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Identification, characterization and expression analysis of ERK2 in Chinese mitten crab *Eriocheir sinensis* after challenge with LPS and *Aeromonas hydrophila*

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ABSTRACT

Farming of *Eriocheir sinensis* was seriously threaten by the infection of opportunistic pathogens, especially the gram-negative bacterium. In this paper, we analyzed the sequence of extracellular signal-regulated kinases 2 (ERK2) of *E. sinensis* (EsERK2) and its expression levels after challenge with LPS and *Aeromonas hydrophila* in both *in vivo* and *in vitro* examination. The full-length cDNA sequence of EsERK2 was 2455 bp in size with an open reading frame (ORF) of 1095 bp, encoding the protein of 365 amino acids. It owned a predicted molecular mass of 42.4 kDa and a theoretical isoelectric point (pI) of 5.93. EsERK2 was distributed in all examined tissues including haemocyte, gonad, hepatopancreas, gill, muscle heart, stomach and intestine, but its expression level was significantly higher in hepatopancreas than it in other examined tissues. The expression level of EsERK2 increased significantly after LPS challenge at 2 h ($P < 0.05$), and then gradually increased and reached highest at 16 h. However, its expression level decreased significantly after *A. hydrophila* challenge at 4 h, and then gradually decreased till 24 h ($P < 0.05$), and returned to its initial value at 36 h. According to the immunofluorescence assay and western blotting assay, EsERK2 was found to be distributed mainly in cytoplasm of haemocyte, and its expression level showed a prominent boost in primary cultured haemocytes after challenge with LPS and *A. hydrophila in vitro*. These results indicated that the expression of EsERK2 was sensitive to the exterior stimulants and its encoding protein might be associated with the signaling transduction in response to exterior pathogens in *E. sinensis*.

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1. Introduction

The Chinese mitten crab *Eriocheir sinensis* is an important commercial aquatic species in China, with annual production approaching 750, 000 tons in 2014. In recent decades, however, the production of this species has been challenged by opportunistic pathogens, including gram-negative bacterium like *Aeromonas hydrophila*, with the rapid development of intensive aquaculture [1].

Lipopolysaccharide (LPS), the major glycolipid of gram-negative bacterial outer membranes, has been demonstrated to be highly antigenic and has endotoxic properties to crustaceans [2]. As a powerful innate immune-stimulant, LPS could significantly enhance phagocytic activity of haemocytes, elicit rapid and massive degranulation, and induce obvious prophenoloxidase release and activation from haemocytes in many crustacean species. However, its toxic effects on haemocytes have been confirmed in previous studies. It could cause serious depletion of circulating haemocytes count in crustacean, such as *Nephrops norvegicus*, *Munida rugosa*, *Paguristes oculatus*, *Pilumnus hirtellus* and *Macropipus vernalis* [3]. Lv also indicated that it could lead total haemocyte counts (THC) to dramatic decline and rapid recovery in *E. sinensis* accompanied with distinct dose-effect relationships between it and haemocytes ratio [4]. In addition, previous studies also found that LPS could induce the production of reactive oxygen species (ROS) in haemocytes and haemocyte apoptosis in *Penaeus monodon* and *E. sinensis* [5]. Similarly, gram-negative bacterium, including *A. hydrophila* and *Vibrio alginolyticus* could cause THC decrease and haemocyte apoptosis also [6–8]. ROS and haemocyte apoptosis play important

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roles in defense mechanism in response to pathogenic infections and various other immune-related challenges. Activation of mitogen activated protein kinases (MAPK) signaling pathways is involved in ROS production and cell apoptosis [9]. In mammalian cells, the MAPK superfamily contains three main protein kinase families: extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs) and p38-family of kinases, which plays important roles in the regulation of intracellular metabolism and gene expression in many biological events, such as proliferation, stress responses, apoptosis and immune defence [10]. ERKs are serine/threonine-specific protein kinases, including ERK1, ERK2, ERK3/4, ERK5 (also named as MAPK7), ERK6 (also known as p38γ) and ERK7/8. Among which, ERK2 contains highly conserved serine/threonine kinases (S_TK) domain, which is activated via phosphorylation on both threonine and tyrosine residues within the conservative TEY sequence and can be activated by many stimuli and environmental stresses [11]. Although the functions of ERK2 involved in the innate immune response to pathogenic infection or pathogen-derived substances have been well studied in many species [12], especially in mammalian cells, there is still limited knowledge about the functions of ERK2 in crustacean. In this study, the characteristics and expressions of *E. sinensis* ERK2 (EsERK2) gene were analyzed *in vivo* and *in vitro* after challenge with LPS and *A. hydrophila*, which will be helpful for better understanding the roles of ERK2 in physiological response to invasive pathogens and exterior immune stimulation in crustacean.

2. Method and material

2.1. Experimental animal

E. sinensis, obtained from a commercial farm in Chongming County, Shanghai, China, were acclimated in a 40 L plastic tank with flowing aerated freshwater at 25 ± 2 °C. Before the experiments, crabs with average body weight of approximately 100 g were fed with fresh clam once a day. Residual clam was cleaned after feeding for 4 h and water was refreshed daily.

2.2. Haemocyte collection

Total 500 μL haemolymph of each individual crab was collected from sinus cavity of pereiopods with a plastic 1-mL syringe containing equal volume of anticoagulant solution [6]. To collect the haemocyte pellets, the haemolymph was centrifuged at 1000 rpm, 4 °C for 3 min and the supernate was removed.

2.3. Sequence analysis

Completed cDNA sequence of ERK2 of *E. sinensis* was deposited in GenBank under accession number GU002542.1. The homology search and structure analysis of protein sequence was conducted with BLAST algorithm and Conserve domain analysis at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast). Signal P 3.0 program was utilized to predict the presence and location of signal peptide in amino acid sequences (http://www.cbs.dtu.dk/services/SignalP). Compute pI/Mw is a tool to predict the theoretical isoelectric point (pI) and molecular weight (Mw) (http://web.expasy.org/compute_pi/). The multiple sequence alignment of crustacean MAPK gene was created using the ClustalW analysis program (DNAman).

2.4. Tissue distribution and expression analysis

The distributions of EsERK2 at different tissues including haemocyte, gonad, hepatopancreas, gill, muscle, heart, intestine and stomach were analyzed by the combination of reverse transcriptase PCR (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR). Total RNA extraction and the first-strand cDNA synthesis were conducted using RNAiso Plus (Takara, Japan) and PrimeScript™ RT Master Mix (Perfect Real Time) (Takara, Japan), respectively, according to the manufacturer's protocols. All the primers used in this study were designed by Primer Premier 5 software and synthesized commercially (Biosune Biotechnology, Hangzhou, China) (Table 1).

2.4.1. RT-PCR

Specific EsERK2 gene primers EsERK2-F and EsERK2-R were designed according to the MAPK nucleotide sequence of *E. sinensis*. Primer sequences are shown in http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0137028 Table 1. A total volume of 25 μL PCR reaction system was set as follow: 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-F and β-actin-R primers were designed as internal control (Table 1).

2.4.2. qRT-PCR

qRT-PCR was carried out using the LightCycler®480 SYBR Green I Master (Roche), and primer sequences (rtERK2-F, rtERK2-R) were provided in Table 1. Each reaction mixture contained 5 μL cDNA template, 1 μL (10 nM) forward primer and 1 μL (10 nM) reverse primer, 3 μL PCR grade water and 10 μL Master Mix. The amplification cycle was 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. Expression data were normalized to β-actin. Each experiment was performed in triplicate. The relative expression level of EsERK2 mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

2.5. Temporal expression of EsERK2 in haemocytes challenged by LPS and Aeromonas hydrophila in vivo

Total 15 crabs were randomly divided into 3 equal groups. The crabs in challenged groups were injected with 100 μL of (1) LPS (100 μg mL^{-1}, Sigma), (2) *A. hydrophila* (1×10^7 mL^{-1}) suspended in saline. The crabs in control group were injected with 100 μL of stroke-physiological saline. At 0, 2, 4, 8, 16, 24 and 36 h, total 1 mL haemolymph was withdrawn from each group (200 μL for each crab) respectively and the haemocyte pellet was collected for qRT-PCR quantification of gene expression.

2.6. Procaryotic expression of EsERK2 protein

2.6.1. Amplification of EsERK2 gene

PCR amplification was performed using primers EsCL-F and EsCL-R (Table 1) with *Eco*RI and *Hind*III sites respectively, which

Table 1
Primers used for RT-PCR, qRT-PCR and procaryotic expression.

Designation	Sequence (5′ → 3′)	Target length
EsERK2-F	ACGGACGGGGAGACTGTGAGGTG	451 bp
EsERK2-R	TGTTCAGGAGGAGGTTTGATGGC	
rtERK2-F	CTTCAGCCTGTCGTACATTGGTG	157 bp
rtERK2-R	TCTGGTTAGAATTTTGATCTCTC	
β-actin-F	GCATCCACGAGACCACTTACA	266 bp
β-actin-R	CTCCTGCTTGCTGATCCACATC	
EsCL-F	CGGAATTCATGTCGGAGGAACAACCCACGGAC	1113 bp
EsCL-R	CCCAAGCTTTCAGTTTTGCTGCATAGGTGTT	

were designed according to the full-length cDNA of EsERK2 gene sequence. The PCR program was performed by using PCR Master Mix (Promega, M7502), and 25 μL 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.

2.6.2. Construction and expression of pET28b-EsERK2 plasmid

The amplified product was gel purified using DNA gel extraction kit (TIANGEN Biotech, Beijing, China) and digested with *Hind*III and *Eco*RI restriction enzymes for 3 h at 37 °C, and then was inserted into prokaryotic expression vector pET28b (Takara, Dalian, China) using T4 DNA ligase. The construct pET28b-EsERK2 was transformed into *Escherichia coli* DH5α competent cells using standard procedures. The plasmid DNA was extracted and transformed into competent *E. coli* BL21 (DE3) cells according to the manufacturer's direction. The recombinant pET28b-EsERK2 was identified and characterized by restriction digestion with *Hind*III/*Eco*RI, PCR amplification and DNA sequencing.

The positive clone of recombinant plasmid pET28b-EsERK2 was picked and inoculated into 20 mL Luria–Bertani (LB) broth containing 50 mg L^{-1} kanamycin. The Bacterial culture was placed in a shaker at 37 °C with a constant speed of 200 rpm. When the OD600 value of the culture reached 0.6, isopropyl-b-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce protein expression. To determine the expression efficiency of BL21 (DE3), 1 mL of bacterial culture was extracted after IPTG added for certain time (0, 1, 2, 3, 4, 5 and 6 h). To collected enough EsERK2 fusing protein, the culture was divided into several aliquots and placed at 26 °C in a shaker at 200 rpm continually for overnight. Soluble body proteins and inclusion body proteins were extracted from BL21 (DE3) by ultrasonic lysis method, and affinity-purified by Ni-NTA Agarose (Qiagen). Purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and was visualized with Coomassie brilliant blue R250. The collected purified protein was used for raising rabbit polyclonal antibody by Huabio (Hangzhou, China).

2.6.3. Western blot analysis

Protein sample (20 μg) was extracted from haemocytes and resolved by SDS-PAGE in a 12% polyacrylamide gel, and then 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-EsERK2 polyclonal antibody (1:1000) 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).

2.7. Expression analysis of EsERK2 in primary cultured haemocyte after challenge of LPS and A. hydrophila in vitro

2.7.1. Cell culture and challenge

The haemocyte pellets, collected as described in Section 2.2, were resuspended with 1 mL L15 medium (Cat.No L5520, Sigma-Aldrich) at densities of 1×10^7 cells mL^{-1}. 0.1 mL of the haemocyte suspension was seeded into 6-well culture plates (Corning-Costar Corp., Corning, New York, USA) containing additional 1.4 mL of L15 medium per well. Haemocytes were incubated at 26 °C without 5% carbon dioxide in a Model incubator (EYELA LTI-700).

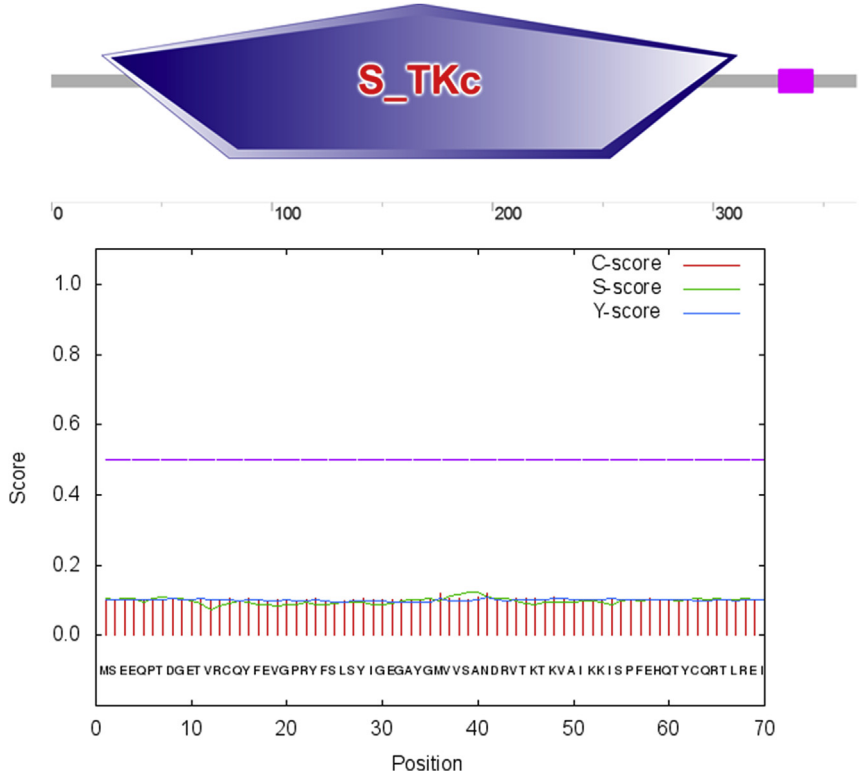

Fig. 1. Prediction of the presence and location of signal peptide cleavage site and conserve domain analysis of EsERK2. No putative signal peptide was found in deduced amino acid sequence of EsERK2, while catalytic domain of ERK 1/2-like Serine/Threonine kinases was detected.

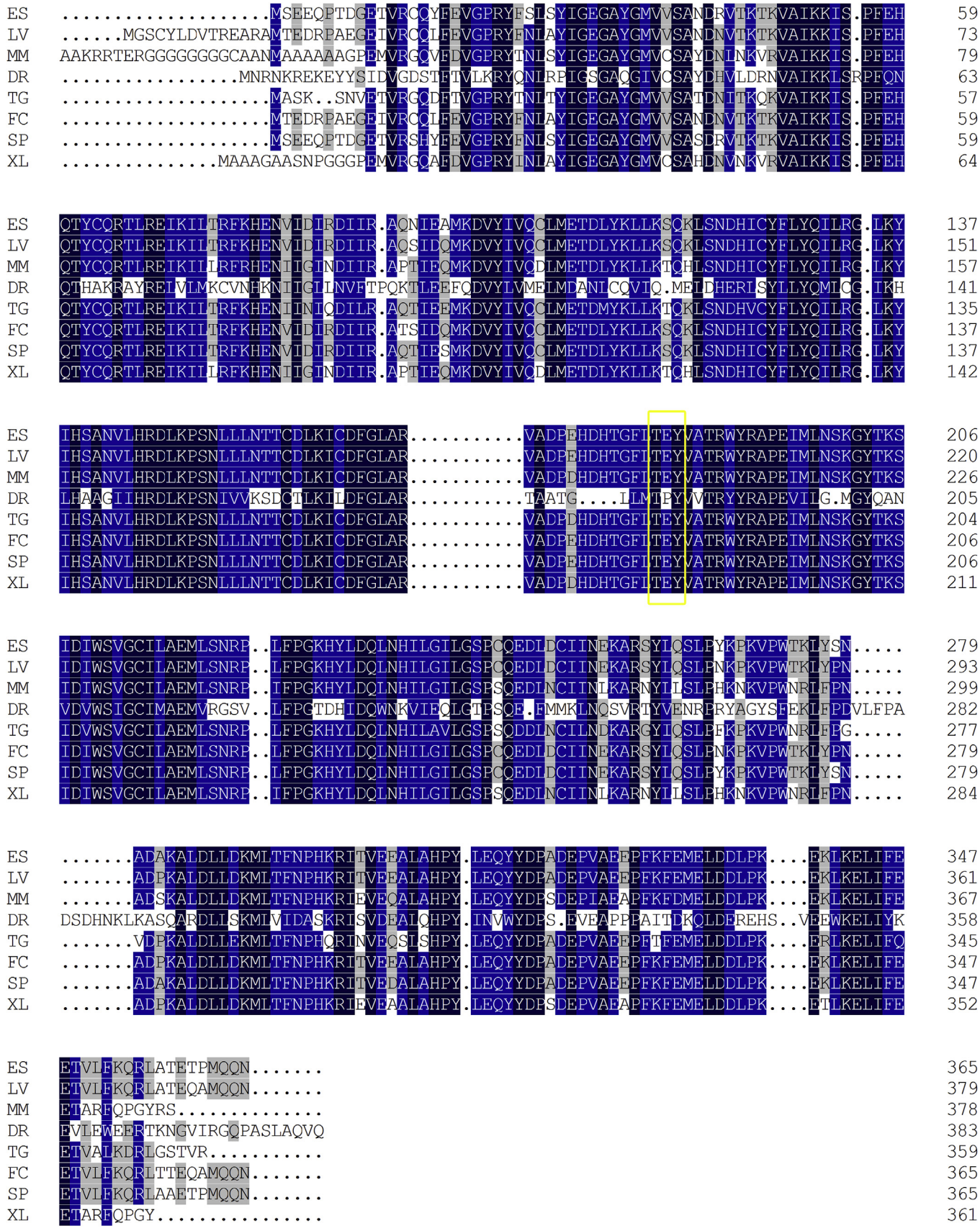

Fig. 2. Multiple amino acid sequence alignments between EsERK2 and MAPK of other crustaceans. The aligned sequences are as follows: ES: *E. sinensis*, ERK2 (MAP kinase) (ADF87942.1); LV: *Litopenaeus vannamei*, ERK (AGS38337.1); MM:*Mus musculus*, MAPK1 (EDK97435.1); DR: *Danio rerio*, MAPK8 (NP_571796.1); TG: *Tegillarca granosa*, ERK2 (AFP57674.1); FC: *Fenneropenaeus chinensis*, MAPK (AHA83424.1); SP: *Scylla paramamosain*, MAPK (ACX32460.1); XL: *Xenopus laevis*, MAPK1 (NP_001083548.1). Identical (black) and similar (blue or gray) residues are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

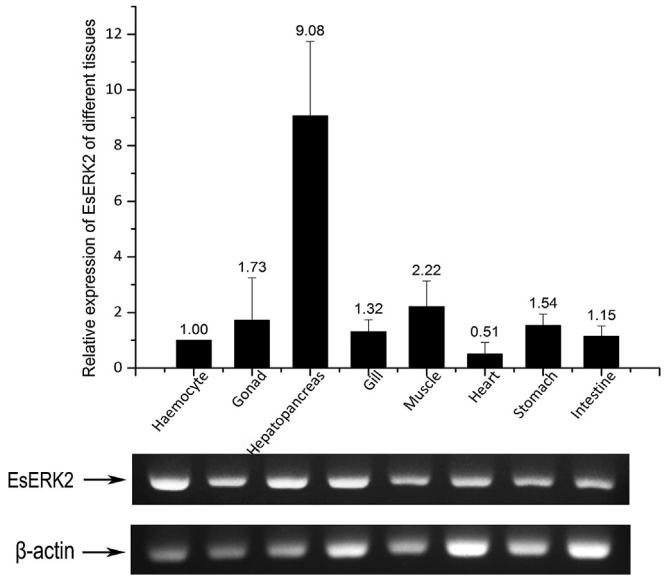

Fig. 3. (A) Real time PCR analysis of EsERK2 in various tissues. β-actin was used as control in all tissues. (B) Expression of EsERK2 mRNA in various tissues. Lane 1, haemocytes; 2, gonad; 3, hepatopancreas; 4, gill; 5, muscle; 6, heart; 7, stomach; 8, intestine.

Until growing to greater than 80% confluence (approximately 12 h), the primary cultured haemocytes were stimulated with 10 μL of LPS (100 μg mL^{-1}) and *A. hydrophila* (1 × 10^7 mL^{-1}), respectively.

After treated for a certain period (0, 2, 4, 6 and 8 h), the haemocytes were harvested and washed with PBS and fixed with 4% paraformaldehyde for the immunofluorescence assay of EsERK2. The other two groups of haemocytes, treated by the same treatment as described above, were collected for the western blotting assay. The primary cultured haemocytes was treated with stroke-physiological saline solution for the same time interval as control.

2.8. Immunofluorescence assay

The haemocytes 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. Afterwards, the haemocytes were permeabilized and blocked with blocking solution (0.5% Triton X-100, 2% BSA, 0.5% rabbit serum in PBS) for 1 h at 20 °C, thoroughly rinsed by PBS, incubated with the rabbit anti-EsERK2 polyclonal antibody (antibody 1:200 in PBS containing 1% BSA and 0.05% Triton X-100) overnight at 4 °C, washed trice with PBS again and incubated 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. The sections were then rinsed in PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in mounting medium. Expression of EsERK2 in haemocytes was observed using confocal laser scanning microscopy with FITC and DAPI emission filters (Zeiss LSM 780, Carl Zeiss, Germany).

2.9. 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 One-way ANOVA and the differences were considered significant as the P is less than 0.05.

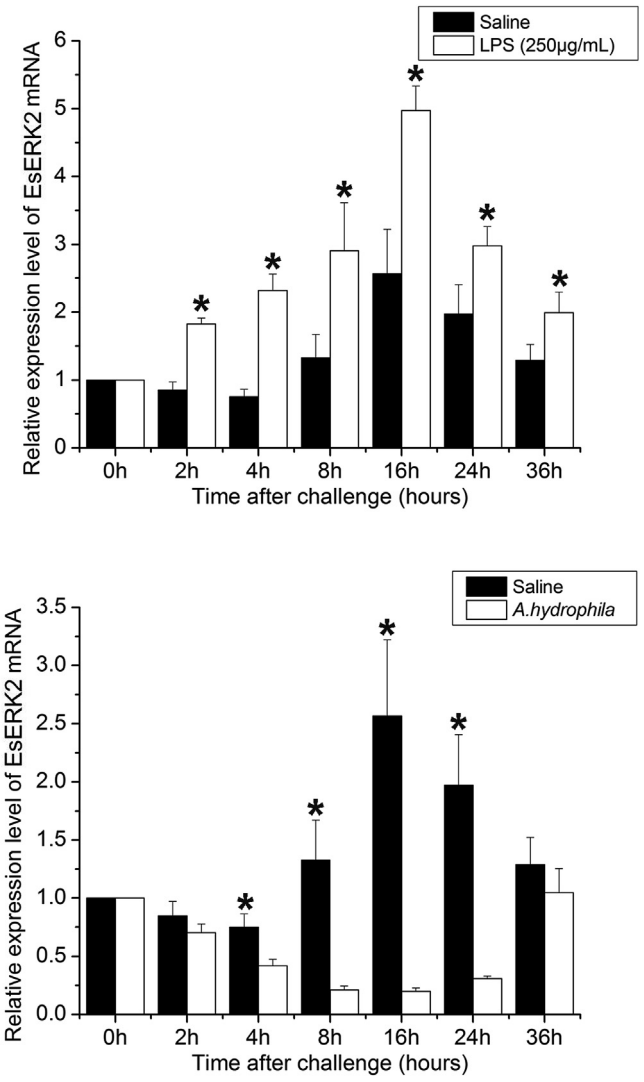

Fig. 4. qRT-PCR analysis of EsERK2 mRNA relative expression in haemocytes treated with saline (control), LPS and *A. hydrophila*. The relative EsERK2 expression level was determined for each group and values were shown as means ± SD, n = 3. The significant differences of EsERK2 expression between the challenged and the control group are indicated with asterisks "*" ($P < 0.05$).

3. Results

3.1. Sequences and similarity analysis of EsERK2

Completed cDNA sequence of EsERK2 is deposited in GenBank under accession number GU002542.1. Sequence analysis revealed that the full-length cDNA was 2455 bp in size with an open reading frame (ORF) of 1095 bp encoding a protein of 365 amino acids with a predicted molecular mass of 42.4 kDa and a theoretical pI of 5.93. The Serine/Threonine protein kinases, catalytic domain (S-TKc) was shown from residue 23 to 311, and the conserved motif TEY was constituted by a threonine (T) residue, a glutamate (E) residue and a tyrosine (Y) residue from 183 to 185, which indicating that EsERK2 was belonged to ERK family. In addition, no predicted signal peptide sequence was found (see Fig. 1).

The Blast P analysis and multiple sequence alignment demonstrated that the EsERK2 displayed high similarity and identity percentage with MAPK from other kinds species including crustacean, insects and mammal, such as the MAPK of *Scylla*

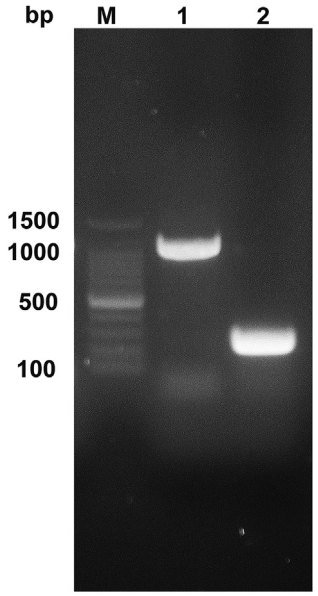

Fig. 5. PCR amplification of ORF of EsERK2 with primers containing *Eco*RI and *Hind*III sites. M: Marker; 1: ORF of EsERK2; 2: β-actin.

paramamosain (98%, ACX32460.1), the ERK1/2 of *Penaeus monodon* (95%, ADT80930.1), the ERK of *Litopenaeus vannamei* (95%, AGS38337.1), the MAPK of *Marsupenaeus japonicus* (95%, BAH86598.1), the MAPK of *Fenneropenaeus chinensis* (95%, AHA83424.1), the extracellular regulated MAP kinase of *Riptortus pedestris* (88%, BAN20448.1), the ERK of *Amphibalanus amphitrite* (87%, AGT02391.1), the MAPK1 of *Bos taurus* (87%, NP_786987.1) and the MAPK1 of *Homo sapiens* (87%, NP_620407.1), respectively (see Fig. 2).

3.2. Tissues distribution of EsERK2

The RT-PCR and qRT-PCR results indicated that the expressions of EsERK2 gene were detected in all of the examined tissues in *E. sinensis*. Its expression level was significantly higher in hepatopancreas, followed by muscle, gonad, stomach, gill, intestine and haemocyte, while lower expression level was detected in heart (Fig. 3).

3.3. Gene expression of EsERK2 in haemocytes after challenge of LPS and A. hydrophila in vivo

The temporal gene expression of EsERK2 in haemocytes challenged by LPS and *A. hydrophila in vivo* is shown in Fig. 4. Its expression level increased significantly after LPS challenge at 2 h ($P < 0.05$), and then gradually increased and reached highest at 16 h. On the contrary, however, its expression level decreased significantly after *A. hydrophila* challenge at 4 h, and then gradually decreased till 24 h ($P < 0.05$), and returned to its initial value at 36 h.

3.4. Expression and purification of the recombinant proteins

The ORF of EsERK2 was successfully amplified with primers containing *Eco*RI and *Hind*III sites at their 5′ ends, respectively (Fig. 5). The sequencing analysis of the amplified product showed that it was 1113 bp in length, which was as anticipated. SDS-PAGE analysis clearly showed a fusion protein was efficiently expressed in *E. coli* BL21 (DE3) cells by IPTG induction at 0.5 mM, and its expression level was found to be increased over time (Fig. 6A). The molecular mass of the protein was about 44.3 kDa, which was consistent with the predicted theoretical value. The protein was expressed in *E. coli* BL21 (DE3) cells as inclusion bodies, and highly purified EsERK2 protein was obtained successfully by Ni-NTA column affinity chromatography (Fig. 6B). Western blotting assay showed that the rabbit polyclonal antibody produced by Huabio (Hangzhou, China) could specifically bind to the purified protein (Fig. 6C).

3.5. Temporal expression of EsERK2 in haemocytes after challenge of LPS and A. hydrophila in vitro

Based on the immunofluorescence assay, EsERK2 was found to be distributed in all haemocytes, and it was mainly distributed in their cytoplasms. After the stimulation of LPS, the expression level of EsERK2 showed a prominent boost at 2 h and kept a relative higher level until 8 h (Fig. 7). 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 EsERK2 in primary cultured haemocytes *in vitro* was also induced after the stimulation of *A. hydrophila*, and its expression level was peaked up at 8 h while the expression level of β-actin in haemocytes decreased obviously (Fig. 8). As it shown in Fig. 9, some *A. hydrophila* were phagocytosed by haemocytes, while the release

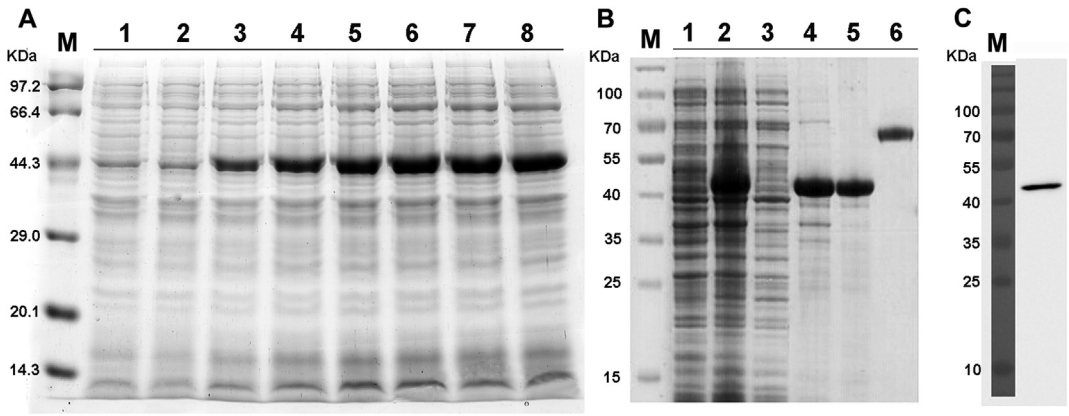

Fig. 6. (A) Analyses of pET28b- EsERK2 products by SDS-PAGE. M: protein marker; 1: mock-vehicle; 2 to 8 indicate the time of 0, 1, 2, 3, 4, 5 and 6 h after challenged with IPTG. (B) Analyses of pET28b-EsERK2 products by SDS-PAGE. M: protein marker; 1: total protein of BL21(DE3) without induction; 2: total protein of BL21(DE3) after induction; 3: ultrasonic supernatant of BL21(DE3) after induction; 4: ultrasonic deposit of BL21(DE3) after induction; 5: purified protein of ultrasonic deposit of BL21(DE3) after induction; 6: BSA control. (C) Identification of EsERK2 protein by SDS-PAGE and Western bloting assay.

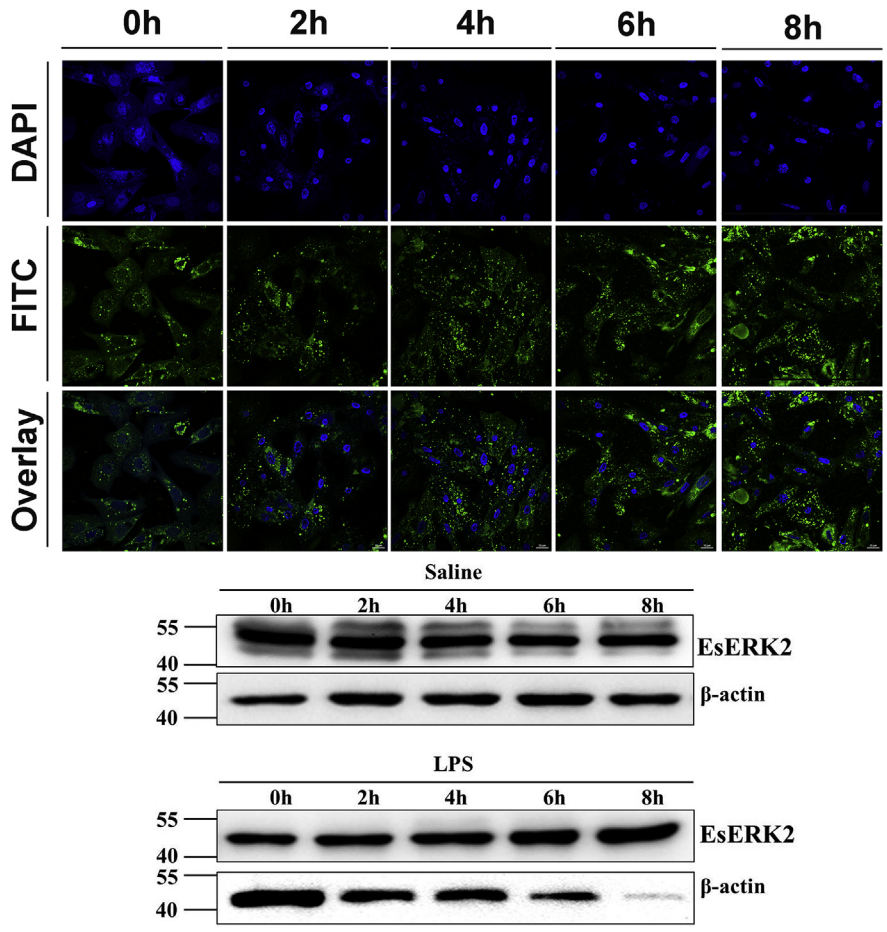

Fig. 7. Temporal expression of EsERK2 in primary cultured haemocytes after challenged with LPS. The haemocytes were fixed and incubated with specific primary antibody and FITC-conjugated secondary antibody, and the nuclei were stained with DAPI post stimulation for different time intervals. Another group were challenged with saline and LPS at the same time points and analyzed by Western blotting.

of granules and haemocytes melanization was occurred simultaneously.

4. Discussion

As the main subfamily of MAPK, ERKs are demonstrated to be effective in regulating many biological activities in previous studies, such as phagocytosis, embryogenesis, cell differentiation, cell proliferation and cell apoptosis [13,14]. In the present study, the amino acid alignments verified that the conserved phosphorylation motif 'TEY' in the kinase activation loop in EsERK2 was at amino acids 183 to 185, and its predicted molecular mass was 42.4 kDa, which indicated that the EsERK2 was belonged to ERK2. Besides, EsERK2 contained no signal peptide indicating that it might be a maturation protein out of transmembrane transportation [13]. Compared MAPK from other organisms, including crustacean, insects and mammal, EsERK2 shares 87%–98% sequence identity. Compared with *S. paramamosain*, *P. monodon*, *L. vannamei*, *M. japonicus* and *F. chinensis*, belonging to the order decapod, the percent identities were high (>95%). And compared with *R. pedestris* and *B. taurus*, which belonging to insects and mammals respectively, high identities were also detected ranged from 87% to 88%. These result further indicated that EsERK2 DNA was highly conserved and gradually evolved in the biological world.

Basing on RT-PCR and qRT-PCR analysis, we found that the EsERK2 gene was expressed in all examined tissues including haemocyte, gonad, hepatopancreas, gill, muscle, heart, stomach and intestine. Its relative high expression level was detected in hepatopancreas, which was consistent with it found in *F. chinensis* and *L. vannamei*. Similarly, both Li and Shi gave a convincing evidence that the expression level of ERK gene was noticeable high in hepatopancreas instead of other tissues [13,15]. The predominant gene expression in hepatopancreas may be related to its tissue-specific roles in metabolize and immunity in *E. sinensis*. In crustacean, hepatopancreas is considered to be functionally homologous to the mammalian liver and pancreas and is responsible for major events on metabolize and immunity. Not only its cells are capable of engulfing cellular debris and soluble bacterial cell components from the gut lumen, but also it is an important source of innate immune molecules involved in the recognition, binding and elimination of cellular pathogens or viruses [16]. Interestingly, the relative expression of EsERK2 gene in haemocytes displayed convert trend after challenge with different stimulants. Injection of LPS into *E. sinensis* led to a significant increase of EsERK2 gene expression level in haemocytes, which was consistent with previous researches. Li and Shi found that the expression level of ERK gene increased obviously in shrimp after challenge of WSSV [13,15]. Similarly, in large yellow croaker (*Larimichthys crocea*) after challenged with poly I:C and flagellin, the expression level of ERK mRNA showed significant up-regulation too [11]. Conversely, the expression of EsERK2 gene in haemocytes decreased significantly after challenge of *A. hydrophila* at 4 h *in vivo*. The contrary response to various exterior stimulants might due to the decline in the counts of haemocytes which expressed EsERK2. In our previous

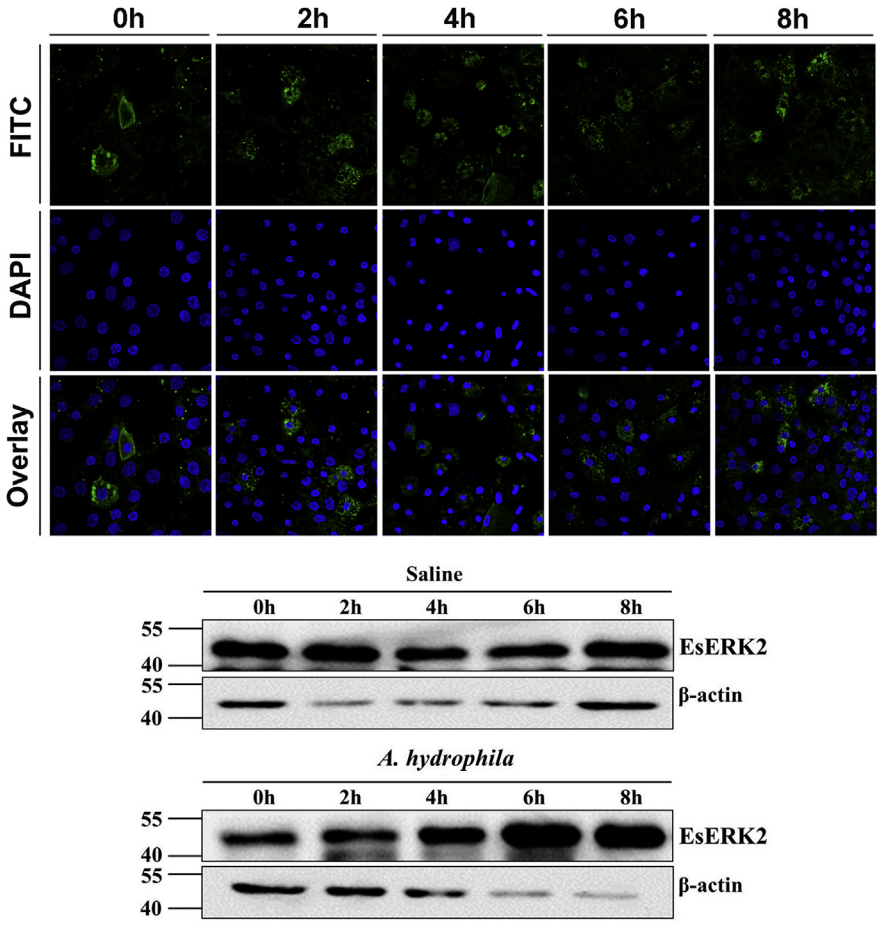

Fig. 8. Temporal expression of EsERK2 in primary cultured haemocytes after challenged with *A. hydrophila*. The haemocytes were fixed and incubated with specific primary antibody and FITC-conjugated secondary antibody, and the nuclei were stained with DAPI post stimulation for different time intervals. Another group were challenged with saline and *A. hydrophila* at the same time points and analyzed by Western blotting.

research, the haemocyte counts of *E. sinensis* experienced drastic decline after challenged with LPS and *A. hydrophila*. The haemocyte counts significantly declined to approximate 2.3% of original counts after *A. hydrophila* challenge at 0.5 h, while the injection of LPS lead to a significant decline of haemocyte counts to approximate 18.3% of total haemocytes [4]. Therefore, the extremely toxic impact of the pathogenic bacteria on circulating haemocytes of *E. sinensis* might be responsible for the down-regulation of EsERK2 after *A. hydrophila* challenge.

In addition, for better understanding of biological properties of the EsERK2 protein, we successfully constructed the expression plasmid pET-28b-EsERK2 and expressed the EsERK2 fusion protein in *E. coli* BL21 (DE3) cells. The rabbit anti-EsERK2 polyclonal antibody was prepared successfully with the purified fusion protein. Western blotting showed a single band of the recombinant fusion with a molecular mass of approximately 42.4 kDa, indicating that the prepared anti-EsERK2 polyclonal antibody specifically recognized the EsERK2 protein. The results of immunofluorescence assay and western blotting showed that EsERK2 protein was distributed in all circulating haemocytes including hyalinocytes, semigranulocytes and granulocytes, and was mainly distributed in their cytoplasms, which was similar to previous reports [15]. And the protein was distinct up-regulation in primary cultured haemocytes after challenged with LPS and *A. hydrophila*. In previous studies, the phagocytic activity of haemocytes in *Lymnaea stagnalis* was proved to be down-regulated after the ERK was inhibited [14],

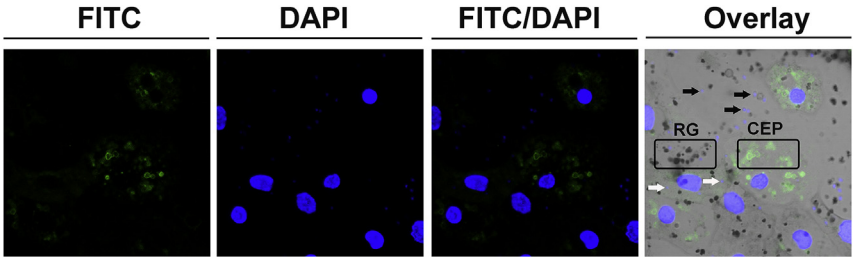

Fig. 9. Temporal expression of EsERK2 in primary cultured haemocytes after challenged with *A. hydrophila* for 6 h. Black arrors and white arrors indicate the extracellular and intracellular *A. hydrophila*, respectively. RG: released granule; CEP: celluar EsERK2 protein.

and Lamprou revealed that MAPK are required for phagocytosis by insect haemocytes basing on flow cytometry experiments [17]. In our experiments, we found that some *A. hydrophila* were phagocytosed by haemocytes and the release of granules and haemocytes melanization was occurred simultaneously. Therefore, the incremental EsERK2 protein in haemocytes after challenged with LPS or *A. hydrophila* might be associated with the signaling transduction in response to exterior stimulation in *E. sinensis*.

In conclusion, the EsERK2 in *E. sinensis* had a conserved motif TEY and belonged to the ERK family. qRT-PCR analysis demonstrated that EsERK2 was found in all examined tissues, and its expression level was significantly higher in hepatopancreas than it in other examined tissues. Challenge of LPS and *A. hydrophila* induced to different gene expression levels of EsERK2 with up-regulated and down-regulated, respectively. Additionally, primary cultured haemocytes after challenged with LPS and *A. hydrophila* displayed prominent boost of EsERK2 protein, indicating it might be associated with the signaling transduction in response to exterior stimulation in *E. sinensis*. However, further studies are needed to uncover its function and mechanism in immune defense to invasive pathogens and exterior stimulation.

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