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Immune response of peroxinectin of Chinese mitten crab *Eriocheir sinensis* to exterior stimulation

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ABSTRACT

Peroxinectin possesses the features of both peroxidase activity and adhesive property and plays important roles in innate immune system of crustaceans. In this study, the sequence of peroxinectin of *Eriocheir sinensis* (EsPX) was analyzed and its expression in response to exterior stimulation was detected in both *in vivo* and *in vitro* examination. We showed that the full-length cDNA sequence was composed of 2701 bp and owned a molecular mass of 85.2 kDa and a theoretical pI (isoelectric point) of 6.91. Real-time PCR revealed that the EsPX was mainly distributed in the muscle, hemocytes and stomach. Furthermore, the EsPX was verified to be located in hyalinocytes, semigranulocytes and granulocytes, and was distributed throughout the cytoplasm and nucleus, especial in cytoplasm. After injected with beads, lipopolysaccharide (LPS) and *Aeromonas hydrophila*, the EsPX mRNA expression was significantly up-regulated and peaked up at 4, 2 and 16 h respectively ($P < 0.05$). In the *in vitro* experiment, the stimulation of LPS and beads also induced a prominent boost of EsPX protein in primary cultured hemocytes. The expression of EsPX was peaked up at 4 and 8 h for LPS and beads challenged groups respectively, followed by remarkable release of the incremental EsPX into the extracellular matrix. These findings suggested that the expression of EsPX was susceptible to exterior stimulation, and that the highly expressional EsPX would be released into extracellular matrix by the exterior stimulus.

1. Introduction

The Chinese mitten crab *Eriocheir sinensis* has been an important economic crustacean species with increasing market demand in China. However, the intensive farming and environmental deterioration have frequently induced infectious diseases through the bacteria like *Aeromonas hydrophila* and *Vibrio parahemolyticus* (Xu et al., 2001; Zhu et al., 2002), viruses like *Es*RV905 and *Es*RNV (Bonami and Zhang, 2011), and spiroplasma (Wang et al., 2004) in the past decades, which resulted in significant economic loss. Due to the lack of specific immunity, crab solely prevents itself from the pathogenic infection by the innate immune system including phagocytosis, melanization, nodulation, encapsulation and the release of relative bactericidal substances (phenoloxidase, antimicrobial peptide) (Jiravanichpaisal et al., 2006).

Peroxinectin, a component of the peroxidase-cyclooxygenase superfamily and characterized of a peroxidase domain and an integrin-binding motif (KGD: Lys-Gly-Asp), is identified to be the cell adhesion factors and migration molecule with peroxidase activity from invertebrates. Peroxinectin was isolated primitively from the freshwater crayfish *Pacifastacus leniusculus* and mainly spread among other crustaceans, such as *Penaeus monodon* (Sritunyalucksana et al., 2001), *Fenneropenaeus chinensis* (Dong et al., 2009), *Litopenaeus vanname* (Liu et al., 2004), *Scylla serrate* (Lin et al., 2010), *Procambarus clarkii* (Dong et al., 2011) and *Scylla paramamosain* (Du et al., 2013). Conventionally, it was synthesized and stored in hemocytes in inactive form and was activated after challenged with bacteria or other pathogens (Lin et al., 2007). The microbial compounds lipopolysaccharide, β-1, 3-glucan and peptidoglycan were demonstrated as the main kinds of signal transduction for the activation of peroxinectin immune system (Johansson et al., 1999; Sritunyalucksana et al., 2001). Activated peroxinectin displays multifunctional antimicrobial activity to the pathogen and is involved in various pathogen-elimination processes, such as hemocyte degranulation (Johansson and Söderhäll, 1989), opsonization (Thörnqvist et al., 1994), cell-adhesion (Johansson and Söderhäll, 1988b), encapsulation (Kobayashi et al., 1990) and phagocytosis (Johansson and Soderhall, 1989), and in the regulation of the expression of antibacterial peptide gene (Dong et al., 2009). In addition, the activated peroxinectin is related to the activation of the prophenoloxidase system and has peroxidase activity to eliminate the encapsulation of microorganisms cooperating with extracellular superoxide dismutase (SOD) (Holmblad and Söderhäll, 1999).

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Although, previous researches have confirmed that the peroxinectin will be upregulated after challenged with exterior stimulants, there is still no research about the immune response of peroxinectin to the exterior stimulants. In the present paper, we report the characteristics and expression of *Eriocheir sinensis* peroxinectin (EsPX) gene and its subcellular location and role in defending against exterior stimulation. The purpose of this study is to determine the function of peroxinectin in *E. sinensis* in hemocytes immune defense to the pathogen invasion and exterior stimulation for better understanding the function of peroxinectin in crustacean innate immunity.

2. Material and method

2.1. Experimental animal and hemolymph collection

Chinese mitten crabs (approximately 100 g each), obtained from a farm in Chongming County, Shanghai, China, were acclimated in a 40 L plastic tank with flowing aerated freshwater at 25 ± 2 °C for a week before the experiment. The crabs were fed with fresh clam once a day and the water was refreshed daily. The hemolymph in the ventral-sinus cavity of each crab was collected into a plastic syringe containing equal volume of anticoagulant solution (100 mM glucose, 26 mM citric acid, 415 mM NaCl, 30 mM sodium citrate, 30 mM EDTA, pH 4.6) (Zhang et al., 2010). To collect the hemocyte pellets, the hemolymph was centrifuged at 1000 rpm, 4 °C for 5 min and the supernate was removed.

2.2. Sequence analysis

The completed cDNA sequence of EsPX was deposited in GenBank under accession number GU002547.1. The homology search of nucleotide and protein sequences was conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast). Compute pI/Mw is a tool to compute the theoretical isoelectric point (pI) and molecular weight (Mw) (http://web.expasy.org/compute_pi/).

2.3. Tissue distribution and expression analysis

Reverse transcriptase PCR (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) were used to analyze tissue distributions of EsPX at the mRNA level in the hemocyte, gonad, hepatopancreas, gill, muscle, heart, intestine and stomach of healthy *E. sinensis*. Total RNA isolated from these tissues was used as the template for first-strand cDNA synthesis using the RNAiso Plus (Takara, Japan) according to the manufacturer's protocols. The first-strand cDNA was synthesized from 2 μg of the total RNA using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara, Japan). All of primers were designed by Primer Premier 5 software and synthesized commercially (Biosune Biotechnology, Hangzhou, China).

2.3.1. RT-PCR

Specific EsPX gene primers EsPX-F (5′-AACCCTGGCTCTCTTCTCCC-3′) and EsPX-R (5′-GAATCTGTCGCCCTTTTTGA-3′) were designed according to the EsPX mRNA sequence. A total volume of 25 μL PCR reaction system was set as follows: 1.5 μL cDNA, 1 μL (10 nM) primer for each, 9.5 μL PCR grade water and 12 μL Mix. The reaction was carried out by denaturing for 5 min at 95 °C, followed by cycling 35 times at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s and finally at 72 °C for 10 min. The primers β-actin forward (5′-GCATCCACGAGACCACTTACA-3′) and β-actin reverse (5′-CTCCTGCTTGCTGATCCACATC-3′) primers were designed as internal control.

2.3.2. qRT-PCR

Tissue expression of the EsPX gene was examined by qRT-PCR using two pairs of gene-specific primers (forward primer: 5′-CACCTCCTGGGCTCGCATTATCT-3′, reverse primers: 5′-AGCACCCG TATCCCTTTCTTGTT-3′) and the LightCycler®480, SYBR Green I Master (Roche). Tissue expression was performed in a 20 μL qRT-PCR reaction system containing 5 μL cDNA, 1 μL (10 nM) forward primer and 1 μL (10 nM) reverse primer, 3 μL PCR grade water and 10 μL Master Mix. The primers β-actin forward and β-actin reverse primers designed in Section 2.3.1 were used as the internal control. The reaction conditions were set-up as follows: pre-incubation step for 10 min at 95 °C, 45 cycles of denaturing (95 °C for 5 s), annealing (59 °C for 20 s), and extending (72 °C for 15 s). Melting curve was run at 95 °C for 10 s and 65 °C for 1 min. All samples were run in triplicate. The relative expression level of EsPX mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

2.4. Temporal expression of EsPX mRNA in hemocyte post injection of beads, LPS and Aeromonas hydrophila

Total 20 crabs were divided into 4 equal groups. The crabs in challenged groups were injected with 100 μL of (1) polystyrene beads solution (approximately 2.7×10^9 beads per mL, cat. No. 8821, Invitrogen), (2) LPS (100 μg mL^{-1}, Sigma), (3) *A. hydrophila* (1×10^7 mL^{-1}) suspended in saline (NaCl 0.21 M, KCl 13.6 mM, H_3BO_3 8.6 mM, NaOH 4.75 mM, $MgSO_4 \cdot 7H_2O$ 20 mM, pH 7.2), respectively. The rest group was set as the control group with each crab injected with 100 μL of saline. At 0, 1, 2, 4, 8, 16, 24 and 32 h, total 1 mL hemolymph was withdrawn from each group (200 μL for each crab) and centrifuged at 1000 rpm, 4 °C for 5 min. The hemocyte pellets were collected and stored at –80 °C for a total RNA extraction and qRT-PCR quantification of EsPX gene expression.

2.5. Subcellular location of E. sinensis peroxinectin on different kinds of hemocyte

2.5.1. Cell culture

The hemocyte pellets, collected as described in Section 2.1, were resuspended with 1 mL L15 medium (Cat. No L5520, Sigma-Aldrich). Hemocyte suspension of 0.1 mL at densities of 1×10^7 cells mL^{-1} was seeded into 6-well culture plates (Corning-Costar Corp., Corning, New York, USA) containing additional 1.4 mL of L15 medium per well. Total 10 wells were used in the following experiments including western blotting assay and immunofluorescence assay. Hemocytes were incubated at 26 °C without 5% carbon dioxide in a Model incubator (EYEL4 LTI-700), and half of the medium by volume was replaced every 2 days. After growing to greater than 80% confluence (approximately 12 h), the primary cultured hemocytes were used for Western blotting and immunofluorescence assay.

2.5.2. Western blotting assay

For the western blotting, total protein extracted from hemocyte was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was placed in a blocking solution (5% fat-free milk in PBS) at 4 °C for 2 h and incubated with rabbit anti-EsPX polyclonal antibody (1:1000, Abmart) and rabbit anti-β-actin antibody (molecular weight 42 kDa, 1:2000, Cat. No. R1207-1, Huabio, China) overnight at 4 °C, followed by incubating for 1 h with goat anti-rabbit IgG conjugated with horseradish peroxidase (Cat. No. 31460, Pierce) at a dilution of 1:2000 in PBS at room temperature. Detection was performed using chemiluminescence luminol reagents (Pierce ECL Western Blotting Substrate, Cat. No. 32106, Pierce) and the immunoreactive bands were scanned using chemiluminescent imaging system (Tanon 5200).

Hemocyte pellets was collected and resuspended with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerinum, 0.5% NP40, pH 7.2) containing PMSF (10 μL per mg pellets) and kept in ice for 10 min. Cytoplasmic proteins in the supernatant were collected after centrifugation at 20,000 *g*, 4 °C for 5 min. The remained pellet containing nucleoproteins was dissolved with lysis buffer and centrifuged at 20,000 *g*, 4 °C for 5 min. The resulting pellet was collected as nucleoproteins after removing the supernate. Gathered cytoplasmic protein and nucleoproteins were analyzed by western blotting.

2.5.3. Immunofluorescence assay

Hemocytes were washed using PBS for three times (each one lasting 5 min), fixed with cooled 4% paraformaldehyde for 15 min, and then washed trice in PBS. After the treatment discussed earlier, the hemocytes were incubated in a permeabilization and blocking solution (0.5% Triton X-100, 2% BSA, 0.5% rabbit serum in PBS) for 1 h at 20 °C. After thoroughly rinsed by PBS, the hemocytes were incubated with the rabbit anti-EsPX polyclonal antibody (antibody 1:200 in PBS containing 1% BSA and 0.05% Triton X-100) overnight at 4 °C, followed by washing in PBS trice and incubating with the goat anti-rabbit IgG (H + L) conjugated with fluorescein isothiocyanate (FITC) (Cat. No. 31635, Pierce) (1:200 in 1% BSA and 0.05% Triton X-100 in PBS) for 1 h at 20 °C in a dark box. Sections were then rinsed in PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in mounting medium. Expression of EsPX in hemocyte was observed using confocal laser scanning microscopy with FITC and DAPI emission filters (Zeiss LSM 780, Carl Zeiss, Germany).

2.6. Expression analysis of EsPX in hemocyte after challenge with LPS and polystyrene beads in vitro

Primary cultured hemocytes were stimulated with 10 μL of LPS (100 μg mL^{-1}) and fluorescent polystyrene bead (2.7×10^8 beads per mL), respectively. After treated for a certain period (0, 1, 2, 4 and 8 h for LPS; 0, 4, 8, 12 and 16 h for polystyrene bead), the hemocytes were washed with PBS and fixed with 4% paraformaldehyde for the immunofluorescence assay of EsPX. The other two groups of hemocytes, treated by the same treatment as described earlier, were collected for the western blotting assay. Two groups of hemocytes treated with saline for the same time interval as LPS and fluorescent polystyrene bead were set as the controls.

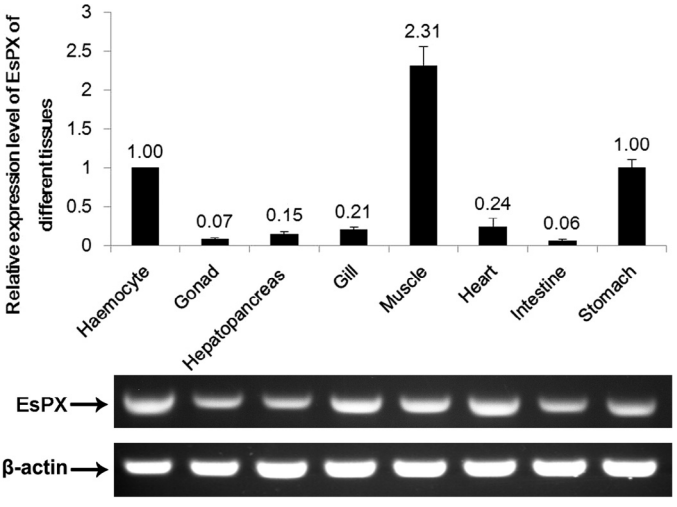

Fig. 1. (A) Real time PCR analysis of EsPX in various tissues. β-Actin was used as control in all tissues. (B) Expression of EsPX mRNA in various tissues. Lane 1, hemocytes; 2, gonad; 3, hepatopancreas; 4, gill; 5, muscle; 6, heart; 7, intestine; 8, stomach.

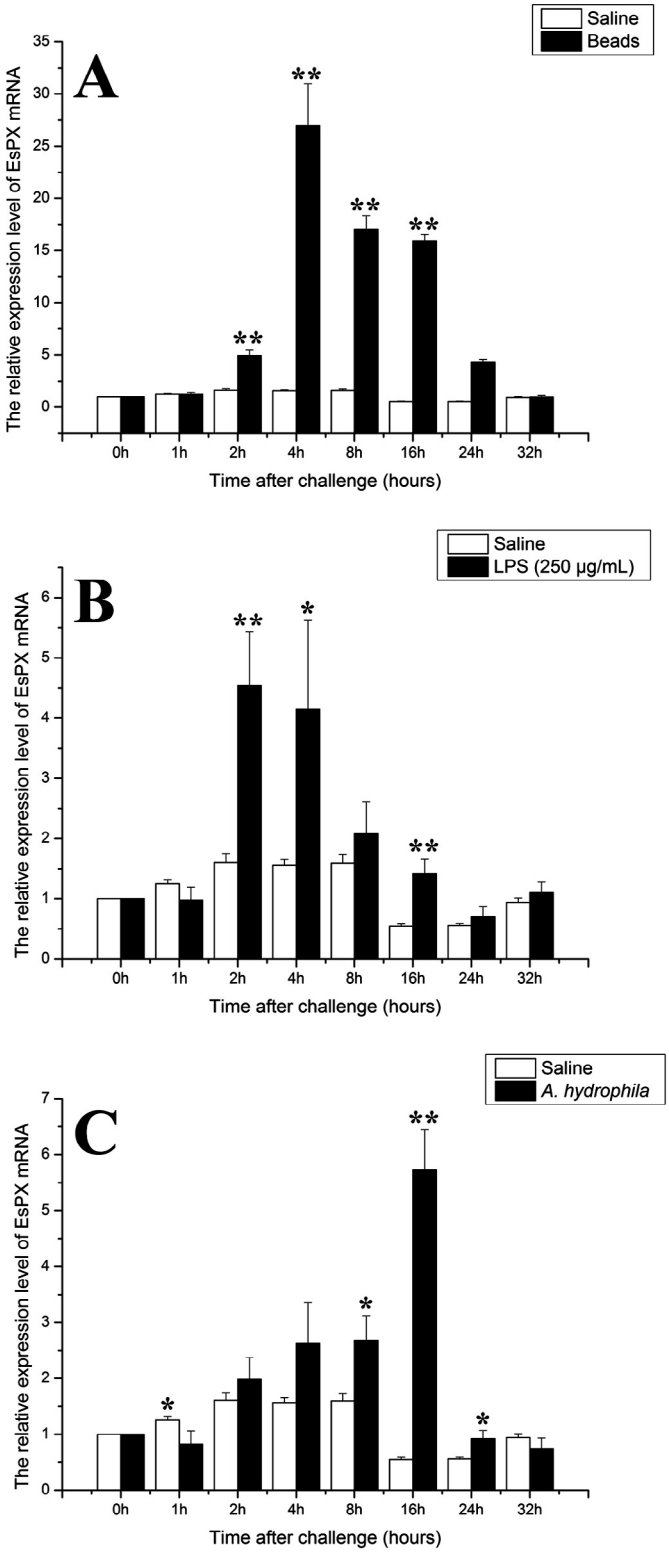

Fig. 2. (A) qRT-PCR analysis of EsPX mRNA relative expression in hemocytes injected with saline (control) and beads. (B) qRT-PCR analysis of EsPX mRNA relative expression in hemocytes injected with saline (control) and LPS. (C) qRT-PCR analysis of EsPX mRNA relative expression in hemocytes injected with saline (control) and *A. hydrophila*. The relative EsPX expression level was determined for each group and values were shown as means ± SD, n = 3. The significant differences of EsPX expression between the challenged and the control group are indicated with asterisks "*" ($P < 0.05$) and asterisks "**" ($P < 0.01$).

2.7. Statistical analysis

Results were presented as means ± SD. SPSS software (11.0) was used for statistical analyses. The effects of treatments were statistically analyzed using analysis of T tests and the differences were considered significant as $P < 0.05$.

3. Results

3.1. Sequences and similarity analysis of EsPX

The completed cDNA sequence of EsPX is deposited in GenBank under accession number GU002547. The full-length cDNA sequence was composed of 2701 bp including an open reading frame (ORF) of 2325 bp, 5′ terminal untranslated region (UTR) of 90 bp and a 3′ terminal UTR of 286 bp with a polyA tail. The putative ORF consists of 775 amino acids and includes a typical signal peptide of 16 amino acids. The putative mature protein was calculated to have a molecular mass of 85.2 kDa and a theoretical pI of 6.91.

The BlastP analysis demonstrated that the EsPX displayed high similarity and identity percentage with other kinds of crustacean, such as *Macrophthalmus japonicas* (82%, AID47197), *Panulirus longipes* (65%, AGO05992), *P. leniusculus* (57%, CAA62752), *P. clarkii* (55%, ADW79421) and *F. chinensis* (55%, ABB55269). A relatively lower similarity of EsPX was found as compared with other invertebrates such as *Crassostrea gigas* (41%, EKC39927), *Camponotus floridanus* (40%, EFN68225) and *Stegodyphus mimosarum* (38%, KFM61817).

3.2. Tissue distribution of EsPX mRNA

Total RNA was extracted from the different tissues of healthy crabs, and the tissue distribution and expression of EsPX mRNA were investigated by RT-PCR and qRT-PCR (Fig. 1). A 496 bp fragment of EsPX cDNA was amplified from different tissues by RT-PCR with specific primers EsPX-F and EsPX-R. The results of qRT-PCR analysis showed that the expression of EsPX was relatively higher in hemocyte, muscle and stomach and relatively lower in other tissues including gonad, hepatopancreas, gill, heart and intestine. Muscle was considered to be the most active tissue in EsPX expression.

3.3. Temporal expression of EsPX mRNA in response to beads, LPS and A. hydrophila challenge

The temporal expression of EsPX mRNA responding to beads, LPS and *A. hydrophila* challenge was detected by qRT-PCR (Fig. 2). All three groups displayed an increasing trend in EsPX mRNA expression and restored to their initial level after challenged at 32 h. The EsPX mRNA expression was significantly up-regulated at 2 hours post injection (hpi) with beads and reached the maximum value at 4 h, followed by a decrease. In spite of the decrease, the EsPX mRNA expression was still significantly higher than the control group during 8–24 hpi ($P < 0.05$). The EsPX mRNA expression increased and reached the peak after challenged with LPS for 2 h, which was significantly higher than the control group ($P < 0.05$), and finally showed a decreasing trend during 4–24 hpi. In the group of *A. hydrophila* challenge, EsPX mRNA expression significantly increased at 8 and 16 hpi ($P < 0.05$).

3.4. Subcellular localization of EsPX in hemocyte

Identified by immunofluorescence assay, the EsPX was determined to be located in all three types of hemocytes, namely hyalinocytes, semigranulocytes and granulocytes (Fig. 3A). In addition, the EsPX was distributed throughout the cell including cytoplasm and nucleus (Fig. 3A) with predominant accumulation

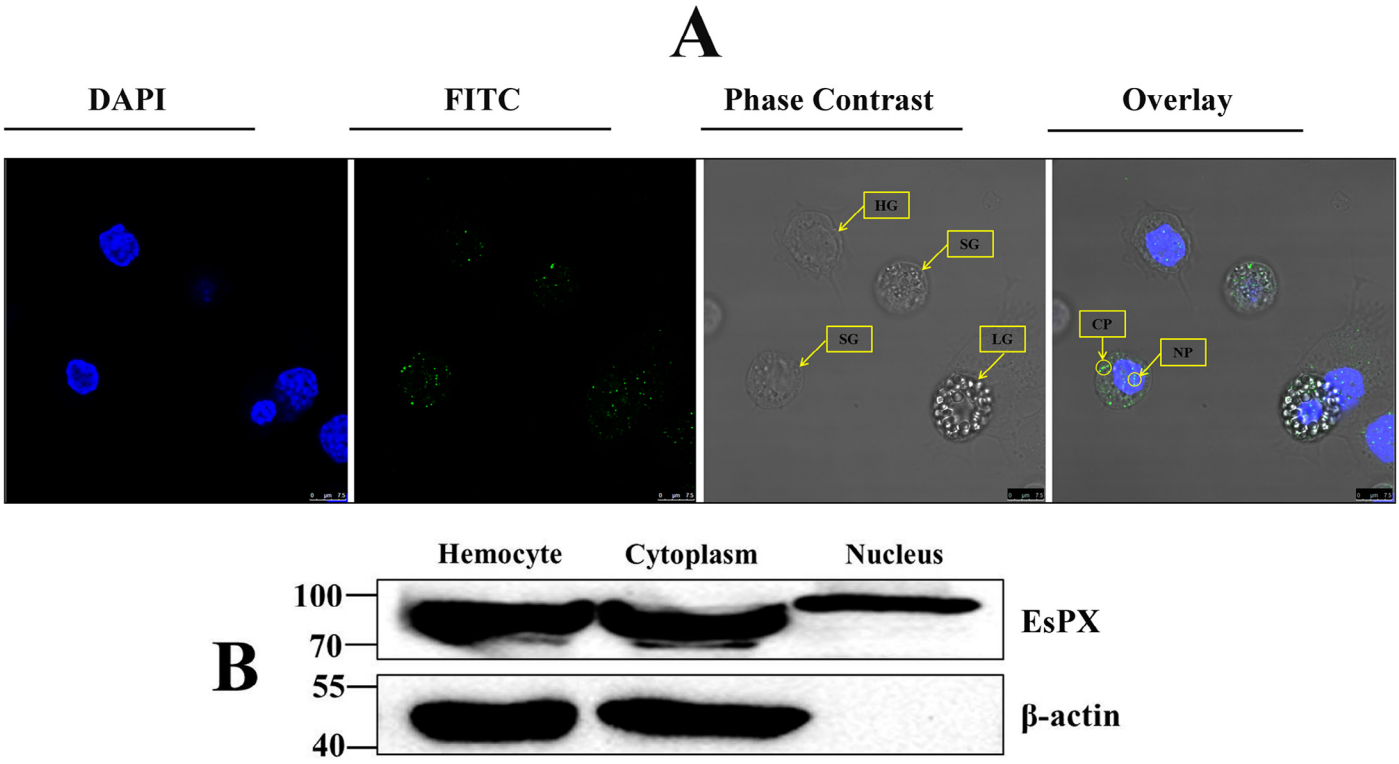

Fig. 3. Subcellular localization of EsPX in different types of hemocyte. (A) EsPX was confirmed to be present in hyalinocytes (HG), semigranulocytes (SG) and large granulocytes (LG) by immunofluorescence. EsPX was distributed throughout the cell of hemocyte including the cytoplasm and the nucleus. (B) EsPX was confirmed to be highly expressed and mainly located in cytoplasm by western blotting assay. CP, cytoplasm peroxinectin; NP, nucleus peroxinectin.

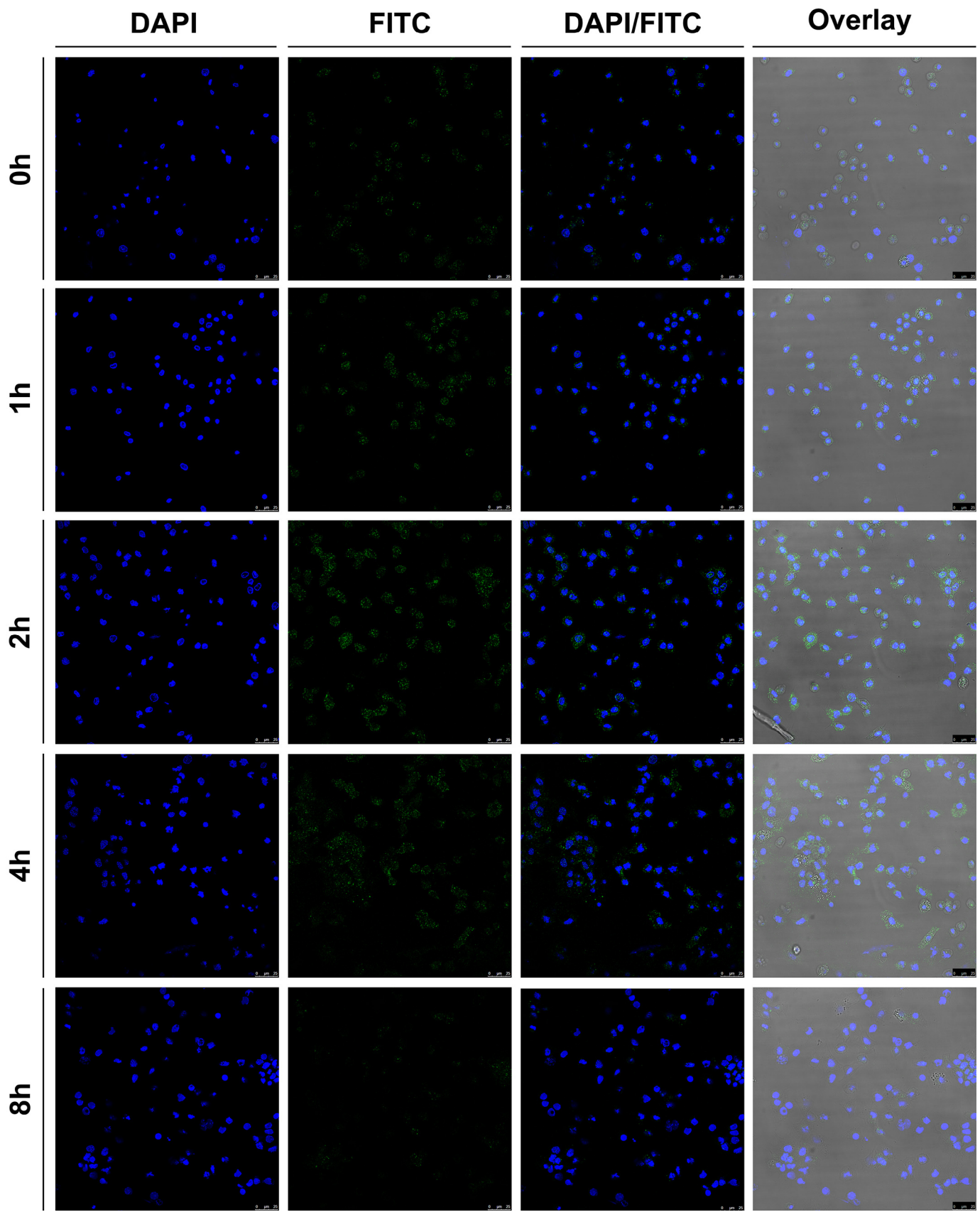

Fig. 4. Temporal expression of EsPX in primary cultured hemocyte after challenged with LPS. The hemocyte were fixed and incubated with specific primary antibody and FITC-conjugated secondary antibody, and the nuclei were stained with DAPI post stimulation for different time intervals. Fluorescent micrographs were obtained by the confocal laser scanning microscope.

in the cytoplasm and relative low concentration in the nucleus region (Fig. 3B).

3.5. *Temporal expression of EsPX in hemocyte after challenged with LPS and beads* in vitro

In order to determine the potential effect of immune activation of EsPX after challenged with LPS (1 μg mL^{-1}), the temporal expression in hemocyte was assessed post stimulation of LPS for 0, 1, 2, 4 and 8 h by immunofluorescence assay. The result indicated that the expression of EsPX showed a prominent boost at 1 h after challenged with LPS by reaching the highest level at 4 h, followed by a significant decrease with few specific fluorescence detected in the hemocyte (Fig. 4). Fig. 5 exhibited that LPS induced a remarkable increase in the expression of EsPX and a release of EsPX from the hemocyte to the extracellular matrix. This process was accompanied by an obvious reduction of β-actin as detected by the western blotting assay.

Similarly, a remarkable increase in the expression of EsPX in hemocyte and in its release to the extracellular matrix was also induced after challenged with beads (Fig. 6). The expression of EsPX in hemocyte was peaked up after 8 h post stimulation, and afterwards displayed an obvious downward trend. A lowest detectable concentration of EsPX was detected after 16 h post stimulation. Fig. 7 showed that the beads were engulfed by hemocyte and EsPX was released from the cell into the extracellular matrix after challenged with beads for 12 h. Separately, by the western blotting assay, the hemocyte did not show obvious change in EsPX and β-actin expression.

4. Discussion

Peroxinectin, firstly isolated from the hemocytes of the freshwater crayfish *P. leniusculus*, is a multifunctional immune component with both cell-adhesive and peroxidase activities, and is demonstrated to play important roles in clearing the invading microbial pathogens in invertebrates. In this research, we analyzed the sequence of EsPX and compared its homology with other known peroxinectin in invertebrates. According to the BLASTP analysis, EsPX showed high similarity in amino acid sequence with the peroxinectin

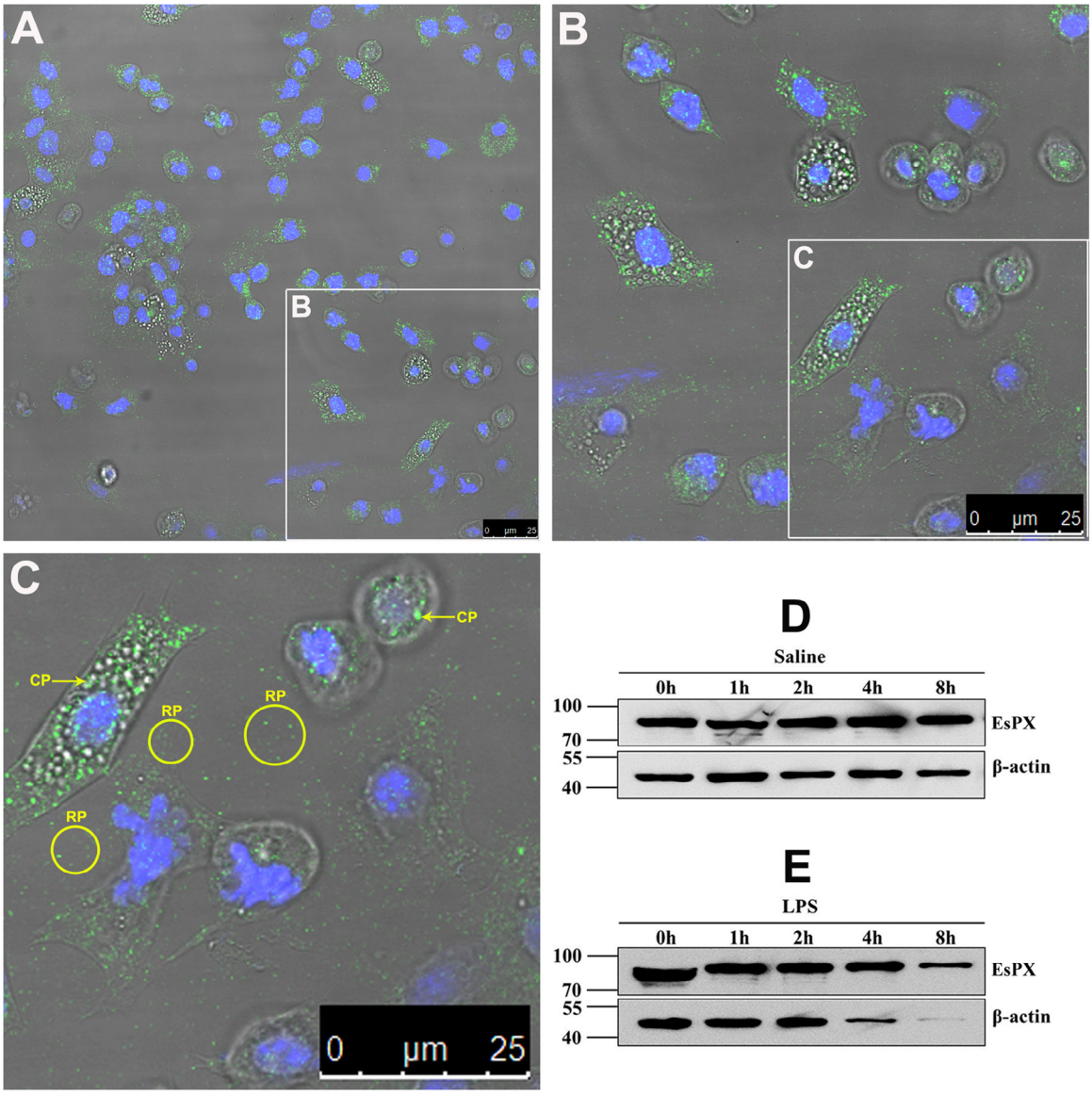

Fig. 5. (A) Release of EsPX in primary cultured hemocytes after challenged with LPS for 4 h. (B) The enlarge micrograph of A. (C) The enlarge micrograph of B. (D and E) Temporal expression of EsPX in primary cultured hemocytes after challenged with saline and LPS by western blotting analysis. CP, cellular peroxinectin; RP, released peroxinectin.

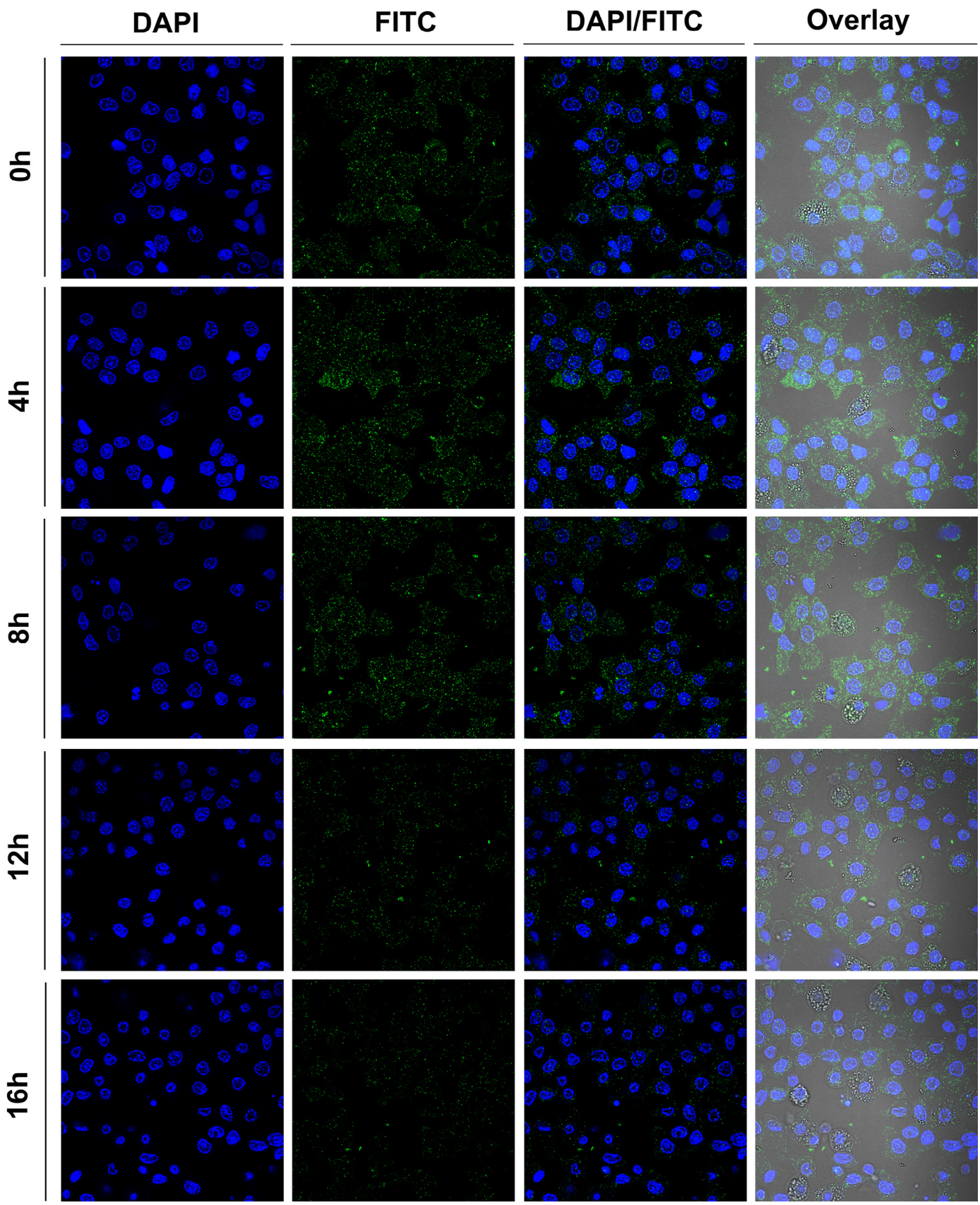

Fig. 6. Temporal expression of EsPX in primary cultured hemocytes after challenged with beads for 0, 4, 8, 12 and 16 h. The hemocytes were fixed and incubated with specific primary antibody and FITC-conjugated secondary antibody, and the nuclei were stained with DAPI post stimulation for different time intervals. Fluorescent micrographs were obtained by the confocal laser scanning microscope.

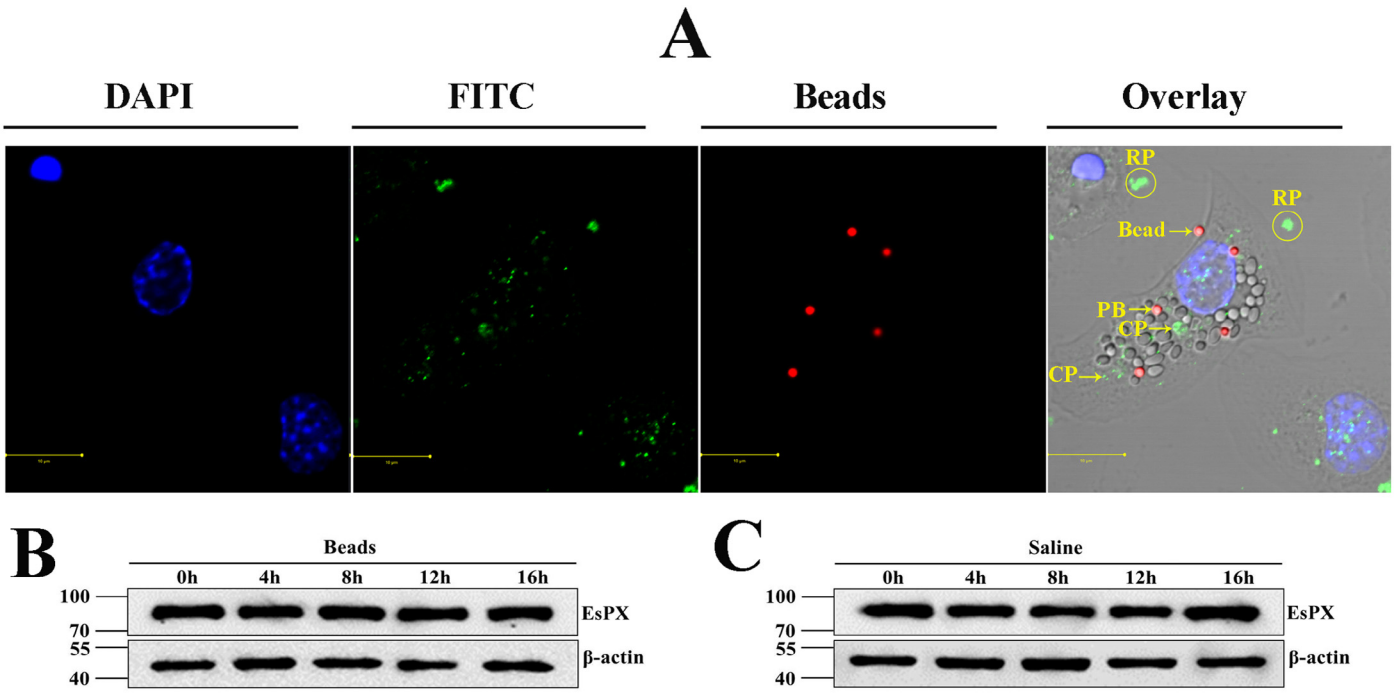

Fig. 7. (A) Release of EsPX in primary cultured hemocytes after challenged with beads for 4 h. (B and C) Temporal expression of EsPX in primary cultured hemocytes after challenged with beads and saline by western blotting analysis. CP, cellular peroxinectin; RP, released peroxinectin; PB, phagocytic beads.

of other kinds of crustaceans, such as *P. monodon*, *S. serrata*, *F. chinensis*, *L. vannamei*, *P. leniusculus* and *P. clarkii*. Given that the immune function of crustacean peroxinectin was verified in previous researches (Dong et al., 2009, 2011; Du et al., 2013), EsPX may work as an essential defensive component for clearing the invading pathogens in *E. sinensis*.

In crustaceans, circulating hemocytes were conventionally recognized as hyalinocytes (HC), semigranulocytes (SGC) and granulocytes (GC), and different types of hemocytes tend to carry out specific immune functions (Ding et al., 2012). In previous research, peroxinectin was demonstrated to be synthesized and stored in the hemocytes and the SGC and GC are considered to be the dominating hemocytes involved in this process (Johansson et al., 2000; Liu et al., 2005; Mangkalanan et al., 2014). However, we observed that the EsPX was present in HC, SGC and GC. Although distributed throughout the cell of hemocytes including the cytoplasm and nucleus, the protein exhibited predominant accumulation in the cytoplasm. This discrepancy in the conclusions is attributed to the multifunctional immunity of EsPX and the evolutional relationship between these three kinds of hemocytes. Peroxinectin was identified to be involved in the phagocytosis and peroxidase release, which was mainly conducted by hyalinocytes and granular-containing hemocytes, respectively (Johansson et al., 2000). Söderhäll found that the peroxinectin owned ability to degranulate crayfish granular cells *in vitro*, and its specific antibodies could also inhibit degranulation within SGC and GC *in vitro* (Johansson and Söderhäll, 1988a). As shown in Fig. 4 (4 h) and Fig. 6 (8 h), partial granular was degranulated from the hemocytes, which was consistent with it observed in *P. leniusculus*. However, not only the SGC and GC, almost all of circulating hemocytes including HC displayed this obvious release of incremental EsPX to the extracellular matrix. This gave further evidence that the peroxinectin was synthesized in HC, SGC and GC. In addition, we confirmed that this protein could be detected in almost all tissues, and the tissue expression assay showed that the muscle owned a relative higher expression than the hemocytes, indicating that the EsPX was also expressed in other tissues not only in the hemocytes in *E. sinensis*.

To elucidate further on the immune function of EsPX, we compared the temporal expression levels of the EsPX gene in the hemocytes between normal and stimulated *E. sinensis*. The expression of EsPX mRNA displayed a trend of significantly fast increase after challenged with beads, LPS and *A. hydrophila*. In the *in vitro* test, the stimulation of LPS and beads also induced a prominent boost of the EsPX protein in the hemocytes. This observation was consistent with previous report on other crustaceans. The expression level of peroxinectin gene was up-regulated in *L. vannamei* after challenged with *V. alginolyticus* at 6 h (Liu et al., 2005), and was up-regulated at 48 h post WSSV challenge in *S. paramamosain* (Du et al., 2013). Intriguingly, the expression of EsPX mRNA was remarkably elevated within 4 h after challenged with beads and LPS, and then down-regulated to the normal level at 32 h, showing a peak after challenged with *A. hydrophila* at 16 h. Previous researches proved that the peroxinectin mRNA expression was conventionally determined by different categories and concentration of immunostimulant (Liu et al., 2007). LPS (a kind of immune inducer) and *A. hydrophila* had been verified to be extremely toxic to the crabs as they induced a serious loss of hemocytes in *E. sinensis* (Lv et al., 2014). Subsequently, incremental EsPX was found to be released into extracellular matrix. It is speculated that corresponding immune response, such as the release of peroxidase and peroxinectin, occurs simultaneously, which results in an upregulation of peroxinectin gene. Additionally, the beads injection induced a more significant enhancement in the peroxinectin gene as compared with LPS and *A. hydrophila*. The beads were solely eliminated by phagocytic process due to the nature of polystyrene, and this process was confirmed to be positive correlate with the upregulated of peroxinectin (Kobayashi et al., 1990). However, some researchers found that the peroxinectin mRNA exhibited a contrary response to exterior stimulation. Sritunyalucksana and Dong found that the injection of laminarin, LPS and inactivated *A. hydrophila* resulted in a decline of

peroxinectin transcript in *P. monodon* and *P. clarkia* (Sritunyalucksana et al., 2001) (Dong et al., 2011). Cerenius et al. (2003) and Liu et al. (2004) found that the laminarin and zymosan did not result in any changes in the peroxinectin transcript of *L. vannamei*, *P. leniusculus* and *Astacus Astacus*, respectively. This discrepancy is considered due to the decline of peroxinectin-expression cells and the proliferation of new hemocytes (Liu et al., 2004). In *E. sinensis*, the circulating hemocytes also experienced drastic decline in count after challenged with LPS, bacterium and beads, which was consistent with other crustaceans (Lv et al., 2014). Cell apoptosis is demonstrated to be the major reason for this decline in circulating hemocytes (unpublished). In the LPS challenge group, we could see that the EsPX protein was boost at 2 h, while it started to be released into the extracellular matrix with fracture nucleus observed in the hemocyte at 4 h. Thus we thought that the upregulation of EsPX protein was prior to the process of hemocytes apoptosis, and the hemocytes apoptosis might be a possible reason for the down-regulation of EsPX protein.

In conclusion, the peroxinectin was confirmed to be located in HC, SGC and GC. We indicated that the *E. sinensis* displayed a trend of obvious outburst and rapid decline of the expression of peroxinectin gene to the exterior stimulants and the syntheses and release of EsPX protein was verified to be the primary cause. Additionally, the EsPX showed a potential possibility of involvement in the phagocytic process of hemocyte. These results provide compelling evidence of the complex involvement of peroxinectin in *E. sinensis* innate immunity.

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