Fish and Shellfish Immunology 87 (2019) 590–599

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Involvement of a novel protein kinase C (nPKC) in immunocompetence in hemocytes of white shrimp, *Litopenaeus vannamei*

Zhong-Wen Chang, Chin-Chyuan Chang*

Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, 91201, Taiwan, ROC

ARTICLE INFO

Keywords:
Litopenaeus vannamei
Novel protein kinase C
Gene silencing
Hypothermal stress
Immunity

ABSTRACT

Complementary (c)DNA encoding novel protein kinase C (PKC) messenger (m)RNA of the white shrimp *Litopenaeus vannamei*, consisted of 2454-bp cDNA containing an open reading frame (ORF) of 2232 bp, belonging to the novel (n)PKC family of proteins characterized by their containing two phorbol ester/diacylglycerol-binding domains (C1 domain), a C2 domain, and a catalytic domain of the serine/threonine kinase, designated LvnPKC. A comparison of amino acid sequences showed that LvnPKC was closely related to arthropod nPKC. LvnPKC cDNA was detected in all tested tissues with a real-time PCR including the hepatopancreas, gills, muscles, subcuticular epithelium, abdominal nerve, thoracic nerve, brain, the stomach, heart, and especially in hemocytes and the intestines. Moreover, significantly upregulated LvnPKC expression was only observed in the eyestalk, brain, and hepatopancreas of shrimp transferred from 28 °C to 18 °C for 30 min. Induction of LvnPKC expression in hemocytes of *L. vannamei* injected with *Vibrio alginolyticus* at 10^5 cfu shrimp^{-1} was detected earlier than in those injected with 10^3 cfu shrimp^{-1}. Shrimp received LvnPKC-dsRNA for 1 days specifically depleted the expression of LvnPKC mRNA in hemocytes compared those of diethylpyrocarbonate water treatment. After that, significantly decreased expressions of lipopolysaccharide - and β-1,3-glucan-binding protein, prophenoloxidase-activating enzyme, peroxinectin, prophenoloxidase I, and prophenoloxidase II in the prophenoloxidase-activating system; lysozyme and cytosolic manganese superoxide dismutase and mitochondrial manganese superoxide dismutase in the antioxidant system were observed. We therefore concluded that LvnPKC is involved in immune defense of *L. vannamei* exposed to hypothermal stress or infected with *V. alginolyticus*.

1. Introduction

Nishizuka and colleagues identified a novel protein kinase in 1977, and the activated form triggered cell responses to signals through phosphorylation of critical targeted proteins following triggering the turnover of inositol phospholipids (IPs), which generates diacylglycerol (DG), mobilizes Ca^{2+}, and leads to cell activation [1–3]. Further, following the dependence on calcium and the regulation by DG and other lipids, at least 10 mammalian PKC isotypes encoded by nine genes were found, and each of them was expressed in a cell type-specific manner [4]. Ohno and Nishizuka [4] reviewed that upon elucidating the primary structure and biochemical properties, three classes including classical or conventional (c)PKC, novel (n)PKC, and atypical (a)PKC isotypes were reported, and the characteristic sequence motif C1 and a serine/threonine-protein kinase domain were detected in all PKC isotypes. cPKC isotypes (α, β, and γ), which share structural motifs C1 and C2, can be activated by DGs or phorbol esters in the presence of Ca^{2+} and acidic phospholipids such as phosphatidylserine (PS). nPKC isotypes (δ, ε, μ, η, and θ) also have the C1 and C2 domains, and the nPKC-C1 domain binds DG and phorbol ester as is the case with cPKCs; however, the sequence of the C2 domain somewhat diverges from that of cPKC-C2. Purified nPKC isotypes can be activated by DGs or phorbol esters in the presence of PS, but this activation does not require Ca^{2+}, in clear contrast to cPKC isotypes. aPKC isotypes share structural motifs C1 and octicosapeptide repeat. The C1 sequences of aPKC isotypes (ζ, and ι/λ) are not repeated, unlike in the cPKC and nPKC isotypes. Purified aPKC isotypes do not bind to nor are they activated by DGs or phorbol esters.

PKC isotypes differ in their tissue distributions and localization within the cell, suggesting that each isotype plays a specific role in specific signal transduction pathways, which indicate they play key regulatory roles in multiple cellular processes that include differentiation, cell growth, secretion and muscle contraction [5–8]. In mollusks, different isotypes of cPKC (DG- and Ca^{2+}-dependent) and nPKC (DG-dependent and Ca^{2+}-independent) were detected in the cytosolic fraction in the adductor and retractor muscles of *Mytilus galloprovincialis*

* Corresponding author.
E-mail address: changcc@mail.npust.edu.tw (C.-C. Chang).

https://doi.org/10.1016/j.fsi.2019.02.005
Received 11 September 2018; Received in revised form 31 January 2019; Accepted 5 February 2019
Available online 08 February 2019

[9], and their roles in the neuroendocrine-immune network in hemocytes were further reviewed [10]. In crustaceans, the role of methyl farnesoate in regulating vitellogenin uptake in the ovary of *Cherax quadricarinatus* mediated by PKCα (a cPKC isotype) activation as identified by rabbit polyclonal *anti*-PKC isoenzyme antibodies was similar to the mode of action suggested for the juvenile hormone in insects [11]. PKCδ (a nPKC isotype) was purified from the hepatopancreas of *Penaeus monodon* using mammalian PKC antibodies [12]. An aPKC of *Macrobrachium rosenbergii* was isolated using designed degenerate primers and the rapid amplification of cDNA ends, which consists of structural domains similar to those of an aPKC homologue [13].

In the innate immune response of mammals, the potential role of PKC isotypes was examined in lots of research. In macrophages, PKCs are purported to regulate processes such as cell adhesion and spread [14], phagocytosis [15], and the production of reactive oxygen/nitrogen intermediates [16,17]. In mollusks, PKCs are also purported to be involved in mechanisms of the hemocyte innate immune response including regulation of phagocytosis [10,18]. Ng et al. [19] reported that extracellular traps (ETs) for phagocytosis were revealed in *Litopenaeus vannamei* responding to phorbol myristate acetate (PMA, a PKC activator), live *Escherichia coli*, and lipopolysaccharide (LPS) stimulation. Furthermore, the generation of human neutrophil ETs was identified via activation of cPKC by various pharmaceuticals [20]. Recently, an aPKC of *M. rosenbergii* was found to be involved in modulation during molting, and in mediation of a signaling pathway responding to epizootic or opportunistic pathogen infections [13]. These isolation procedures revealed that antibodies against mammalian PKCs are cross-reactive in a very diverse group of organisms, and also demonstrate similar modes of action, which strongly point to conservation of PKC sequences in evolution and their importance in living organisms from invertebrates to vertebrates.

The culture of penaeid shrimps had been limited by epidemic infectious diseases such as *Vibrio alginolyticus*, which resulted in whitish musculature in diseased *L. vannamei* to cause mortality [21]. Water temperature fluctuation was the most general stressor of aquatic organisms, and hypothermal stress was found to induce the catecholamine biosynthesis then weaken immunocompetence in *L. vannamei* [22–24], and the same phenomena were observed in *V. alginolyticus* infected shrimp [24]. Zhang et al. [25] indicated that tyrosine hydroxylase activity, the first and rate-limiting enzyme in the catecholamine biosynthetic pathway, and dopamine synthesis were modulated through PKC. These suggested that PKC might play crucial role in shrimp immunocompentence under stress. PKC has been a model for analyzing cellular signal transduction mechanisms [10,14–18], however, less emphasis on its potential role in immunocompetence of shrimp. The aims of the present study were to present a characterization of *L. vannamei* nPKC (LvnPKC), and evaluate its expression in hemocytes after they were challenged with *V. alginolyticus* or exposed to hypothermal stress. Furthermore, LvnPKC gene silencing was conducted to investigate its possible roles in immune modulation of shrimp.

2. Materials and methods

2.1. Animals

White shrimp *L. vannamei* (10–20 g) obtained from a shrimp farm in Pingtung, Taiwan were acclimated in 10 recirculating water tanks. Fifteen shrimp in each tank containing 120 L of seawater were fed commercial pellet feed (Shinta Feed, Pingtung, Taiwan) daily for 2 weeks. Only shrimp in the intermolt stage were used for the study. The molt stage was determined by examining the uropods, in which partial retraction of the epidermis could be distinguished [26]. During the acclimation period, salinity was maintained at 20‰, and the water temperature was 28 ± 1 °C.

There were 127 shrimp in total sampled in this study, and they were divided into four parts. (1) One shrimp was used for cloning potential PKC isotypes from hemocytes, and six shrimp were used for the tissue distribution test under hypothermal stress. (2) Sixty shrimp were used to analyze LvnPKC gene expression of hemocytes in *L. vannamei* challenged with *V. alginolyticus*. (3) Forty-eight shrimp were sampled to evaluate the efficiency and specificity of LvnPKC gene silencing. (4) Twelve shrimp were used for an assessment of immune gene expressions in hemocytes of LvnPKC-silenced shrimp.

2.2. Cloning and characterization of LvnPKC

2.2.1. Sampling

Hemolymph (500 μl) from one shrimp was withdrawn, and hemocytes were collected according to a previously described method [27]. The resulting hemocyte pellet was homogenized in liquid nitrogen, and re-suspended in Tris-EDTA buffer (50 mM Tris-HCl and 1 mM EDTA; pH 7.4) to extract and purify total RNA. Total RNA was extracted and further purified using the Quick-RNA™ MiniPrep Kit (R1055, Zymo Research, USA) following the manufacturer's instructions. First-strand complementary (c)DNA in reverse transcription was synthesized using a Tool Script MMLV RT kit (TGKRA04, Tools Biotechnology, New Taipei City, Taiwan). Reaction conditions recommended by the manufacturer were followed. A polymerase chain reaction (PCR) and subcloning of LvnPKC cDNA were performed. Full-length LvnPKC cDNA of *L. vannamei* was obtained by a reverse-transcription (RT)-PCR, and SMARTer® RACE 5′/3′kit (cat. no. 634858, Takara, USA) following the manufacturer's methods. Degenerate primers were designed based on the highly conserved PKC amino acid sequences of *Apis mellifera* (NP_001128420), *Drosophila melanogaster* (AAA28817), *Zootermopsis nevadensis* (XP_021920016), and *Lucilia cuprine* (KNC31888) in the GenBank database [28] using the Clustal program [29]. Amplification primer pairs for LvnPKC cDNA are shown in Table 1. Primers of PKC1F and PKC1R were used for the first fragment of partial LvnPKC cDNA, and the first cloned LvnPKC fragment was used to designate specific primer sets named Lv-Q-nPKC1F and Lv-Q-nPKC1R for rapid amplification of cDNA (RACE) methods and a real-time PCR assay with Primer Express Software (vers. 3.0., Applied Biosystems, Lincoln, NE, USA). Lv-Q-nPKC1R and PKC1R were used for the 5′-RACE, and Lv-Q-nPKC1F and PKC1F were used for the 3′-RACE. Details of the procedures were described previously [30], and PCRs were performed as follow: 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C (65 °C was used for RACE) for 45 s, and elongation at 72 °C for 70 s, followed by a 10-min extension at 72 °C and cooling at 4 °C.

The PCR products from the previous step were purified using the Zymoclean Gel DNA recovery (Zymo Research, CA, USA) and cloned into a pGEM T-Easy vector (Promega, WI, USA) following the manufacturer's instructions. The ligation product was transformed into *Escherichia coli* strain JM 109 on Luria Bertani (LB) agar containing ampicillin (0.01 g ml^{-1}), X-gal-IPTG ready solution (Amresco, Inc. USA). The positive clones containing target inserts were extracted using a ZR Plasmid Miniprep-classic (Zymo Research, CA, USA) and then was used as a template for DNA sequencing.

2.2.2. Nucleotide sequence analysis

A nucleotide sequence analysis was performed using the dideoxynucleotide chain termination method [31] on a DNA sequencer (Model 373A, Applied Biosystems). Plasmid DNA (1 μg) was used for sequencing with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and was subjected to electrophoresis on 6% denaturing gels. Clones were sequences with the SP6 and T7 primers. The LvnPKC gene sequence was analyzed and compared using the BLASTX and BLASTP search programs (htt://blast.genome.ad.jp) with a GenBank database search. Multiple sequence alignment of the LvnPKC gene was created using the Clustal W analysis program (http://searchlauncher.bcm.tmc.edu). The calculated molecular mass and theoretical isoelectric point were predicated with the ExPASy proteomics server (http://tw.expasy.

Table 1
Degenerate and specific primers, and sequences for gene silencing used in the experiment.

Items/genes	Primer name	Sequence 5′-3′	Amplicon	Reference/GenBank
LvnPKC cloning	PKC1F	AAAGTCCTNGGMAAAGGCAGTTTTGG		Present study
	PKC1R	ACGCCNAGWGCCCACCAGTCNA		
	Lv-Q-nPKCF	CCTTACGTGACGCTGGAGGT		
	Lv-Q-nPKCR	GATCTTGAGGCCGAGCTGGA		
Gene silencing	609-dsRNA	GGUCACAAAUUCAUGGCAATT UUGCCAUGAAUUUGUGACCTT		Present study
	1208-dsRNA	GGACAAGAAGAUGAAGGAATT UUCCUUCAUCUUCUUGUCCTT		
	2348-dsRNA	GCACAGUACAAAGUGCUAATT UUAGCACUUUGUACUGUGCTT		
	GAPDH-dsRNA	UGACCUCAACUACAUGGUUTT AACCAUGUAGUUGAGGUCATT		
LGBP	Lv-Q-LGBP-F	CGGCAACCAGTACGGAGGAAC	115 bp	[66]
	Lv-Q-LGBP-R	GTGGAAATCATCGGCGAAGGAG		
ppAE	Lv-Q-ppA-F	CTAGAGACGTCGGTGTCA TCACC	151 bp	[67]
	Lv-Q-ppA-R	AACTTGCCGTCCGAAGTGCG		
α2-M	Lv-Q-α2M-F	GCACGTAATCAAGATCCG	204 bp	[67]
	Lv-Q-α2M-R	CCCATCTCATTAGCACAAAC		
PE	Lv-Q-PE-F	ATCCAGCAGCCAGGTATG	147 bp	[54]
	Lv-Q-PE-R	CAGACTCATCAGATCCATTCC		
proPOI	Lv-Q-proPOI-F	ACGTCACTTCCGGCAAGCGA	156 bp	[67]
	Lv-Q-proPOI-R	CCTCCTTGTGAGCGTTGTCAG G		
proPOII	Lv-Q-proPOII	ACCACTGGCACTGGCACCTCG TCTA	161 bp	[67]
	Lv-Q-proPOII	TCGCCAGTTCTCGAGCTTCTGCAC		
cytMnSOD	Lv-Q-cytMnSOD-F	TGACGAGAGCTTTGGATCATTCC	155 bp	[67]
	Lv-Q-cytMnSOD-R	TGATTTGCAAGGGATCCTGGTT		
mtMnSOD	Lv-Q-mtMnSOD-F	CAGACTTGCCCTACGATTAC	216 bp	[67]
	Lv-Q-mtMnSOD-R	AGATGGTGTGATTGATGTGAC		
Lysozyme	Lv-Q-Lys-F	GGACTACGGCATCTTCCAGA	97 bp	[68]
	Lv-Q-Lys-R	ATCGGACATCAGATCGGAAC		
β-actin	Lv-Q-βactinF	GCTCGTCCGCCCTTTTAGTA	91 bp	AF300705
	Lv-Q-βactinR	ACAATGGCTCCGGCATGTGCAA		

LvnPKC: *Litopenaeus vannamei* novel protein kinase C; PE: peroxinectin; ppAE, prophenoloxidase activating enzyme; proPO, prophenoloxidase; α2-M: α2-macroglobulin; cytMnSOD, cytosolic manganese superoxide dismutase; mtMnSOD, mitochondrial MnSOD; LGBP, lipopolysaccharide- and β-1,3-glucan-binding protein.

org/tools/pi_tool.html). Potential domains were searched for using Conserved Domain Search Service (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.2.3. Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of amino acid differences (p-distances) by the Neighbor-joining (NJ) method [32] using MEGA 4 software [33]. To construct the phylogenetic tree, indels were removed from multiple alignments. The reliability of the tree obtained was assessed by boot-strapping using 1000 bootstrap replications [34]. The following proteins were used in the alignment: novel protein kinase C of *L. vannamei* (MF683955), *Daphnia magna* (KZS12886), *Z. nevadensis* (XP_021920016), *Aplysia californica* (AAA27771), *Caenorhabditis elegans* (NP_001256396), *Oryctolagus cuniculus* (AAA31426), *Mus musculus* (BAA14288); classical protein kinase C of *Apis mellifera* (NP_001128420), *Neogonodactylus oerstedii* (AUC64088), *D. melanogaster* (AAA28817), *Bos taurus* (AAA30706), *Rattus norvegicus* (alpha type, NP_001099183); atypical protein kinase C of *M. rosenbergii* (ARV85772), *D. melanogaster* (NP_001036541), *A. californica* (NP_001191516), *Danio rerio* (AAK91291), *R. norvegicus* (iota type, NP_114448), *Xenopus laevis* (iota type, NP_001084068); different isoform of PKC in *Homo sapiens* including epsilon type (NP_005391), eta type (NP_006246), gamma type (NP_001303258), beta type (NP_997700) and iota type (NP_002731).

2.2.4. Quantification of LvnPKC gene expression in tissues of shrimp under hypothermal stress by a real-time RT-PCR

The tissue distribution of expression was analyzed with a real-time RT-PCR. Twelve shrimp were placed in a 0.5-metric ton fiberglass-reinforced plastic (FRP) tank containing 0.4 metric tons of recirculating aerated seawater at 20‰ salinity and 28 ± 0.5 °C for 3 days, and then were randomly transferred to 18 or 28 °C for 30 min of exposure. To assess LvnPKC expression in tissues under hypothermal stress, each of two thermal regimes consisted of three tanks as triplicates, and each replicate was comprised of two shrimp kept in a 60-L glass aquarium containing 40 L of recirculating aerated seawater at 20‰ salinity, which was adjusted with a heater or cooler to the desired temperatures of 18 and 28 °C. Two groups (two temperature exposures regimes for 30 min) were conducted, and each group was carried out on three shrimp. In this test, there were six shrimp sampled for determining LvnPKC mRNA expression. After 30 min of exposure, LvnPKC mRNA transcription levels in different tissues were determined. The eyestalk, stomach, muscle, brain, hepatopancreas, heart, intestines, abdominal nerve, gills, subcuticular epithelium, thoracic nerve, and hemocytes were excised, homogenized in liquid nitrogen, and resuspended in Tris EDTA buffer (50 M Tris Cl and 1 M EDTA; pH 7.4), and total RNA was extracted. LvnPKC transcript levels were measured using an SYBR green I real-time RT-PCR assay in an ABI 48-well StepOne™ Real-Time System (Applied Biosystems), and checked following directions of Bustin et al. [35]. A real-time PCR was performed in a total volume of 20 μl containing 10 μl of 2 × power SYBR Green PCR Master mix (Applied Biosystems), 0.4 μl (each) of forward/reverse primers (10 μM), 2 μl of a cDNA template, and 7.2 μl diethylpyrocarbonate water (DEPC-H_2O, AMRESCO). The thermal profile for the SYBR Green PCR was initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing at 60 °C for 1 min. The specific primers of Lv-Q-nPKC1F and Lv-Q-nPKC1R, and Lv-Q-βactinF and Lv-Q-βactinR (Table 1) were used for the quantitative RT-PCR. After amplification, data acquisition and analysis were performed using StepOne™ software (vers. 2.0, Applied Biosystems).

The $2^{-\Delta\Delta CT}$ method was chosen as the calculation method [36]. The difference in the cycle threshold (CT) value of the LvnPKC gene and its housekeeping gene (β-actin), called ΔCT, was calculated. In tissue expression, ΔΔCT = (ΔCT of shrimp for the LvnPKC gene in each sample tissue at 18 or 28 °C treatment) - (ΔCT of those in the hepatopancreas at 28 °C). The LvnPKC mRNA expression was expressed as multiples of change compared to the hepatopancreas of shrimp exposed to 28 °C (mean, $n = 3$).

2.3. LvnPKC gene expression of hemocytes in shrimp challenged with V. alginolyticus

Litopenaeus vannamei in the intermolt stage was injected with 20 μl of a bacterial suspension at 5×10^6 and 5×10^4 colony-forming units (cfu) ml^{-1} of *V. alginolyticus* into the ventral sinus, resulting in 10^5 and 10^3 cfu $shrimp^{-1}$, respectively. The preparation and injection of *V. alginolyticus* followed descriptions by Liu et al. [27] and Yeh et al. [37]. After the injection, shrimp were placed in six tanks (six replicates). Each aquarium contained 100 L of aerated seawater at 28 ± 1 °C and a salinity of 20‰, and ten shrimp were reared in each one. The same treatment of shrimp injected with 20 μl of saline served as the control. At 15, 30, and 60 min after the injection, six shrimp were sampled from each of the six tanks, and another six shrimp without injection served as the initial control. LvnPKC mRNA expression of hemocytes was then determined. Hemocytes were sampled, and total RNA was isolated and measured using an SYBR green I real-time RT-PCR assay as described above. In this test, 60 shrimp were sampled to determine LvnPKC mRNA expression.

The $2^{-\Delta\Delta CT}$ method was used as described above. In the challenge test, ΔΔCT = (ΔCT of bacteria- or saline-injected shrimp for the LvnPKC gene at each time point) - (ΔCT of the initial control). The LvnPKC mRNA expression was expressed as the multiples of change compared to hemocytes of shrimp injected with saline (mean, $n = 6$).

2.4. Immune gene expressions in LvnPKC-silenced L. vannamei

2.4.1. Double-stranded (ds)RNA preparation and gene silencing

Three dsRNAs corresponding to LvnPKC (609-, 1208-, and 2348-dsRNA) transcripts and the control which consisted of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-dsRNA were chemically synthesized and purified with high-performance liquid chromatography (HPLC) by GenePharma (Shanghai, China). The sequence information is shown in Table 1. The three potential LvnPKC-dsRNA, GAPDH-dsRNA, and DEPC-H_2O treatments were conducted, and shrimp without treatment served as the initial group. Hemocytes were collected from silenced shrimp (15 ± 0.2 g) injected with different dsRNAs (Table 1) at a concentration of 15 μg $shrimp^{-1}$ before and 1, 2, and 3 days after the injection. The efficiency and specificity of LvnPKC gene knockdown were subsequently examined using a real-time RT-PCR as described above. Three shrimp were used for each dsRNA fragment test and the controls. The three fragments of 609-, 1208-, and 2348-dsRNA specifically suppressed the expression of LvnPKC mRNA at day 1 post-injection by 52.1%, 29.5%, and 23.8%, respectively, and therefore, 2348-dsRNA was used for the LvnPKC mRNA silencing gene test (Supplementary Fig. 1). Forty-eight shrimp were sampled for the evaluation of efficiency and specificity of LvnPKC gene silencing.

2.4.2. Effect of silencing of LvnPKC on immune gene expressions by hemocytes of L. vannamei

Litopenaeus vannamei (15 ± 0.3 g) received DEPC-H_2O, GAPDH-dsRNA, and 2348-dsRNA following the same method described above. Another three shrimp without treatment served as the initial control. There were four treatments, and each treatment was carried out on three shrimp. After the injection, shrimp were kept in a separate glass aquaria containing 40 L of aerated seawater at 20‰ and 28 ± 1 °C. There were 12 shrimp sampled for the gene expression assessment.

At 1 day after the injection, hemolymph (200 μl) was individually withdrawn, and care was taken to avoid any contamination from sea water or generation of air-bubbles in the hemolymph. Hemolymph added to a 1-ml sterile syringe (25 gauge) containing 0.8 ml of an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, at pH 7.55, with the osmolality adjusted with NaCl to 780 mOsm kg^{-1}) was used to assess immune gene expressions. The hemocyte pellet for the subsequent test was prepared from a hemolymph mixture centrifuged at 400 × *g* and 4 °C for 20 min followed by two washes with saline.

The immune gene mRNA transcriptions in hemocytes, including the prophenoloxidase (proPO)-activating system (prophenoloxidase I (proPOI), proPOII, lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP), peroxinectin (PE), prophenoloxidase-activating enzyme (ppAE), and α2 macroglobulin (α2M)), antioxidant system (cytosolic manganese superoxide dismutase (cytMnSOD) and mitochondrial SOD (mtMnSOD), and an antimicrobial agent (lysozymes) were measured using an SYBR green I real-time RT-PCR assay as described above. Specific primer pairs of immune genes and a housekeeping gene (Table 1) were used for the quantitative real-time PCR, and data acquisition, analysis, and calculation method were as described above. $\Delta\Delta CT$ = (ΔC_T of dsRNA-injected shrimp for the test gene) - (ΔC_T of the initial control).

2.5. Statistical analysis

A *t*-test was conducted to examine significant differences in LvnPKC expression between 18 and 28 °C for each sampled tissue. Data of the multiple of change in LvnPKC expression are presented as the mean ± standard error (SE) and were analyzed with a one-way analysis of variance (ANOVA). When significant differences were indicated at the 0.05 level, then a multiple-comparison (Tukey's) test was conducted to examine significant differences among treatments at the same sampling time in challenge tests, evaluation of the efficiency and specificity of gene silencing, and immune gene expressions in LvnPKC-silenced shrimp, using Sigmastat computer software (Jandel Scientific,CA, USA).

3. Results

3.1. cDNA sequence analysis of LvnPKC

cDNA encoding PKC mRNA of the white shrimp *L. vannamei*, was obtained from hemocytes by an RT-PCR and RACE (accession number: MF683955). The 2454-bp cDNA contained an open reading frame (ORF) of 2232 bp and encoded a 744-amino acid (aa) protein with a predicted molecular mass of 84.4 kDa and a p*I* of 6.25. A search of functional domains revealed the presence of two phorbol ester/diacylglycerol-binding domains (C1 domain), a C2 domain, and a catalytic domain of the serine/threonine kinase, which were as found in nPKC (Fig. 1), and was therefore designated LvnPKC.

3.2. Lv-nPKC amino acid sequence comparison and phylogenetic analysis

Sequence comparison of the LvnPKC deduced amino acids (MF683955) showed identities of 63.4%, 61.8%, 56.7%, 55.2%, and 52.7% to nPKCs of *D. magna* (KZS12886), *A. californica* (AAA27771), *M. musculus* (BAA14288), *Z. nevadensis* (XP_021920016) and *H. sapiens* (NP_005391), respectively (Supplementary Fig. 2). A molecular phylogenetic tree was constructed to further analyze evolutionary relationships among these animal LvnPKC sequences. Based on a MEGA 4 analysis, members of the LvnPKC protein can be classified into three groups of nPKCs, aPKCs, and cPKCs, and the parallel evolution between PKCs of shrimp and other animals was statistically supported. Genetic distances among the penaeid LvnPKC clade were rather short and close to arthropod nPKCs (Fig. 2).

```
                                                                                              cggct    5
tacagtcacttttgccccatttccctatgcactactgggacctattggtgaggtgagacggggcttgtgagcacctgagcagagagaacacgggcg  101
atgttcacgggcagccttaaagtgaaaatatgcgaagctaccgatctccgccttaccgactgtatgacccgctatgtcggagtagcaggggtggga  197
 M  F  T  G  S  L  K  V  K  I  C  E  A  T  D  L  R  L  T  D  C  M  T  R  Y  V  G  V  A  G  V  G   32
aaaggtcctcaagaccaaaccctggatccttacgtgacgctggaggtggacgaggtacactggaccaagacgcaggcccgacagaagaccttcacg  293
 K  G  P  Q  D  Q  T  L  D  P  Y  V  T  L  E  V  D  E  V  H  W  T  K  T  Q  A  R  Q  K  T  F  T   64
cccatatggaacgagagctttgagcaagacgtgatgggcgccgtccagctcggcctcaagatcttccatgactccgcggtcggcaacgatgacttc  389
 P  I  W  N  E  S  F  E  Q  D  V  M  G  A  V  Q  L  G  L  K  I  F  H  D  S  A  V  G  N  D  D  F   96
gtggccgacgcttccttgctcttcgaggaaatatgtgccgagaaccagacccatgccgacatctgggtggacttagaacctcaggggaagctacat  485
 V  A  D  A  S  L  L  F  E  E  I  C  A  E  N  Q  T  H  A  D  I  W  V  D  L  E  P  Q  G  K  L  H  128
gtggtgattgaactaaaatgggcaccaccagaagatggaggcgtccgcccacgagaattcagggaacggcagggcttcaaccggcgacggggtgcc  581
 V  V  I  E  L  K  W  A  P  P  E  D  G  G  V  R  P  R  E  F  R  E  R  Q  G  F  N  R  R  R  G  A  160
atgaggaggcgggttcatcaggtcaatggtcacaaattcatggcaacgttcctacgacaacctacatcctgctcgcattgtcgagaattcatatgg  677
 M  R  R  R  V  H  Q  V  N  G  H  K  F  M  A  T  F  L  R  Q  P  T  S  C  S  H  C  R  E  F  I  W  192
ggtctagggaaacaaggatatcaatgtcaagtatgtacttgcgtagtccacaagcgctgccaccaatcggttgtcacacgatgtccgggaagcaag  773
 G  L  G  K  Q  G  Y  Q  C  Q  V  C  T  C  V  V  H  K  R  C  H  Q  S  V  V  T  R  C  P  G  S  K  224
agtgaaaccatcaatgaggaaccgtacagtccagggaagttgtcaaccacgattcaacgtcaatgtgccacatcggttttctgttcactcctcaaa  869
 S  E  T  I  N  E  E  P  Y  S  P  G  K  L  S  T  T  I  Q  R  Q  C  A  T  S  V  F  C  S  L  L  K  256
cggtttacattctgtgaccattgtggctcattactttatggccttattcgtcagggtctccaatgtgaagtatgcaacatgaatgttcacaagaga  965
 R  F  T  F  C  D  H  C  G  S  L  L  Y  G  L  I  R  Q  G  L  Q  C  E  V  C  N  M  N  V  H  K  R  288
tgccaaaagaatgtagccaacaattgtgggatagacgtaaaacagttatcagaaattctagccaccatgggtatacggccccaagatgaagcggcc 1061
 C  Q  K  N  V  A  N  N  C  G  I  D  V  K  Q  L  S  E  I  L  A  T  M  G  I  R  P  Q  D  E  A  A  320
aaacgcaagaagaagtctgttagtgagacaaggttatcatcggccaccgcccctgttagtgtcccaggaatcatcggagaagtggaagtcccaggt 1157
 K  R  K  K  K  S  V  S  E  T  R  L  S  S  A  T  A  P  V  S  V  P  G  I  I  G  E  V  E  V  P  G  352
gaaatgaatgaggaagagctaagactaaggattgaagctcagcgtatcatggacaagaagatgaaggaacgctgtgcagaagaaggactagatgcc 1253
 E  M  N  E  E  E  L  R  L  R  I  E  A  Q  R  I  M  D  K  K  M  K  E  R  C  A  E  E  G  L  D  A  384
aagtcaaagccacacgatacttctttagatgtcatgcttggtgggaagaaggtagacctggatgacttctcattcattaaagtgcttggcaaagga 1349
 K  S  K  P  H  D  T  S  L  D  V  M  L  G  G  K  K  V  D  L  D  D  F  S  F  I  K  V  L  G  K  G  416
agttttggaaaagtaatgctagctgaactgaaagggacagatgaagtctatgccataaaggtgcttaagaaagatgtgatcctacaagatgatgat 1445
 S  F  G  K  V  M  L  A  E  L  K  G  T  D  E  V  Y  A  I  K  V  L  K  K  D  V  I  L  Q  D  D  D  448
gtagaatgtacaatgactgagcggcggatcttagcgatggctgcccaccaccccttcctcactgccctccactcttgctttcaaaccaaggatcga 1541
 V  E  C  T  M  T  E  R  R  I  L  A  M  A  A  H  H  P  F  L  T  A  L  H  S  C  F  Q  T  K  D  R  480
ttattctttgttatggaatatgttaatggtggcgacctaatgttccaaattcagaaggctcgcaagttcacagagtcacgtgcaagattttatgca 1637
 L  F  F  V  M  E  Y  V  N  G  G  D  L  M  F  Q  I  Q  K  A  R  K  F  T  E  S  R  A  R  F  Y  A  512
gctgaggtcacgttggccttacagttcctccataaaaatggtgttatttacagagatttgaagttagataatattctactagacagtgaaggacat 1733
 A  E  V  T  L  A  L  Q  F  L  H  K  N  G  V  I  Y  R  D  L  K  L  D  N  I  L  L  D  S  E  G  H  544
tgtaaaatagctgattttggtatgtgtaaggaaggcatcagggataatattacaacaaccaccttctgtggaacaccagattacattgcgccagag 1829
 C  K  I  A  D  F  G  M  C  K  E  G  I  R  D  N  I  T  T  T  T  F  C  G  T  P  D  Y  I  A  P  E  576
attcttaaagagttggactatggtgcaagtgttgactggtgggcactaggagttctcatgtatgaaatgatggcaggacagccaccatttgaggct 1925
 I  L  K  E  L  D  Y  G  A  S  V  D  W  W  A  L  G  V  L  M  Y  E  M  M  A  G  Q  P  P  F  E  A  608
gataatgaagatgatctctttgaatccatcctgcatgaagaagttttatatccagtgtggctttcaaaggaagcagtttctatactgaaagggttt 2021
 D  N  E  D  D  L  F  E  S  I  L  H  E  E  V  L  Y  P  V  W  L  S  K  E  A  V  S  I  L  K  G  F  640
atgacaaaggaaccatccaagcggttgggctgtgtcgcagagcgtggaggagaattggcaattcgtaaccacaaattcttccgagagatagactgg 2117
 M  T  K  E  P  S  K  R  L  G  C  V  A  E  R  G  G  E  L  A  I  R  N  H  K  F  F  R  E  I  D  W  672
gaggcccttgagcagcgcaaagttaaaccaccatttacacctaagataaagggacgtaaggatacagtcaactttgatgccgagtttactaaggaa 2213
 E  A  L  E  Q  R  K  V  K  P  P  F  T  P  K  I  K  G  R  K  D  T  V  N  F  D  A  E  F  T  K  E  704
gagccaactctcacctttattaatgaagaagttgttcgtgctatcgaccaggacgagtttagaggattttcttttgttaatcgtgaatttaagtcc 2309
 E  P  T  L  T  F  I  N  E  E  V  V  R  A  I  D  Q  D  E  F  R  G  F  S  F  V  N  R  E  F  K  S  736
atggcggctgcaccatgtcaaacctaagtgcaaatgcagcacagtacaaagtgctaaagtctccccaggaagcttgatgtgcagactatatgagag 2405
 M  A  A  A  P  C  Q  T                                                                          744
gattctttagggaagaaagcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa                                                2454
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Litopenaeus vannamei* novel protein kinase C (LvnPKC). C2 domain in protein kinase C epsilon is underline. Two phorbol esters/diacylglycerol binding domains (C1 domain) are shaded grey with black bold letter. Catalytic domain of the serine/threonine kinase is in box.

3.3. Tissue distribution of LvnPKC in hypothermally stressed shrimp

Tissue expressions of LvnPKC in various tissues of *L. vannamei* were examined after transfer from 28 to 28 °C and from 28 to 18 °C for 30 min. In the real-time PCR study, the specific primer pair, Lv-Q-nPKC1F and Lv-Q-nPKC1R, was used to amplify 112-bp fragments of cDNA from hemocytes, the hepatopancreas, gills, muscles, subcuticular epithelium, intestines, abdominal nerve, thoracic nerve, brain,

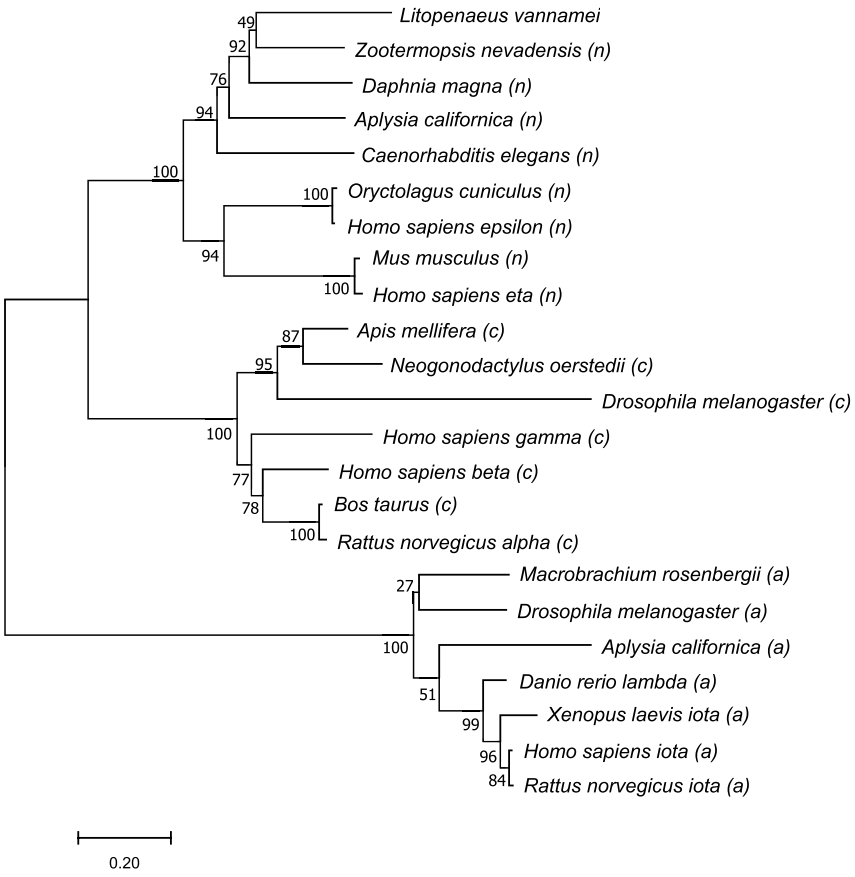

Fig. 2. Phylogenetic tree for *Litopenaeus vannamei* novel protein kinase C (LvnPKC) and 22 other members of the PKC family based on ClustalW alignment of amino acid sequences including novel protein kinase C of *L. vannamei* (MF683955), *Daphnia magna* (KZS12886), *Zootermopsis nevadensis* (XP_021920016), *Aplysia californica* (AAA27771), *Caenorhabditis elegans* (NP_001256396), *Oryctolagus cuniculus* (AAA31426), *Mus musculus* (BAA14288); classical protein kinase C of *Apis mellifera* (NP_001128420), *Neogonodactylus oerstedii* (AUC64088), *Drosophila melanogaster* (AAA28817), *Bos taurus* (AAA30706), *Rattus norvegicus* (alpha type, NP_001099183); atypical protein kinase C of *Macrobrachium rosenbergii* (ARV85772), *D. melanogaster* (NP_001036541), *A. californica* (NP_001191516), *Danio rerio* (AAK91291), *Rattus norvegicus* (iota type, NP_114448), *Xenopus laevis* (iota type, NP_001084068); different isoform of PKC in *Homo sapiens* including epsilon type (NP_005391), eta type (NP_006246), gamma type (NP_001303258), beta type (NP_997700) and iota type (NP_002731) in Genbank. The values for each internal branch determined by bootstrap analysis with 1000 replications is based on the Neighbor-joining method. The numbers at the nodes are bootstrap values in percentage. (n), (c) and (a) represent novel, classical and atypical protein kinase C, respectively.

Table 2
The LvnPKC expression in different tissues of *Litopenaeus vannamei* exposed to 28 °C and 18 °C including eyestalks (E), stomach (ST), muscle (M), hepatopancrea (HP), intestine (I), heart (H), abdominal nerve (AN), gills (G), subcuticular epithelium (SE), thoracic nerve (TN), brain (B), hemocyte (HC) (n = 3). The relative expression of tissues detected with SYBR green real-time PCR using a $2^{-\Delta\Delta CT}$ calculation method are compared to hepatopancrea. Data with different letters among tissues at the same temperature and with asterisk between different temperatures in the same tissue significantly differ ($p < 0.05$).

Tissues/Temperature	28°C	18°C
E	869.1 ± 58.4 c*	3206.20 ± 754.6 x
ST	1498.3 ± 367.1 b*	13.95 ± 2.9 y
M	1501.3 ± 271.5 b*	5.93 ± 2.4 y
HP	1.0 ± 0.3 e	6.45 ± 1.1y*
I	2378.0 ± 97.4 a*	9.92 ± 0.9 y
H	44.3 ± 13.6 e*	13.32 ± 1.5 y
AN	536.5 ± 118.6 cd*	38.54 ± 4.4 y
G	366.2 ± 63.1 de*	101.31 ± 12.8 y
TN	991.5 ± 77.1 c*	5.43 ± 1.3 y
B	43.8 ± 14.9 e	78.42 ± 10.0 y*
HC	2290.9 ± 233.6 a*	11.93 ± 1.2 y
SE	343.5 ± 68.2 de*	81.70 ± 22.1 y

stomach, and heart, and β-actin was used as a positive control. LvnPKC mRNA was widely distributed in all tissues of shrimp exposed to 28 °C. Hypothermally stressed shrimp exhibited a significant increase in LvnPKC expression in the brain, eyestalks, and hepatopancreas, and a significant decrease levels in the stomach, muscles, intestines, heart, abdominal nerve, gills, thoracic nerve, brain, hemocytes, and subcuticular epithelium, compared to those transferred from 28 to 28 °C. LvnPKC expression in intestines and hemocytes of shrimp transferred from 28 to 28 °C and in the eyestalk of those transferred from 28 to 18 °C showed significantly higher levels than the other tissues, respectively (Table 2).

3.4. LvnPKC expressions in hemocytes of shrimp challenged with V. alginolyticus

Significantly increased LvnPKC expression was observed in hemocytes of shrimp infected with 10^3 cfu shrimp^{-1} for 30 and 60 min revealing 1.3- and 1.6-fold changes or with 10^5 cfu shrimp^{-1} for 15 min at a 1.4-fold change, compared to the control at each sampling time (Fig. 3).

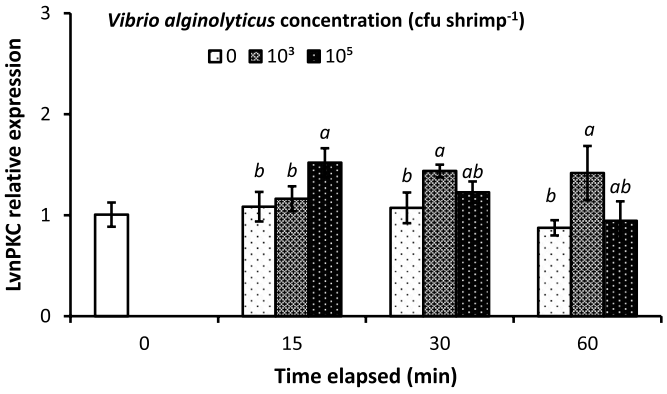

Fig. 3. *Litopenaeus vannamei* novel protein kinase C (LvnPKC) expression in hemocytes of shrimp injected with 0, 10^3 and 10^5 cfu shrimp^{-1} of *Vibrio alginolyticus* for 0, 15, 30 and 60 min. Each bar represents the mean value from 6 shrimp with the standard error. Bars with different letters significantly differ ($p < 0.05$) among treatments.

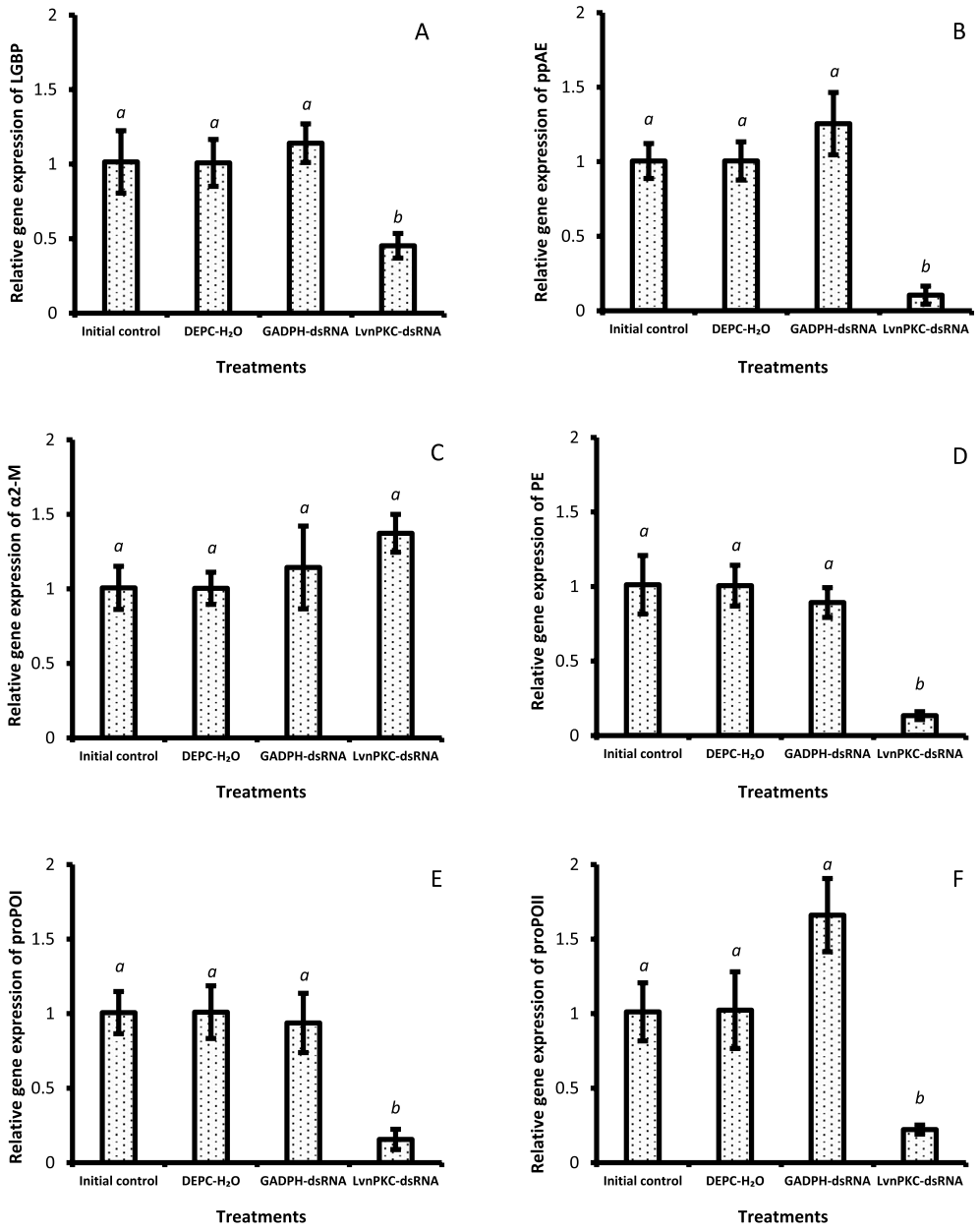

Fig. 4. Lipopolysaccharide- and β-glucan-binding protein (LGBP) (A), prophenoloxidase activating enzyme (ppAE) (B), and α2-macroglobulin (α2-M) (C), peroxinectin (PE) (D), prophenoloxidase I (proPOI) (E), proPOII (F), gene expression in hemocytes of *Litopenaeus vannamei* after receiving DEPC-H_2O, glyceraldehyde-3-phosphate dehydrogenase dsRNA (dsGAPDH), and dsLvnPKC for 1 day. Statistical descriptions are the same as those in Fig. 3.

3.5. Immune gene expressions in hemocytes of LvnPKC-silenced shrimp

In all the immune gene expression assessments, there was no significant difference among the initial control, DEPC-H_2O, and GADPH-dsRNA treatments. Expressions of genes involved in the proPO system, including LGBP, ppAE, PE, proPOI, and proPOII significantly decreased in LvnPKC-silenced shrimp compared to those in the initial control or those injected with DEPC-H_2O and GADPH-dsRNA (Fig. 4A–F). In the antioxidant system of LvnPKC-silenced shrimp, cytMnSOD and mtMnSOD expressions had significantly decreased by 0.30- and 0.31-fold, respectively, compared to those injected with DEPC-H_2O (Fig. 5A and B). The antimicrobial agent, lysozyme, revealed significant decreases in LvnPKC-silenced shrimp compared to the other treatments (Fig. 6).

4. Discussion

The PKC family of serine/threonine kinases can be subdivided into three groups including classical isotypes (calcium and diacylglycerol dependent), novel isotypes (calcium independent and diacylglycerol dependent), and atypical isotypes (nonresponsive to diacylglycerol) [4]. A receptor for activated PKC (RACK), required in protein-protein interactions between each activated isozyme and a specific, isozyme-selective anchoring protein, must be involved in targeting activated PKCs and other signaling proteins and in localization of each activated PKC isozyme [38], which possesses diverse functions including in cell signaling pathways, cell development, cell growth, cell adhesion and movement, and immune responses [39]. In *L. vannamei*, RACK1 was identified and found to be involved in immune defense and signaling transduction in hemocytes, when they were infected with *V. alginolyticus* [30]. In the present study, a potential PKC isotype possessing a phorbol ester/diacylglycerol-binding domain (C1 domain), a C2

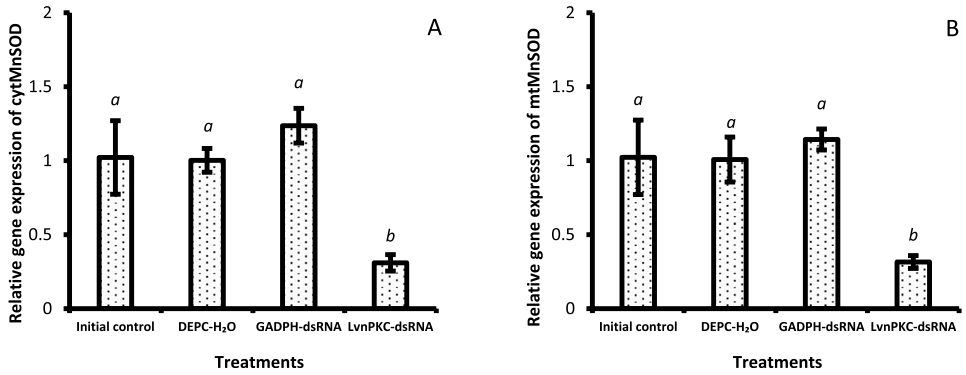

Fig. 5. Cytosolic manganese superoxide dismutase (cytMnSOD) (A) and mitochondrial (mt)MnSOD (B) expression in hemocytes of *Litopenaeus vannamei* after receiving DEPC-H_2O, glyceraldehyde-3-phosphate dehydrogenase dsRNA (dsGAPDH), and dsLvnPKC for 1 day. Statistical descriptions are the same as those in Fig. 3.

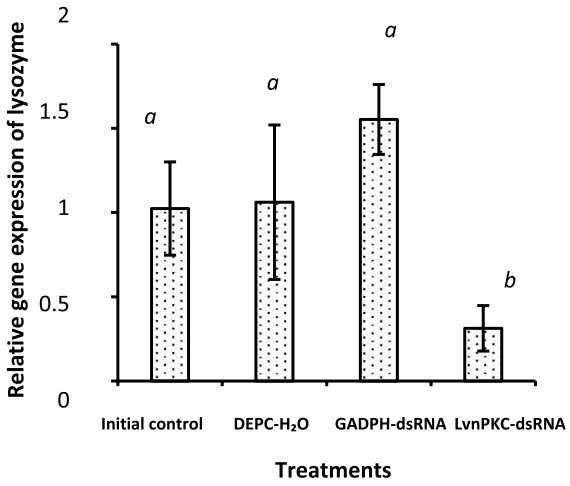

Fig. 6. Lysozyme expression in hemocytes of *Litopenaeus vannamei* after receiving DEPC-H_2O, glyceraldehyde-3-phosphate dehydrogenase dsRNA (dsGAPDH), and dsLvnPKC for 1 day. Statistical descriptions are the same as those in Fig. 3.

domain, and a catalytic domain of the serine/threonine kinase was isolated from hemocytes of *L. vannamei* as LvnPKC, which shared 52.7% similarity with the epsilon type of PKC (nPKC) of *H. sapiens*. Furthermore, in a phylogenic analysis, LvnPKC was found to be involved in a group of nPKCs, and similar to those in invertebrates. These results suggested that LvnPKC possessed well-preserved catalytic and regulatory domains revealed in nPKCs of vertebrates or invertebrates, indicating the potential function in mediating signaling pathways of physiological and immunological responses.

In the assessment of tissue distribution, LvnPKC was detected in all tested tissues in shrimp exposed to 28 or 18 °C, and those in the eyestalk at 18 °C treatment and intestines and hemocytes at 28 °C treatment were significantly higher than levels in other tissues. Furthermore, except to eyestalk, hepatopancreas and brain, when *L. vannamei* was exposed to hypothermal stress, LvnPKC expressions in the other sampled tissues significantly decreased compared to those at 28 °C. These results implied that LvnPKC possesses a dual role in mediating signaling pathways against a stressful environment; however, its role in regulating physiological or immunological responses remains largely unknown. Interestingly, in the present study, significantly upregulated LvnPKC expressions were observed in the eyestalk, hepatopancreas, and brain of hypothermally stressed shrimp in the meantime. In decapods, the crustacean hyperglycemic hormone (CHH, a neuropeptide), which is synthesized, stored, and released in the brain and eyestalk, participates in several mechanisms including glucose level regulation [40–42]. The hyperglycemic effect of CHH secreted from eyestalk is correlated with glycogenolysis primarily in hepatopancrea [43,44]. Those results suggested that upregulation of LvnPKC in the eyestalk, brain, and hepatopancreas of hypothermally stressed shrimp might be part of the induction of stress responses to hyperthermia. Redundancy of function in different isotypes of PKCs in various tissues hampered the identification of definitive roles of individual family members, and therefore, to highlight the essential non-redundant roles of LvnPKC in different systems necessarily should be clarified in future studies.

In the present study, within 60 min of *V. alginolyticus* being injected into *L. vannamei*, LvnPKC expression was upregulated in hemocytes, and the increased expression in those receiving a high challenge dosage occurred earlier than with a low dose. Lipopolysaccharides (LPS) are found in the outer membrane of gram-negative bacteria. In mammalian macrophages, PKC was suggested to respond to LPS challenge [45,46], and in mollusks, Walker and Plows [47] reported an LPS-mediated PKC pathway in molluscan immune cells (hemocytes). In response to pathogenic stimulation, extracellular traps (ETs) are formed to trap invading pathogens and then kill them extracellularly by vertebrate immune cells [48] or *L. vannamei* hemocytes [19]. Ng et al. [19] reported that ET formation was strongly stimulated by PMA by activating the PKC signal pathway in a dose-dependent manner in hemocytes of *L. vannamei*. Those results indicated that *V. alginolyticus* inducing LvnPKC expression in hemocytes play crucial roles in mediating shrimp cellular defense against invading pathogens.

In a review, Amparyup et al. [49] stated that the proPO-activating system as a non-self-recognition system participates in innate immune responses accompanying cellular responses via hemocyte attraction and induces phagocytosis, melanization, cytotoxic reactant production, particle encapsulation, and the formation of nodules and capsules, and these therefore revealed that cellular defense plays an important role in crustacean immunity. In invertebrates, phagocytosis is a process carried out by hemocytes (phagocytes) that have the capacity to recognize and ingest non-self molecules, such as bacteria, spores, or senescent cells of the organism itself; this process had been preserved during evolution as a precursor of vertebrate innate immunity [50]. PKCs in macrophages were reported to mediate cell adhesion and spread, phagocytosis, and the production of reactive oxygen/nitrogen intermediates in mammals [14–17]. Castrillo et al. [17] reported that PKCε, a novel PKC, was critically involved at an early stage of LPS-mediated signaling in activated macrophages of mice, and an increased incidence of mortality was observed in mice deficient for PKCε against bacterial infection. In a review, Ramos Martínez et al. [10] stated that in molluscan hemocytes, phagocytosis, activation of respiratory bursts, and synthesis of antimicrobial peptides employed protein phosphorylation/dephosphorylation as a mechanism to transmit information between different cellular compartments to elicit distinct physiological responses, implicating the roles of PKC enzymatic activities. RACK1, an anchoring protein as a receptor for activated PKC, was identified in *L. vannamei*, and was claimed to potentially be involved in cellular immunity

signaling transduction [30]. To clarify the roles of LvnPKC in signaling pathways of immune modulation, hemocytes from LvnPKC-silenced shrimp were further applied in the present study.

In *L. vannamei*, Phupet et al. [51] reported that LGBP functioned as a pattern recognition receptor to recognize microbial pathogens to activate shrimp immune defense against invading pathogens through the agglutination, binding, enhancing encapsulation and the activation of PO activity in proPO system in the hemocytes. After binding to the ligands, LGBP engages in activation of the proPO system that plays a prominent role in non-self recognition, hemocyte communication, and melanin production. Following activation, hemocytes are degranulated and release granules containing proPO, which is converted to its active form as PO by a serine proteinase (also termed ppAE) [52]. In addition, PE, a protein associated with the proPO system for mediating hemocyte attachment and spread, is a multifunctional protein and is essential in crustacean cellular defense reactions for enhancing encapsulation [53,54]. The alpha-2-macroglobulin, a broad range serine proteinase inhibitor, regulating the proPO-activating system in *L. vannamei* [55] showed no significant difference on gene expression in hemocytes of LvnPKC silenced shrimp in the present study. These might indicate that alpha-2-macroglobulin was not regulated through LvnPKC. The significantly reduced LGBP, ppAE, PE, proPOI, and proPOII expressions involving in the proPO-activating system were detected in hemocytes of LvnPKC-silenced shrimp, which implied that the proPO system in hemocytes was modulated through LvnPKC. Furthermore, the obvious decrease of lysozyme expression in hemocytes of LvnPKC-silenced shrimp was also investigated. These indicated that not only proPO system but also lysozyme in hemocytes of *L. vannamei* might be regulated via LvnPKC. Xie et al. [56] reported that in *L. vannamei*, LPS induced an increase in degranulation and extracellular PO activity as well as a decrease in intracellular PO activity, all of which were mediated through a PKC signaling pathway. Lysozyme were found to be released within semigranular and granular hemocytes in many marine invertebrates following a process of degranulation of hemocytes during the immune response [57,58], and then they assist in breaking down foreign materials through membrane destabilization [59–61]. These studies implied that proPO system, lysozyme and/or degranulation might be modulated through PKC pathway. Direct evidence of the relation between LvnPKC and degranulation in hemocytes should be clarified in future studies.

SODs, including iron (Fe)SOD, MnSOD, and copper/zinc (Cu,Zn) SOD, are classified depending on the metal content [62], and are essential antioxidant enzymes that rapidly eliminate reactive oxygen species (ROS) and reactive oxygen intermediates (ROIs) resulting from oxidative stress or during defense reactions in oxygen-respiring organisms [63]. In human lung adenocarcinoma cells, the tumor-promoting TPA and tumor necrosis factor (TNF)-α were implicated in stimulating expression of the mitochondrial manganese-dependent isoform of SOD (MnSOD) mediated through PKC signaling pathways [64]. Cell signaling in oxidation and activation through PKC was reviewed by Cosentino-Gomes et al. [65]. In the present study, cyMnSOD and mtMnSOD expressions significantly decreased in hemocytes of LvnPKC-silenced shrimp, and these results implied that LvnPKC plays an important role in mediating oxidation to maintain homeostasis.

In conclusion, a potential PKC isotype containing the classical phorbol ester/diacylglycerol-binding domain (C1 domain), C2 domain, and catalytic domain of the serine/threonine kinase was cloned from *L. vannamei* termed LvnPKC, sharing similarity with novel PKCs in vertebrates and invertebrates. LvnPKC expression in eyestalks, hepatopancreas, and brain of shrimp exposed to hypothermal stress significantly increased, and those in stomach, muscle, intestines, heart, abdominal nerve, gills, thoracic nerve, hemocytes, and subcuticular epithelium significantly decreased. In addition, LvnPKC expression in hemocytes of shrimp injected with *V. alginolyticus* at 10^5 cfu shrimp^{-1} was significantly elevated earlier than those at 10^3 cfu shrimp^{-1}. Moreover, after silencing LvnPKC in hemocytes, the downregulated proPO system- and antioxidant system-related genes as well as lysozyme were investigated. These results suggest that LvnPKC is crucially involved in shrimp immunity against invading pathogens and hypothermal stress, and plays an important role in mediating the proPO and antioxidant systems.

Acknowledgements

This research was supported by a grant from the Ministry of Science and Technology (MOST 106-2313-B-020 -009 - and MOST 107-2313-B-020 -007 -MY3), Taiwan, Republic of China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.005.

References

[1] M. Inoue, A. Kishimoto, Y. Takai, Y. Nishizuka, Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain, J. Biol. Chem. 252 (1977) 7610–7616.
[2] Y. Takai, A. Kishimoto, U. Kikkawa, T. Mori, Y. Nishizuka, Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system, Biochem. Biophys. Res. Commun. 91 (1979) 1218–1224.
[3] M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, Y. Nishizuka, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, J. Biol. Chem. 257 (1982) 7847–7851.
[4] S. Ohno, Y. Nishizuka, Protein kinase C isotypes and their specific functions: prologue, J. Biochem. 132 (2002) 509–511.
[5] Y. Nishizuka, Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607–614.
[6] Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular responses, FASEB J. 9 (1995) 484–496.
[7] M.P. Walsh, A. Horowitz, O. Clement-Chomienne, J.E. Andrea, B.G. Alien, K.G. Morgan, Protein kinase C mediation of Ca^{2+} -independent contractions of vascular smooth muscle, Biochem. Cell Biol. 74 (1996) 485–502.
[8] J.P. Liu, Protein kinase C and its substrates, Mol. Cell. Endocrinol. 116 (1996) 1–29.
[9] R. Barcia, J.M. Lopez-Garcia, J.E. Girardini, J.I. Ramos-Martínez, Identification of protein kinases C isoforms in marine mussels, Mol. Biol. Int. 42 (1997) 1241–1248.
[10] J.I. Ramos Martínez, M. González-Riopedre, R. Barcia, Role of protein kinases C (PKC) in the relationship between the neuroendocrine and immune systems in marine mussels: the model of *Mytilus galloprovincialis* Lamark (1819), Ital. J. Zool. 79 (2012) 162–168.
[11] Y. Soroka, A. Sagi, I. Khalaila, U. Abdu, Y. Milner, Changes in protein kinase C during vitellogenesis in the crayfish *Cherax quadricarinatus*–possible activation by methyl farnesoate, Gen. Comp. Endocrinol. 118 (2000) 200–208.
[12] C.F. Huang, N.N. Chuang, Purification of the delta isoenzyme of protein kinase C from the hepatopancreas of the shrimp *Penaeus monodon* with phosphorylation on tyrosine residues, J. Exp. Zool. 272 (1995) 258–265.
[13] Y.C. Yeh, C.C. Chang, P.P. Lee, W. Cheng, The transcription of atypical protein kinase C in hemocytes of the giant freshwater prawn, *Macrobrachium rosenbergii*, during the molt stage and injection of pathogen-associated compounds, Fish Shellfish Immunol. 69 (2017) 52–58.
[14] E.A. Clarke, J.S. Brugge, Integrins and signal-transduction pathways-the road taken, Science 268 (1995) 233–239.
[15] L.H. Allen, A.A. Aderem, A role for MARCKS, the α isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages, J. Exp. Med. 182 (1995) 829–840.
[16] E.C. Larsen, J.A. DiGennaro, N. Saito, S. Mehta, D.J. Loegering, J.E. Mazurkiewicz, M.R. Lennartz, Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells, J. Immunol. 165 (2000) 2809–2817.
[17] A. Castrillo, D.J. Pennington, F. Otto, P.J. Parker, M.J. Owen, L. Bosca, Protein kinase C is required for macrophage activation and defence against bacterial infection, J. Exp. Med. 194 (2001) 1231–1242.
[18] L.D. Plows, R.T. Cook, A.J. Davies, A.J. Walker, Phagocytosis by *Lymnaea stagnalis* haemocytes: a potential role for phosphatitidylinositol 3-kinase but not protein kinase A, J. Invertebr. Pathol. 91 (2006) 74–77.
[19] T.H. Ng, S.H. Chang, M.H. Wu, H.C. Wang, Shrimp hemocytes release extracellular traps that kill bacteria, Dev. Comp. Immunol. 41 (2013) 644–651.
[20] R.D. Gray, C.D. Lucas, A. Mackellar, F. Li, K. Hiersemenzel, C. Haslett, D.J. Davidson, A.G. Rossi, Activation of conventional protein kinase C (PKC) is critical in the generation of human neutrophil extracellular traps, J. Inflamm. 10 (2013) 12.
[21] C.H. Liu, W. Cheng, J.P. Hsu, J.C. Chen, *Vibrio alginolyticus* in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing, Dis. Aquat. Org. 61 (2004) 169–174.

[22] W. Cheng, Y.W. Ka, C.C. Chang, Dopamine beta-hydroxylase participate in the immunoendocrine responses of hypothermal stressed white shrimp, *Litopenaeus vannamei*, Fish, Shellfish Immunol. 59 (2016) 166–178.
[23] R. Mapanao, W. Cheng, Cloning and characterization of tyrosine hydroxylase (TH) from the pacific white leg shrimp *Litopenaeus vannamei*, and its expression following pathogen challenge and hypothermal stress, Fish Shellfish Immunol. 56 (2016) 506–516.
[24] R. Mapanao, C.C. Chang, W. Cheng, K.F. Liu, Silencing tyrosine hydroxylase retards depression of immunocompetence of *Litopenaeus vannamei* under hypothermal stress, Fish Shellfish Immunol. 72 (2018) 519–527.
[25] D. Zhang, A. Kanthasamy, Y. Yang, V. Anantharam, A. Kanthasamy, Protein kinase C delta negatively regulates tyrosine hydroxylase activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons, J. Neurosci. 27 (2007) 5349–5362.
[26] L. Robertson, W. Bray, J. Leung-Truillo, A. Lawrenve, Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*, J. World Aquacult. Soc. 18 (1987) 180–185.
[27] C.H. Liu, M.C. Tseng, W. Cheng, Identification and cloning of the antioxidant enzyme, glutathione peroxidase, of white shrimp, *Litopenaeus vannamei*, and its expression following *Vibrio alginolyticus* infection, Fish Shellfish Immunol. 23 (2007) 34–45.
[28] D.A. Benson, M. Boguski, D.J. Lipman, J. Ostell, GenBank, Nucleic Acids Res. 22 (1994) 3441–3444.
[29] D.G. Higgins, P.M. Sharp, CLUSTAL: a package for performing multiple alignment on a microcomputer, Gene 73 (1998) 237–244.
[30] Z.W. Chang, C.C. Chang, Roles of receptor for activated protein kinase C1 for modulating immune responses in white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 46 (2015) 753–764.
[31] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 5463–5467.
[32] N. Saitou, M. Nei, The Neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[33] K.J. Kumar, K. Tamura, M. Nei, MEGA: molecular evolutionary genetics analysis MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.
[34] J. Felsenstein, Confidence limits on phylogenies: and approach using the bootstrap, Evolution 39 (1985) 783–791.
[35] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol. 25 (2000) 169–193.
[36] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method, Methods 25 (2001) 402–408.
[37] M.S. Yeh, W.L. Tsai, W. Cheng, Identification and cloning of the second type transglutaminase from *Litopenaeus vannamei*, and its transcription following pathogen infection and in relation to the haemolymph coagulation, Fish Shellfish Immunol. 35 (2013) 1613–1623.
[38] D. Mochly-Rosen, H. Khaner, J. Lopez, Identification of intracellular receptor proteins for activated protein kinase C, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3997–4000.
[39] A. Mccahill, J. Warwicker, G.B. Bolger, M.D. Houslay, S.J. Yarwood, The RACK1 scaffold protein: a dynamic cog in cell response mechanisms, Mol. Pharmacol. 62 (2002) 1261–1273.
[40] C.M. Kuo, Y.H. Yang, Hyperglycaemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*, J. Comp. Physiol. 169B (1999) 49–54.
[41] M.L. Fanjul-Moles, Biochemical and functional aspects of crustacean hyperglycemic hormone in decapods crustaceans: review and update, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 142 (2006) 390–410.
[42] S.G. Webster, R. Keller, H. Dircksen, The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction, Gen. Comp. Endocrinol. 175 (2012) 217–233.
[43] E.A. Santos, R. Keller, Crustacean hyperglycemic hormone (CHH) and the regulation of carbohydrate metabolism: current perspectives, Comp. Biochem. Physiol. A Physiol. 106 (1993) 405–411.
[44] J.S. Chung, N. Zmora, H. Katayama, N. Tsutsui, Crustacean hyperglycemic hormone (CHH) neuropeptides family: functions, titer, and binding to target tissues, Gen. Comp. Endocrinol. 166 (2010) 447–454.
[45] M.J. Sweet, D.A. Hume, Endotoxin signal transduction in macrophages, J. Leukoc. Biol. 60 (1996) 8–13.
[46] K.J. Procyk, M.R. Rippo, R. Testi, F. Hofmann, P.J. Parker, M. Baccarini, Lipopolysaccharide induces jun N-terminal kinase activation in macrophages by a novel Cdc42/Rac-independent pathway involving sequential activation of protein kinase C zeta and phosphatidylcholine-dependent phospholipase C, Blood 96 (2000) 2592–2598.
[47] A.J. Walker, L.D. Plows, Bacterial lipopolysaccharide modulates protein kinase C signalling in *Lymnaea stagnalis* haemocytes, Biol. Cell 95 (2003) 527–533.
[48] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria, Science 303 (2004) 1532–1535.
[49] P. Amparyup, W. Charoensapsri, A. Tassanakajon, Prophenoloxidase system and its role in shrimp immune responses against major pathogens, Fish Shellfish Immunol. 34 (2013) 990–1001.
[50] L. Mudlarz, L.E. Jones, C.D. Harvell, Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates, Annu. Rev. Ecol. Evol. Syst. 37 (2006) 251–288.
[51] B. Phupet, T. Pitakpornpreecha, N. Baowubon, P. Runsaeng, P. Utarabhand, Lipopolysaccharide- and β-1,3-glucan-binding protein from Litopenaeus vannamei: purification, cloning and contribution in shrimp defense immunity via phenoloxidase activation, Dev. Comp. Immunol. 81 (2018) 167–179.
[52] K. Sritunyalucksana, K. Söderhäll, The proPO and clotting system in crustaceans, Aquaculture 191 (2000) 53–69.
[53] M.W. Johansson, K. Söderhäll, Isolation and purification of a cell adhesion factor from crayfish blood cells, J. Cell Biol. 106 (1988) 1795–1803.
[54] C.H. Liu, W. Cheng, C.M. Kuo, J.C. Chen, Molecular cloning and characterisation of a cell adhesion molecule, peroxinectin from the white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 17 (2004) 13–26.
[55] S. Ponprateep, T. Vatanavicharn, C.F. Lo, A. Tassanakajon, V. Rimphanitchayakit, Alpha-2-macroglobulin is a modulator of prophenoloxidase system in pacific white shrimp Litopenaeus vannamai, Fish Shellfish Immunol. 62 (2017) 68–74.
[56] P. Xie, L. Pan, W. Xu, F. Yue, The roles of serine protease, intracellular and extracellular phenoloxidase in activation of prophenoloxidase system, and characterization of phenoloxidase from shrimp haemocytes induced by lipopolysaccharide or dopamine, Chin. J. Oceanol. Limnol. 31 (2013) 1018–1027.
[57] N.A. Ratcliffe, A.F. Rowley, S.W. Fitzgerald, C.P. Rhodes, Invertebrate immunity: basic concepts and recent advances, Int. Rev. Cytol. 97 (1985) 183–350.
[58] H.H. Sung, R. Sun, Intrahaemocytic activity of lysosomal enzymes in *Penaeus monodon* and *Macrobrachium rosenbergii*, Fish Shellfish Immunol. 9 (1999) 505–508.
[59] C. Hauton, L.E. Hawkins, S. Hutchson, Response of haemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L.), Fish Shellfish Immunol. 11 (2001) 143–153.
[60] M. Faurschou, N. Borregaard, Neutrophil granules and secretory vesicles in inflammation, Microb. Infect. 5 (2003) 1317–1327.
[61] S. Nicholson, Lysosomal membrane stability, phagocytosis and tolerance to emersion in the mussel *Perna viridis* (Bivalvia: mytilidae) following exposure to acute, sublethal, copper, Chemosphere 52 (2003) 1147–1151.
[62] D. Bordo, K. Djinovic, M. Bolognesi, Conserved patterns in the Cu,Zn superoxide dismutase family, J. Mol. Biol. 238 (1994) 366–386.
[63] K. Bell, V. Smith, Occurrence and distribution of antioxidant enzymes in the haemolymph of the shore crab *Carcinus maenas*, Mar. Biol. 123 (1994) 829–836.
[64] Y.W. Chung, H.K. Kim, I.Y. Kim, M.B. Yim, P.B. Chock, Dual function of protein kinase C (PKC) in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced manganese superoxide dismutase (MnSOD) expression: activation of CREB and FOXO3a by PKC-alpha phosphorylation and by PKC-mediated inactivation of Akt, respectively, J. Biol. Chem. 286 (2011) 29681–29690.
[65] D. Cosentino-Gomes, N. Rocco-Machado, J.R. Meyer-Fernandes, Cell signaling through protein kinase C oxidation and activation, Int. J. Mol. Sci. 13 (2012) 10697–10721.
[66] W. Cheng, C.H. Liu, C.T. Tsai, J.C. Chen, Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and β-1,3-glucan binding protein (LGBP)from the white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 18 (2005) 297–310.
[67] Y.C. Lin, J.C. Chen, Y.Y. Chen, C.H. Liu, W. Cheng, C.H. Hsu, W.C. Tsui, Characterization of white shrimp *Litopenaeus vannamei* integrin β and its role in immunomodulation by dsRNA-mediated gene silencing, Dev. Comp. Immunol. 40 (2013) 167–169.
[68] Y.C. Wang, P.S. Chang, H.Y. Chen, Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 23 (2007) 1161–1177.