinović, J. Kurtz, Immune memory in invertebrates, Semin. Immunol. 28 (4) (2016) 328–342.
[8] J.L. Sánchez-Salgado, M.A. Pereyra, C. Agundis, O. Vivanco-Rojas, C. Sierra-Castillo, J.J. Alpuche-Osorno, E. Zenteno, Participation of lectins in crustacean immune system, Aquacult. Res. 48 (8) (2017) 4001–4011.
[9] L. Cerenius, B.L. Lee, K. Söderhäll, The proPO-system: pros and cons for its role in invertebrate immunity, Trends Immunol. 29 (6) (2008) 263–271.
[10] M. Liu, L. Pan, L. Li, D. Zheng, Molecular cloning, characterization and recombinant expression of crustacean hyperglycemic hormone in white shrimp *Litopenaeus vannamei*, Peptides 53 (2) (2014) 115–124.
[11] L. Wang, H. Chen, J. Xu, Q. Xu, M. Wang, D. Zhao, L. Wang, L. Song, Crustacean hyperglycemic hormones directly modulate the immune response of hemocytes in shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 62 (2017) 164–174.
[12] Y. Guo, L. Wang, Z. Zhou, M. Wang, R. Liu, L. Wang, Q. Jiang, L. Song, An opioid growth factor receptor (OGFR) for [Met5]-enkephalin in *Chlamys farreri*, Fish Shellfish Immunol. 34 (5) (2013) 1228–1235.
[13] A.F. Rowley, The immune system of Crustaceans, Encyclopedia of Immunobiology 1 (2016) 437–453.
[14] T. Luo, H. Yang, F. Li, X. Zhang, X. Xu, Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp, Dev. Comp. Immunol. 30 (7) (2006) 607–617.

[15] Z. Ying, W. Leilei, W. Lingling, W. Ning, Z. Zhi, S. Linsheng, An integrin from shrimp *Litopenaeus vannamei* mediated microbial agglutination and cell proliferation, PLoS One 7 (7) (2012) e40615.
[16] J. Lu, Z. Yu, C. Mu, R. Li, W. Song, C. Wang, Characterization and functional analysis of a novel C-type lectin from the swimming crab *Portunus trituberculatus*, Fish Shellfish Immunol. 64 (2017) 185–192.
[17] H. Li, H. Zhang, S. Jiang, W. Wang, L. Xin, H. Wang, L. Wang, L. Song, A single-CRD C-type lectin from oyster *Crassostrea gigas* mediates immune recognition and pathogen elimination with a potential role in the activation of complement system, Fish Shellfish Immunol. 44 (2) (2015) 566–575.
[18] Y. Xiu, Y. Wang, J. Bi, Y. Liu, M. Ning, H. Liu, S. Li, W. Gu, W. Wang, Q. Meng, A novel C-type lectin is involved in the innate immunity of *Macrobrachium nipponense*, Fish Shellfish Immunol. 50 (2016) 117–126.
[19] R. Wongpanya, P. Sengprasert, P. Amparyup, A. Tassanakajon, A novel C-type lectin in the black tiger shrimp *Penaeus monodon* functions as a pattern recognition receptor by binding and causing bacterial agglutination, Fish Shellfish Immunol. 60 (2017) 103–113.
[20] G.D. Brown, J.A. Willment, L. Whitehead, C-type lectins in immunity and homeostasis, Nat. Rev. Immunol. 18 (6) (2018) 374–389.
[21] A.N. Zelensky, J.E. Gready, The C-type lectin-like domain superfamily, FEBS J. 272 (24) (2005) 6179–6217.
[22] W.I. Weis, M.E. Taylor, K. Drickamer, The C-type lectin superfamily in the immune system, Immunol. Rev. 163 (1) (2010) 19–34.
[23] I.E. Liener, 10-Nutritional significance of lectins in the diet, Lectins 1 (6) (1986) 527–552.
[24] P. Kwankaew, R. Praparatana, P. Runsaeng, P. Utarabhand, An alternative function of C-type lectin comprising low-density lipoprotein receptor domain from *Fenneropenaeus merguiensis* to act as a binding receptor for viral protein and vitellogenin, Fish Shellfish Immunol. 74 (2017) 295–308.
[25] M. Šereš, L. Pavlíková, Z. Sulová, A. Breier, Lectin detection of cell surface saccharides remodeling induced by development of P-glycoprotein mediated multidrug resistance phenotype in L1210 leukemia cells, Acta Chim. Slovaca 7 (1) (2014) 52–56.
[26] K.P. Bene, D.W. Kavanaugh, C. Leclaire, A.P. Gunning, D.A. Mackenzie, A. Wittmann, I.D. Young, N. Kawasaki, E. Rajnavolgyi, N. Juge, Lactobacillus reuteri surface mucus adhesins upregulate inflammatory responses through interactions with innate C-type lectin receptors, Front. Microbiol. 8 (321) (2017) 321.
[27] W.I. Weis, M.S. Quesenberry, M.E. Taylor, K. Bezouska, W.A. Hendrickson, K. Drickamer, Molecular mechanisms of complex carbohydrate recognition at the cell surface, Cold Spring Harbor Symp. Quant. Biol. 57 (57) (1992) 281–289.
[28] B.T. Heron, A. Sateriale, J.E. Teixeira, C.D. Huston, Evidence for a novel Entamoeba histolytica lectin activity that recognises carbohydrates present on ovalbumin, Int. J. Parasitol. 41 (2) (2011) 137–144.
[29] K. Drickamer, C-type lectin-like domains, Curr. Opin. Struct. Biol. 9 (5) (1999) 585–590.
[30] E.L. Stattin, F. Wiklund, K. Lindblom, P. Onnerfjord, B.A. Jonsson, Y. Tegner, T. Sasaki, A. Struglics, S. Lohmander, N. Dahl, A missense mutation in the aggrecan C-type lectin domain disrupts extracellular matrix interactions and causes dominant familial osteochondritis dissecans, Am. J. Hum. Genet. 86 (2) (2010) 126–137.
[31] B. Pees, W. Yang, A. Záratepotes, H. Schulenburg, K. Dierking, High innate immune specificity through diversified C-type lectin-like domain proteins in invertebrates, Journal of Innate Immunity 8 (2) (2015) 129–142.
[32] M. Li, C. Li, C. Ma, H. Li, H. Zuo, S. Weng, X. Chen, D. Zeng, J. He, X. Xu, Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 46 (2) (2014) 231–240.
[33] H.T. Ma, J.A.H. Benzie, J.G. He, S.M. Chan, PmLT, a C-type lectin specific to hepatopancreas is involved in the innate defense of the shrimp *Penaeus monodon*, J. Invertebr. Pathol. 99 (3) (2008) 332–341.
[34] X.Q. Yu, M.R. Kanost, Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria, J. Biol. Chem. 275 (48) (2000) 37373–37381.
[35] X.Q. Yu, H. Gan, M.R. Kanost, Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase, Insect Biochem. Mol. Biol. 29 (7) (1999) 585–597.
[36] Z.Y. Fang, D. Li, X.J. Li, X. Zhang, Y.T. Zhu, W.W. Li, Q. Wang, A single CRD C-type lectin from *Eriocheir sinensis* (EsLecB) with microbial-binding, antibacterial prophenoloxidase activation and hem-encapsulation activities, Fish Shellfish Immunol. 50 (2016) 175–190.
[37] L. Vázquez, A. Pérez, D. Millán, C. Agundis, G. Martin, E.L. Cooper, R. Lascurain, E. Zenteno, Morphology of hemocytes from the freshwater prawn *Macrobrachium rosenbergii*, J. Morphol. 234 (2) (1997) 147–153.
[38] C. Sierra, A. Perez, C. Agundis, E. Zenteno, L. Vazquez, Subcellular organization of a seric lectin in haemocytes from *Macrobrachium rosenbergii* (Decapoda, nanantia) and its role in phagocytosis, Crustaceans and the biodiversity crisis 1 (1999) 961–970.
[39] M. Nalini, P. Mullainadhan, M. Arumugam, Characterization of a natural hemagglutinin in the serum of a freshwater crab *Parathelphusa hydrodromus* (Herbst), Arch. Int. Physiol. 102 (5) (1994) 259–264.
[40] X.W. Wang, W.T. Xu, X.W. Zhang, X.F. Zhao, X.Q. Yu, J.X. Wang, A C-type lectin is involved in the innate immune response of Chinese white shrimp, Fish Shellfish Immunol. 27 (4) (2009) 556–562.
[41] X.K. Jin, S. Li, X.N. Guo, L. Cheng, M.H. Wu, S.J. Tan, Y.T. Zhu, A.Q. Yu, W.W. Li, Q. Wang, Two antibacterial C-type lectins from crustacean, *Eriocheir sinensis*, stimulated cellular encapsulation in vitro, Dev. Comp. Immunol. 41 (4) (2013) 544–552.
[42] A. Gajewska, E. Wolinskawitort, M. Zielinska, A. Krawczynska, H. Antushevich, Cugnrh and pacap-induced cAMP/PKA and cGMP/PKG pathways activity in anterior pituitary cells *in vitro*, 15th International & 14th European Congress of Endocrinology 29 (2012) 1114.
[43] E. Bollen, D. Puzzo, K. Rutten, L. Privitera, J.D. Vry, T. Vanmierlo, G. Kenis, A. Palmeri, R. D'Hooge, D. Balschun, Improved long-term memory via enhancing cGMP-PKG signaling requires cAMP-PKA signaling, Neuropsychopharmacology Official Publication of the American College of Neuropsychopharmacology 39 (11) (2014) 2497–2505.
[44] J.P. Gainor, C.A. Morton, J.T. Roberts, P.A. Vincent, F.L. Minnear, Platelet-conditioned medium increases endothelial electrical resistance independently of cAMP/PKA and cGMP/PKG, Am. J. Physiol. Heart Circ. Physiol. 281 (5) (2001) H1992–H2001.
[45] K.S. Murthy, H. Zhou, J.R. Grider, G.M. Makhiouf, Inhibition of RhoA-dependent sustained smooth muscie contraction by cAMP-and cGMP-dependent protein kinase (PKA and PKG), Gastroenterology 120 (5) (2001) A201-A201.
[46] C.P. Chuo, S.M. Liang, H.H. Sung, Signal transduction of the prophenoloxidase activating system of prawn haemocytes triggered by CpG oligodeoxynucleotides, Fish Shellfish Immunol. 18 (2) (2005) 149–162.
[47] D. Strasser, K. Neumann, H. Bergmann, M.J. Marakalala, R. Guler, A. Rojowska, K.P. Hopfner, F. Brombacher, H. Urlaub, G. Baier, Syk kinase-coupled C-type lectin receptors engage protein kinase C-δ to elicit Card9 adaptor-mediated innate immunity, Immunity 36 (1) (2012) 32–42.
[48] E. Solon, A.P. Gupta, R. Gaugler, Signal transduction during exocytosis in Limulus polyphemus granulocytes, Dev. Comp. Immunol. 20 (5) (1996) 307–321.
[49] E.C. Khor, T. Abel, J. Tickner, S.M. Chim, C. Wang, T. Cheng, B. Ng, P.Y. Ng, D.A. Teguh, J. Kenny, Loss of protein kinase C-δ protects against LPS-induced osteolysis owing to an intrinsic defect in osteoclastic bone resorption, PLoS One 8 (8) (2013) e70815.
[50] Q. Zhao, L. Pan, Q. Ren, L. Wang, J. Miao, Effect of salinity on regulation mechanism of neuroendocrine-immunoregulatory network in *Litopenaeus vannamei*, Fish Shellfish Immunol. 49 (2016) 396–406.
[51] L. Zhang, L. Pan, L. Xu, L. Si, Effects of ammonia-N exposure on the concentrations of neurotransmitters, hemocyte intracellular signaling pathways and immune responses in white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 75 (2018) 48–57.
[52] L. Zhang, L. Pan, L. Xu, L. Si, Independent and simultaneous effect of crustacean hyperglycemic hormone and dopamine on the hemocyte intracellular signaling pathways and immune responses in white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 83 (2018) 262–271.
[53] T. Yamada, K. Matsuda, M. Uchiyama, Atrial natriuretic peptide and cGMP activate sodium transport through PKA-dependent pathway in the urinary bladder of the Japanese tree frog, J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 176 (3) (2006) 203–212.
[54] D. Demirbas, O. Ceyhan, A.R. Wyman, F.D. Ivey, C. Allain, L. Wang, M.N. Sharuk, S.H. Francis, C.S. Hoffman, Use of a Schizosaccharomyces pombe PKA-repressible reporter to study cGMP metabolising phosphodiesterases, Cell. Signal. 23 (3) (2011) 594–601.
[55] F. Demaugre, Y. Philippe, S. Sar, B. Pileire, L. Christa, C. Lasserre, C. Brechot, HIP/PAP, a C-type lectin overexpressed in hepatocellular carcinoma, binds the RIIα regulatory subunit of cAMP- dependent protein kinase and alters the cAMP-dependent protein kinase signalling, FEBS J. 271 (19) (2004) 3812.
[56] D.M. Godin, W.H. Carr, G. Hagino, F. Segura, J.N. Sweeney, L. Blankenship, Evaluation of a fluorescent elastomer internal tag in juvenile and adult shrimp *Penaeus vannamei*, Aquaculture 139 (3) (1996) 243–248.
[57] L.M. Perazzolo, R. Gargioni, P. Ogliari, M.A.A. Barracco, Evaluation of some hemato- immunological parameters in shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress, Aquaculture 214 (1) (2002) 19–33.
[58] L. Robertson, W. Bray, J. Leung-Trujillo, A. Lawrence, Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*, J. World Aquacult. Soc. 18 (3) (2010) 180–185.
[59] Y. Zhang, L. Qiu, L. Song, H. Zhang, J. Zhao, L. Wang, Y. Yu, C. Li, F. Li, K. Xing, Cloning and characterization of a novel C-type lectin gene from shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 26 (1) (2009) 183–192.
[60] Y.U. Jinhong, L. Pan, H. Zhang, Prokaryotic expression of C-type lectin gene of *Litopenaeus vannamei* and immunologic activity evaluation of recombinant protein, J. Fish. China 37 (8) (2013) 1183–1191.
[61] H. Zhang, L. Chen, J. Qin, D. Zhao, P. Wu, C. Qin, N. Yu, E. Li, Molecular cloning, characterization and expression of a C-type lectin cDNA in Chinese mitten crab, *Eriocheir sinensis*, Fish Shellfish Immunol. 31 (2) (2011) 358–363.
[62] H.Z. Guo, P.F. Zou, J.P. Fu, Z. Guo, B.K. Zhu, P. Nie, M.X. Chang, Characterization of two C-type lectin-like domain (CTLD)-containing proteins from the cDNA library of Chinese mitten crab Eriocheir sinensis, Fish Shellfish Immunol. 30 (2) (2011) 515–524.
[63] F. Vargas-Albores, M.A. Guzmán, J.L. Ochoa, An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*), Comp. Biochem. Physiol. Part A Physiology 106 (2) (1993) 299–303.
[64] H. Zih-Jhan, K. Shih-Ting, L. Jiann-Horng, C. Li-Li, Endocytic pathway is indicated for white spot syndrome virus (WSSV) entry in shrimp, Fish Shellfish Immunol. 35 (3) (2013) 707–715.
[65] L. Pan, P. Xie, F. Hu, Responses of prophenoloxidase system and related defence parameters of *Litopenaeus vannamei* to low salinity, J. Ocean Univ. China 9 (3) (2010) 273–278.
[66] V.J. Smith, K. Söderhäll, A comparison of phenoloxidase activity in the blood of marine invertebrates, Dev. Comp. Immunol. 15 (4) (1991) 251–261.
[67] Y. Su-Tuen, C. Jiann-Chu, White shrimp *Litopenaeus vannamei* that received the hot-water extract of Gracilaria tenuistipitata showed earlier recovery in immunity after a *Vibrio alginolyticus* injection, Fish Shellfish Immunol. 26 (5) (2009) 724–730.

[68] D. Hultmark, H. STEINER, T. Rasmuson, H.G. Boman, Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*, Eur. J. Biochem. 106 (1) (1980) 7–16.
[69] M.J. Robinson, D. Sancho, E.C. Slack, S. Leibundgutlandmann, e.S.C. Reis, Myeloid C-type lectins in innate immunity, Nat. Immunol. 7 (12) (2006) 1258–1265.
[70] L. Renwrantz, A. Stahmer, Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from *Mytilus edulis*, J. Comp. Physiol. 149 (4) (1983) 535–546.
[71] R. Yang, T.P. Yoshino, Immunorecognition in the freshwater bivalve, *Corbicula fluminea*. II. Isolation and characterization of a plasma opsonin with hemagglutinating activity, Dev. Comp. Immunol. 14 (4) (1990) 397–404.
[72] A.M. Kerrigan, G.D. Brown, C-type lectins and phagocytosis, Immunobiology 214 (7) (2009) 562–575.
[73] C. Chen, H.J. Durrant, R.P. Newton, N.A. Ratcliffe, A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus discoidalis*, Biochem. J. 310 (1) (1995) 23–31.
[74] X. Wei, L. Wang, W. Sun, Z. Ming, H. Ma, Y. Zhang, X. Zhang, S. Li, C-type lectin B (Sp CTL-B) regulates the expression of antimicrobial peptides and promotes phagocytosis in mud crab *Scylla paramamosain*, Dev. Comp. Immunol. 84 (2018) 213–229.
[75] X.W. Wang, X.F. Zhao, J.X. Wang, C-type lectin binds to beta-integrin to promote hemocytic phagocytosis in an invertebrate, J. Biol. Chem. 289 (4) (2014) 2405–2414.
[76] L. Cerenius, K. Söderhäll, The prophenoloxidase-activating system in invertebrates, Immunol. Rev. 198 (1) (2004) 116–126.
[77] M. Ashida, Recent advances in research on the insect prophenoloxidase cascade, Res. Immunol. 141 (8) (1990) 908–910.
[78] K. Sastry, R.A. Ezekowitz, Collectins: pattern recognition molecules involved in first line host defense, Curr. Opin. Immunol. 5 (1) (1993) 59–66.
[79] Y. Romeo, B. Lemaitre, Drosophila immunity, Methods Mol. Biol. 415 (2008) 379–394.
[80] X.Z. Shi, X.Q. Yu, The extended loop of the C-terminal carbohydrate-recognition domain of *Manduca sexta* immulectin-2 is important for ligand binding and functions, Amino Acids 42 (6) (2012) 2383–2391.
[81] X.W. Wang, H.W. Zhang, X. Li, X.F. Zhao, J.X. Wang, Characterization of a C-type lectin (PcLec2) as an upstream detector in the prophenoloxidase activating system of red swamp crayfish, Fish Shellfish Immunol. 30 (1) (2011) 241–247.
[82] M.J. Berridge, Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol, Biochem. J. 212 (3) (1983) 849–858.
[83] M.J. Berridge, R. Dawson, C. Downes, J. Heslop, R. Irvine, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides, Biochem. J. 212 (2) (1983) 473–482.
[84] D. Baines, R.G.H. Downer, Octopamine enhances phagocytosis in cockroach hemocytes: involvement of inositol trisphosphate, Arch. Insect Biochem. Physiol. 26 (4) (1994) 249–261.
[85] J.Q. Huang, S.P. Nie, S.S. Zhang, D.F. Huang, K.X. Zhu, M.Y. Xie, Effect of a polysaccharide from ganoderma atrum on cAMP/PKA,IP3/Cā(2+) and DAG/PKC signaling pathways in *peritoneal macrophages* of S-180 tumor-bearing mice, Food Sci. (N. Y.) 35 (1) (2014) 204–208.
[86] N.D. Charalambidis, C.G. Zervas, M. Lambropoulou, P.G. Katsoris, V.J. Marmaras, Lipopolysaccharide-stimulated exocytosis of nonself recognition protein from insect hemocytes depend on protein tyrosine phosphorylation, Eur. J. Cell Biol. 67 (1) (1995) 32–41.
[87] H.H. Sung, P.H. Chen, C.L. Liu, Initiation of signal transduction of the respiratory burst of prawn haemocytes triggered by CpG oligodeoxynucleotides, Fish Shellfish Immunol. 24 (6) (2008) 693–700.
[88] S. Tsukada, J. Fukuda, Nitric oxide induced neurite extension through cGMP-PKA pathway, Neurosci. Res. 31 (98) (1998) S291.