The responsive expression of a chitinase gene in the ridgetail white prawn *Exopalaemon carinicauda* against *Vibrio anguillarum* and WSSV challenge

Yafei Duan · Ping Liu · Jitao Li · Jian Li · Yun Wang ·
Ping Chen

Received: 21 October 2013 / Revised: 23 November 2013 / Accepted: 25 November 2013 / Published online: 10 January 2014
© Cell Stress Society International 2014

Abstract Chitinases are essential enzymes for crustaceans and participates in several biological processes, such as nutrient digestion, morphogenesis, pathogenesis, and pathogen defense. In the present study, the full-length cDNA of Chi (named EcChi) was cloned from the hemocytes of ridgetail white prawn *Exopalaemon carinicauda* by rapid amplification of cDNA ends methods. The full-length cDNA of EcChi was 1,319 bp, including contains a 5'-untranslated region (UTR) of 42 bp, 3'-UTR of 101 bp with a poly (A) tail, an open-reading frame of 1,176 bp, encoding a 391-amino acid polypeptide with the predicted molecular weight of 43.71 kDa and estimated isoelectric point of 4.78. Sequence analysis revealed that the conserved chitinases family 18 active site was predicted in the amino acid sequence of EcChi. BLAST analysis revealed that amino acids of EcChi shared high identity (61–77 %) with that of other crustaceans. Quantitative real-time PCR analysis indicated that EcChi could be detected in all the tested tissues, and strongly expressed in hepatopancreas of *E. carinicauda*. After challenged with *Vibrio anguillarum* and WSSV, EcChi transcripts both in hemocytes and hepatopancreas increased significantly in the first 3 h, respectively. These results indicated that EcChi might be involved in the innate immune responses to *V. anguillarum* and WSSV in *E. carinicauda*.

Keywords *Exopalaemon carinicauda* · Chitinase (Chi) · *Vibrio anguillarum* · White spot syndrome virus (WSSV) · Immune response

Introduction

The ridgetail white prawn, *Exopalaemon carinicauda*, is an economically important shrimp species naturally distributed in the coasts of the Yellow Sea and the Bohai Sea, China, which contributes to one third of the gross output of the polyculture ponds in eastern China (Duan et al. 2013a). *E. carinicauda* have good reproductive performance, fast growth and wide environmental adaptability, which make it a good experimental animal candidate for shrimp (Li et al. 2012; Wang et al. 2013; Duan et al. 2013c). However, with the development of intensive culture and the ecologic environmental deterioration, various diseases caused by bacteria and viruses have frequently occurred in cultured *E. carinicauda*, causing economic losses to commercial shrimp aquaculture (Duan et al. 2013b; Xu et al. 2010). Shrimp lack an adaptive immune system and their defense mechanisms mainly rely on innate immune responses to protect them against invaders. Therefore, a better understanding of the innate immune abilities and immune defense mechanisms of shrimp will be beneficial to the development of health management and disease control in shrimp aquaculture.

Chitin, a linear homopolymer composed of β -(1, 4)-*N*-acetyl-*D*-glucosamine (GlcNAc), are the major components in the cell walls of fungi, the exoskeleton of arthropod, the shell and radulae of mollusks, the microfilarial sheath of parasitic nematodes, and the lining of the digestive tracts of many insects (Rocha et al. 2012; Zhang et al. 2012). Chitin can provide mechanical rigidity to extracellular structure in animal such as insect exoskeletons and shells of crustaceans

Y. Duan · P. Liu (✉) · J. Li · J. Li · P. Chen
Key Laboratory of Sustainable Development of Marine Fisheries,
Ministry of Agriculture, Yellow Sea Fisheries Research Institute,
Chinese Academy of Fishery Sciences, Qingdao 266071,
People's Republic of China
e-mail: liuping@ysfri.ac.cn

Y. Duan · Y. Wang
Key Laboratory of South China Sea Fishery Resources Exploitation
and Utilization, Sustainable Development of Marine Fisheries,
Ministry of Agriculture, South China Sea Fisheries Research
Institute, Chinese Academy of Fishery Sciences,
Guangzhou 510300, People's Republic of China

(Okada et al. 2013; Flach et al. 1992) and form a physical barrier against invading microorganisms (Mali et al. 2004).

Chitinase (Chi; EC 3.2.1.14) is a glycosyl hydrolase are enzymes that randomly hydrolyze the β -1,4-glycosidic bonds of chitin and produce *N*-acetylchitooli-gosaccharides (GlcNAc)_n (Flach et al. 1992). Chis are found not only in chitin-containing organisms, but also in widely diverse organisms that are not composed of chitin (Mali et al. 2004). During the molting cycle of arthropods, Chi dissolves chitin in the old exoskeleton into more soluble forms, which can then be partially reabsorbed into the body and used to synthesize the new exoskeleton (Huang et al. 2010). Previous studies have demonstrated that Chi plays an important role in the molting process of invertebrates, the digestion of chitinous food, and defense against chitin-bearing pathogens (Tan et al. 2000; Duo-Chuan 2006; Mali et al. 2004; Dahiya et al. 2006; Kramer and Muthukrishnan 1997). In mammals, Chi is closely related to allergic inflammation and other immunity (Kawada et al. 2007). In invertebrates, Chi is involved in early embryonic development and functions as immunity effectors in *Crassostrea gigas* (Badariotti et al. 2007), and the expression of Pjchi-3 was upregulated in the hepatopancreas of white spot syndrome virus (WSSV)-resistant shrimp in *Penaeus japonicus* using suppression subtractive hybridization (Pan et al. 2005). They are involved in degradation of chitin-containing pathogens as part of host defense mechanism.

To date, Chi gene have been isolated from some crustaceans (Salma et al. 2012; Rocha et al. 2012; Proespraiwong et al. 2010; Zhang et al. 2010; Huang et al. 2010; Watanabe et al. 1998). However, little is known about the potential role of Chi in ridgetail white prawn *E. carinicauda* against pathogens like *Vibrio anguillarum* and WSSV. In the present study, a full-length cDNA of Chi was cloned from hemocytes of *E. carinicauda* and the gene structure was analyzed. In addition, the expression pattern of Chi in various tissues of *E. carinicauda* were investigated, and expression profiles in hemocytes and hepatopancreas of *E. carinicauda* after *V. anguillarum* and WSSV challenge were evaluated. These results will be essential to better understand the physiological function of Chi in the shrimp immune response to bacterial and viral infection.

Materials and methods

Animal materials

Healthy adult *E. carinicauda*, averaging weight 1.19 ± 0.32 g, were collected randomly from a commercial farm in Qingdao, China, which included female and male prawns, and almost every prawns were not in the moult stage because the cuticle and setae of them were very hard. They were cultured in filtered aerated seawater (salinity 20‰, pH 8.2) at 18 ± 0.5 °C for 7 days before processing. There were 30 prawns

in each group. The prawns were fed with formulated pellet feed (Tongyi, Taiwan; 42 % protein) twice daily at 06:00 and 18:00 hours, with a ration of 10 % of body weight. Two thirds of the water in each group was renewed once daily during the culture period.

RNA extraction and cDNA synthesis

Hemocytes was collected with a syringe which contained an equal volume of anticoagulant solution (Söderhäll and Smith 1983), and centrifuged at $800 \times g$, 4 °C for 15 min. Total RNA was extracted from hemocytes using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA samples were analyzed in 1.0 % agarose electrophoresis and quantitated at 260 nm, all OD₂₆₀/OD₂₈₀ were between 1.8 and 2.0. The 3' and 5' ends rapid amplification of cDNA ends (RACE) cDNA template were synthesized using SMART™ cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

Cloning the full-length cDNA of EcChi

An EST sequence was obtained from *E. carinicauda* hemocytes cDNA library of our laboratory, and has been reported by Duan et al. (2013b). BLAST analysis showed that they had high identifies with Chis of other crustaceans. According to the EST sequence, a gene specific primer F1 was designed for 3' RACE, and primer R1 was designed for 5' RACE (Table 1).

Based on the EST sequence data of Chi, its 3' and 5' ends were obtained using SMART RACE cDNA Amplification Kit (Clontech, USA). For 3' RACE, the PCR reaction was performed using the primer F1 and the anchor primer UPM

Table 1 Primer sequences used in this study

Primer name	Sequence (5'–3')
EcChi	
F1 (forward)	CTGCTTGCTTCGCTCCTC
R1 (reverse)	ACCATACAAGGGGATACC
F2 (forward)	CTCACCTCGTCTACGCCTTC
R2 (reverse)	GAGAGTGTCCTCCAGTGTCGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT CTAATACGACTCACTATAGGGC
18S rRNA	
18S-HF	TATACGCTAGTGGAGCTGGAA
18S-HR	GGGGAGGTAGTGACGAAAAAT
WSSV	
F3 (forward)	ACAATGGTCCCGTCCTCATC
R3 (reverse)	TGCCTTGCCGGAATAGTGTG
Probe (T)	TET-CAGAAGCCATGAAGAATGCCGTCTATCAC-TAMRA

(Table 1). The PCR reaction conditions were 5 cycles of 94 °C for 30 s, 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; and 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. For 5' RACE, the PCR reaction was performed using the primer R1 and the anchor primer UPM (Table 1). The PCR reaction conditions were the same as those described above.

The PCR fragments were subjected to electrophoresis on 1.0 % agarose gel to determine length differences, and the target band was purified by PCR purification kit (Promega, USA). The purified products were cloned into PMD18-T vector, following the instructions provided by the manufacturer (TaKaRa, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

Sequence analysis

The nucleotide and deduced amino acid sequences of EcChi cDNA were analyzed and compared using the BLAST search programs (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>). The signal peptide was predicted by SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>). The multiple sequence alignment of Chi amino acid sequences was performed using the programs of Vector NTI advance 10.3 (Invitrogen). A phylogenetic NJ tree of Chis was constructed by the MEGA 4.0 software (Tamura et al. 2007).

Tissue expression of EcChi

Eight tissues including hemocytes, gill, hepatopancreas, muscle, ovary, eyestalk, stomach and intestine were dissected from unchallenged *E. carinicauda*. The mRNA expressions of EcChi in different tissues were determined by quantitative real-time PCR (RT-PCR). Total RNA was extracted as described above. The RNA samples were analyzed in 1.0 % agarose electrophoresis and quantitated at 260 nm, all OD₂₆₀/OD₂₈₀ were between 1.8 and 2.0. Total RNA (5 µg) was reverse transcribed using the PrimeScript™ Real-Time PCR Kit (TaKaRa, Japan) for quantitative RT-PCR analysis. 18S rRNA gene of *E. carinicauda* (GenBank accession number, GQ369794) was used as an internal control. 18S rRNA gene is abundant and stable in cells, and hardly affected by the external regulation. The expression of 18S rRNA gene is invariant in the present experimental conditions, and was used as an internal control in gene expression analysis of crustaceans (Li et al. 2010, 2012; Wang et al. 2013).

Experimental design of *V. anguillarum* and WSSV challenge

The experiments were divided into the bacterial (*V. anguillarum*) challenged group, the virus (WSSV) challenged

group and the control group. *V. anguillarum* strains was obtained from Germplasm Resources and Genetic Breeding Laboratory, Yellow Sea Fisheries Research Institute (China), activating on marine agar 2611E. The pathogenicity of the *V. anguillarum* which used in the present study was checked in a preliminary experiment before the *V. anguillarum* challenged treatment, and the median lethal dose of *V. anguillarum* in 48 h was 2×10^8 CFU prawn⁻¹. To better analysis of EcChi expression level between 72 h after *V. anguillarum* challenged, the injected dose of *V. anguillarum* was 2×10^8 CFU prawn⁻¹. WSSV crude extract was obtained from 10 g WSSV-infected tissue from *Litopenaeus vannamei*, which was provided by the Mariculture Disease Control and Pathogenic Molecular Biology Laboratory, Yellow Sea Fisheries Research Institute, the methods referred to Xie et al. (2005). Quantitative detection of WSSV was performed by RT-qPCR on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA), the methods referred to Durand and Lightner (2002). The RT-qPCR was carried out in a total volume of 20 µL, containing 12.5 µL of Perfect Real-Time premix (1×; RR039A, TaKaRa), 50–100 ng of virus DNA, 0.25 µmol/L each of F3 and R3 primer, 0.125 µmol/L of probes, and added sterile ddH₂O to total volume of 20 µL. Recombinant plasmid PUCm-T/WSSV69 containing purpose fragment was used as standard. The PCR program was 94 °C for 10 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s.

For *V. anguillarum* challenged group, prawns were injected individually with 20 µL live *V. anguillarum* suspension (2×10^9 CFU/mL) in sterile 0.9 % saline solution, resulting in 2×10^8 CFU prawn⁻¹. For WSSV challenged group, prawns were injected individually with 20 µL live WSSV crude extract as described above. The control group received individually an injection of 20 µL sterile 0.9 % saline solution. Then both the challenged and control prawns were returned to the PVC tanks of aerated seawater and fed at 25 °C as described above. The seawater waste was dealt with safely to make it harmless for the environment. Through the separation and identification of pathogens from prawns in the *V. anguillarum* and WSSV challenged group, respectively, the results showed that the shrimp was infected with particular pathogen. Previous studies have demonstrated that the gene expression level of crustaceans changed rapidly at early stage after bacterial and viral infections, and the choice of the sampling times was referred to the previous studies (Anghong et al. 2010; Tian et al. 2011). Hemocytes and hepatopancreas of six prawns from each treatment (the challenged group and the control group) were randomly sampled at 0, 3, 6, 12, 24, 48, and 72 h postinjection, respectively; then, the samples were snap-frozen in liquid nitrogen. There were three replicates for each time point. Total RNA was extracted and the first strand cDNA was synthesized as described above.

Expression of EcChi after *V. anguillarum* and WSSV challenge

Quantitative RT-PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) to investigate the expression of EcChi. For the mRNA expression of EcChi, the pair of specific primers F2 and R2 (Table 1) were used to amplify a PCR product of 593 bp. Two primers 18S-HF and 18S-HR (Table 1) were used to amplify an 18S rRNA gene of 147 bp as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The RT-PCR was carried out in a total volume of 20 μ L, containing 10 μ L SYBR® Premix Ex Taq™ II (2 $\times$; TaKaRa), 2 μ L of the 1:5 diluted cDNA, 0.8 μ L each of F2 and R2 primer (or 18S-HF and 18S-HR to amplify the 18S rRNA), 0.4 μ L ROX Reference Dye II (50 $\times$)*3 and 6 μ L DEPC-treated water. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. DEPC-treated water for the replacement of template was used as negative control.

RT-PCR data from three replicate samples were analyzed with the ABI 7300 system SDS Software (Applied Biosystems, USA), for estimating transcript copy numbers for each sample. The comparative C_T method was to analyze the relative expression levels of EcChi. The C_T for the target amplified products of EcChi and internal control 18S rRNA was determined for each sample. The difference in the C_T between the target and the internal control (called ΔC_T) was calculated to normalize the differences in the amount of template and the efficiency of the RT-PCR. In the same challenge time, the ΔC_T of the control group was used as the calibrator, and the difference between the ΔC_T of the challenged group and the control group was called $\Delta\Delta C_T$. The expression level of EcChi was calculated by the $2^{-\Delta\Delta C_T}$ comparative C_T method (Livak and Schmittgen 2001). There were three replicates in each treatment group, respectively, and three samples were analyzed per conditions during the different experimentations. Statistical analysis was performed using SPSS software (Ver 11.0). The normality of data distribution and the variance homoscedasticity were tested before performing a parametric statistic analysis, and the statistical analysis methods of our data were consistent with the previous studies (Chen et al. 2011; Li et al. 2012). Statistical significance was determined using one-way ANOVA (González-Rodríguez et al. 2012) and post hoc Duncan multiple range tests. Significance was set at $P < 0.05$.

Results

Analysis of the EcChi sequence and the predicted protein

A full-length 1,319 bp cDNA of Chi gene (designated EcChi) was obtained from the hemocytes of *E. carinicauda* by RACE

method, and the nucleotide and deduced amino acid sequences were shown in Fig. 1. It contained 42 bp of 5'-untranslated region (UTR), 1176 bp of open-reading frame (ORF), 101 bp of 3'-UTR including a stop codon (TGA) polyadenylation signal (AATTAAA) and a poly A tail. The EcChi cDNA sequence and deduced amino acid sequence has been submitted to the GenBank (accession number, KF548041).

The ORF of EcChi encoded a protein of 391 amino acids. SignalP software predicted that the EcChi contained a putative signal peptide of 19 amino acids, cleaved between G¹⁹ and E²⁰ to generate a 372 amino acid mature protein. The calculated molecular mass was 43.71 kDa, and the estimated isoelectric point (pI) of this protein was 4.78. InterProScan program analysis revealed that Chi active site, ¹³⁷FDGLDMDWE¹⁴⁵, was existed in the EcChi sequence (Fig. 1). In addition, a glycoside hydrolase catalytic domain and a Chi insertion domain were also observed in the EcChi sequence (Fig. 1).

Multiple sequences alignment and phylogenetic analysis

Sequence analysis with the BLASTP program revealed that the deduced amino acid sequence of EcChi exhibited a high identification with Chi of *Pandalopsis japonica* (77 %), *Scylla serrata* (65 %), *L. vannamei* (62 %), *Fenneropenaeus chinensis* (62 %), *Marsupenaeus japonicus* (62 % to Chi3), and *Penaeus monodon* (61 % to Chi3), respectively. Multiple sequence alignment of EcChi of *E. carinicauda* with Chi of other animals showed high conservation in the Chi active site (FDGLDMDWE) (Fig. 2).

Based on the sequences of Chis, a neighbor-joining phylogenetic tree was constructed for phylogenetic analysis (Fig. 3). Based on the MEGA 4.0 analysis, plant and animal Chis were separated and formed two distinct branches in the tree. In the animal branch, all the crustaceans were clustered together and formed three branches (Chi1, Chi2, and Chi3 isoform groups). The relationships displayed in the phylogenetic tree were in good agreement with the concept of traditional taxonomy. EcChi of *E. carinicauda* belongs to the crustacean Chi3s subgroup and is closely related to Chi3 of *P. japonica*.

Tissue expression of EcChi gene

To evaluate the abundance of EcChi mRNA in the tissues, total RNA was extracted from hemocytes, gill, hepatopancreas, muscle, ovary, intestine, stomach, and eyestalk. Quantitative RT-PCR was employed to quantify the EcChi expression in different tissues (Fig. 4). The amplification specificity for EcChi and 18S rRNA was determined by analyzing the dissociation curves. Only one peak presented in the dissociation curves for both EcChi and 18S rRNA gene, which could indicate that the amplifications were specific. EcChi transcript was most abundant in hepatopancreas, with

Fig. 3 Phylogenetic tree of different species Chis based on the amino acid sequence using neighbor-joining distance analysis. The protein sequences used for phylogenetic analysis were as follows: Chi of *C. japonica* (AFF59213), *Arabidopsis thaliana* (BAA82818), *Dioscorea oppositifolia* (BAC56863), *Nicotiana tabacum* (AAB23374), and *Zea mays* (ACX37090); Chi1 of *L. vannamei* (EU883591), *M. japonicus* (BAA12287), *P. monodon* (AAD40313), *F. chinensis* (ABB85237), *S. serrata* (ACG60512), and *P. japonica* (JF694836); Chi2 of *L. vannamei* (EU861222), *M. japonicus* (BAA14014), *P. monodon* (ADG22164), *S. serrata* (ACZ53950), and *P. japonica* (JN982965); and Chi3 of *L. vannamei* (AAN74647), *M. japonicus* (BAA22854), *P. monodon* (ADG22163), *F. chinensis* (DQ000159), and *P. japonica* (JF694838). The numbers at the forks indicated the bootstrap

Fig. 4 Tissue specific expression of EcChi mRNA related to hepatopancreas expression by the real-time PCR. The reference gene is 18S rRNA. Vertical bars represent the mean $\pm$ SD ($N=3$)

Expression profiles of EcChi in hemocytes and hepatopancreas after WSSV challenge

The temporal expression of EcChi mRNA in hemocytes and hepatopancreas of *E. carinicauda* after WSSV challenge was shown in Fig. 6. Compared with the control group, WSSV challenge significantly induced EcChi transcripts in hemocytes at 3 h (2.72-fold of the control group, $P < 0.05$), then it reduced to the lowest level at 6 h (0.08-fold of the control group, $P < 0.05$). Afterwards, the transcript level of EcChi increased gradually and reached to the second peak level at 24 h (4.63-fold of the control group, $P < 0.05$), then it decreased gradually and recovered to the control level at 72 h (Fig. 6a). In hepatopancreas, the EcChi mRNA expression level was strongly induced by WSSV challenge and reached to the highest level at 3 h (3.80-fold of the control group, $P < 0.05$), then it decreased gradually from 6 to 12 h and reached to the lowest level at 24 h (0.40-fold of the control group, $P < 0.05$). Afterwards, it upregulated again to the second peak level at 48 h (3.01-fold of the control group, $P < 0.05$), then it recovered to the control level at 72 h (Fig. 6b).

Discussion

Chi plays a major role in the molting cycle of arthropods, dissolving chitin in the old exoskeleton into more soluble forms, which can then be partially resorbed into the body and used to synthesize the new exoskeleton (Huang et al. 2010). In the present study, a new full-length 1,319 bp of Chi cDNA (EcChi) was isolated and sequenced from *E. carinicauda*. The deduced amino acid sequence of EcChi showed high identities (61–77 %) with Chis of other crustaceans. Multiple sequence alignment analysis revealed that the

Fig. 5 The mRNA expression levels of EcChi in hemocytes (a) and hepatopancreas (b) of *E. carinicauda* at different time intervals after *V. anguillarum* challenge treatment. The reference gene is 18S rRNA. Vertical bars represented the mean $\pm$ SD ($N=3$). Significant differences ($P<0.05$) in EcChi expression between the challenged and the control group were indicated with asterisks

Chi-active site ($^{137}\text{FDGLDMDWE}^{145}$) are highly conserved in all of the Chis investigated. The domain structure and conservation of active site signature suggested that the Chi has chitinolytic activity. Phylogenetic analysis revealed that EcChi of *E. carinicauda* was clustered with Chi3s of other crustaceans and closely related to Chi3 of *P. japonica*. The facts suggested that EcChi was more closely related to the invertebrate Chi3 isoform.

Quantitative RT-PCR revealed that EcChi was expressed in all the tested tissues of *E. carinicauda*. The EcChi mRNA level was highest in hepatopancreas as compared with other seven tested tissues (hemocytes, gill, muscle, ovary, intestine, stomach, and eyestalk), and EcChi is specifically expressed in hepatopancreas. Zhang et al. (2010) have demonstrated that hepatopancreas is a site where large quantities of Chi are synthesized for the degradation of the absorbed chitin from

Fig. 6 The mRNA expression levels of EcChi in hemocytes (a) and hepatopancreas (b) of *E. carinicauda* at different time intervals after WSSV challenge treatment. The reference gene is 18S rRNA. Vertical bars represented the mean $\pm$ SD ($N=3$). Significant differences ($P<0.05$) in EcChi expression between the challenged and the control group were indicated with asterisks

the diet. The hepatopancreas specific expression of Chi in *E. carinicauda* is almost in agreement with those found in *P. japonica* (Salma et al. 2012), *F. chinensis* (Zhang et al. 2010), and *L. vannamei* (Huang et al. 2010).

Virulent pathogens have a major impact on aquaculture. *V. anguillarum* and WSSV are both extremely virulent pathogens that seriously affect shrimp aquaculture (Toranzo et al. 2005; Lightner 2011; Spann and Lester 1997). When pathogens enter the body of the shrimp, they will encounter the innate immune

system (Soonthornchai et al. 2010). To know whether EcChi is related to the immune response of shrimp, the transcriptional profile of EcChi was analyzed in hemocytes and hepatopancreas when shrimp were injected with *V. anguillarum* or WSSV. Previous studies have demonstrated that hemocytes and hepatopancreas are the main cells and tissue involved in the immune response, and the major site for the synthesis of immune defense molecules involved in eliminating pathogens or other particulate matter in crustaceans (Lemaitre and Hoffmann

2007; Bianchini and Monserrat 2007; Iwanaga and Lee 2005). Information on the expression profile of the EcChi gene in these tissues after *V. anguillarum* and WSSV challenge would be helpful to better understand its immunological function.

V. anguillarum is a major virulent pathogen for cultured shrimp worldwide (Toranzo et al. 2005). As far as we know, the effect of *V. anguillarum* on the expression of Chi has not been evaluated in crustaceans. In the present study, live *V. anguillarum* were chosen to challenge the shrimp, so that the shrimps' health would be affected severely by the production of *V. anguillarum*. In our study, the expression of EcChi increased at 3 h significantly in hemocytes and hepatopancreas respectively, which indicated that EcChi could be induced by *V. anguillarum* and the cellular requirement for more of EcChi protein to repair damaged proteins. As time progressed, the expression of EcChi dropped to a low level at 6 h in hemocytes and 24 h in hepatopancreas, perhaps because infection progress brought more bacteria, and destroyed the normal function of the shrimps' cells and finally caused the expression of EcChi in the challenged group to decrease gradually (Chen et al. 2011). The results showed that EcChi might be involved in an immune response to *V. anguillarum* stimulation. The expression profiles of the EcChi gene with *V. anguillarum* challenge indicated that it was inducible and might be involved in the immune response against bacterial infection in *E. carinicauda*.

White spot syndrome is a lethal disease that affects cultured shrimp species (Lightner 2011). WSSV is a large DNA virus with a broad host range among shrimps and the causative agent of White Spot disease of shrimp, causing mass mortalities and economic losses in shrimp aquaculture (Lightner 2011; Escobedo-Bonilla et al. 2008; Leu et al. 2009). However, there are still no effective preventive and therapeutic measures against WSSV infection. In this study, the temporal up and downregulation of the EcChi transcripts in hemocytes and hepatopancreas indicated that the EcChi is potentially involved in the acute response against invading WSSV in *E. carinicauda*. This result was in agreement with earlier reports in *F. chinensis* challenged with WSSV (Zhang et al. 2010). Previous studies have demonstrated that Chi3 are likely to be related to resistance against WSSV in *P. japonicus* (Pan et al. 2005). The expression profiles of EcChi in hemocytes and hepatopancreas after *V. anguillarum* and WSSV challenge were different, it might be caused by the different function of hemocytes and hepatopancreas in the immune defense system. It has been proved that hemocytes are key cells for invertebrate's innate defense reactions (Vazquez et al. 2009; Iwanaga and Lee 2005) and play an important role in the host immune functions when the organism is attacked by bacterias or viruses (Lee and Söderhäll 2002; Lavine and Strand 2002).

In summary, a full-length cDNA sequence of Chi gene (EcChi) was cloned from hemocytes of *E. carinicauda*, and

it was constitutively expressed in the tissues of hemocytes, gill, hepatopancreas, muscle, ovary, intestine, stomach, and eyestalk. The expression of EcChi in hemocytes and hepatopancreas changed rapidly and dynamically in response to infection *V. anguillarum* and WSSV, which indicated that EcChi was inducible and might be involved in the immune response against bacterial and viral infection in the ridgetail white prawn *E. carinicauda*. More information about the exact mechanism of this phenomenon was required to better understand the EcChi function in immune defense mechanisms of *E. carinicauda* in the future.

Acknowledgments The authors are grateful to all the laboratory members for experimental material preparation and technical assistance. This study was supported by the earmarked fund for National "863" Project of China (No. 2012AA10A409), Modern Agro-industry Technology Research System (No. CARS-47), Special Fund for Independent Innovation of Shandong province (No. 2013CX80202) and Special Fund for Agro-scientific Research in the Public Interest (No. 201103034).

References

- Angthong P, Watthanasurorot A, Klinbunga S, Ruangdej U, Söderhäll I, Jiravanichpaisal P (2010) Cloning and characterization of a melanization inhibition protein (PmMIP) of the black tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol 29:464–468
- Badariotti F, Thuau R, Lelong C, Dubos MP, Favrel P (2007) Characterization of an atypical family 18 chitinase from the oyster *Crassostrea gigas*: evidence for a role in early development and immunity. Dev Comp Immunol 31:559–570
- Bianchini A, Monserrat JM (2007) Effects of methyl parathion on *Chasmagnathus granulatus* hepatopancreas: protective role of sesamol. Ecotoxicol Environ Saf 67:100–108
- Chen P, Li JT, Gao BQ, Liu P, Wang QY, Li J (2011) cDNA cloning and characterization of peroxiredoxin gene from the swimming crab *Portunus trituberculatus*. Aquaculture 322–323:10–15
- Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782
- Duan YF, Liu P, Li JT, Li J, Chen P (2013a) Expression profiles of selenium dependent glutathione peroxidase and glutathione S-transferase from *Exopalaemon carinicauda* in response to *Vibrio anguillarum* and WSSV challenge. Fish Shellfish Immunol 35:661–670
- Duan YF, Liu P, Li JT, Li J, Chen P (2013b) Immune gene discovery by expressed sequence tag (EST) analysis of hemocytes in the ridgetail white prawn *Exopalaemon carinicauda*. Fish Shellfish Immunol 34: 173–182
- Duan YF, Liu P, Li JT, Wang Y, Li J, Chen P (2013c) A farnesoic acid O-methyltransferase (FAMeT) from *Exopalaemon carinicauda* is responsive to *Vibrio anguillarum* and WSSV challenge. Cell Stress Chaperones. doi:10.1007/s12192-013-0464-5
- Duo-Chuan L (2006) Review of fungal chitinases. Mycopathologia 161: 345–360
- Durand SV, Lightner DV (2002) Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. J Fish Dis 25:381–389
- Escobedo-Bonilla CM, Alday-Sanz V, Wille M, Sorgeloos P, Pensaert MB, Nauwynck HJ (2008) A review on the morphology, molecular

- characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J Fish Dis* 31:1–18
- Flach J, Pilet PE, Jollès P (1992) What's new in chitinase research? *Experientia* 48:701–716
- González-Rodríguez G, Colubi A, Gil MÁ (2012) Fuzzy data treated as functional data: a one-way ANOVA test approach. *Comput Stat Data Anal* 56:943–955
- Huang QS, Yan JH, Tang JY, Tao YM, Xie XL, Wang Y, Wei XQ, Yan QH (2010) Cloning and tissue expressions of seven chitinase family genes in *Litopenaeus vannamei*. *Fish Shellfish Immunol* 29:75–81
- Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. *J Biochem Mol Biol* 38:128–150
- Kawada M, Hachiya Y, Arihiro A, Mizoguchi E (2007) Role of mammalian chitinases in inflammatory conditions. *Keio J Med* 56:21–27
- Kramer KJ, Muthukrishnan S (1997) Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem Mol Biol* 27:887–900
- Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol* 32:1295–1309
- Lee S, Söderhäll K (2002) Early events in crustacean innate immunity. *Fish Shellfish Immunol* 12:421–437
- Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 25:697–743
- Leu JH, Yang F, Zhang X, Xu X, Kou GH, Lo CF (2009) Whispovirus. *Curr Top Microbiol Immunol* 328:197–227
- Li FH, Wang DD, Li SH, Yan H, Zhang JK, Wang B, Zhang JQ, Xiang JH (2010) A dorsal homolog (FcDorsal) in the Chinese shrimp *Fenneropenaeus chinensis* responsive to both bacteria and WSSV challenge. *Dev Comp Immunol* 34:874–883
- Li JT, Han JY, Chen P, Chang ZQ, He YY, Liu P, Wang QY, Li J (2012) Cloning of a heat shock protein 90 (HSP90) gene and expression analysis in the ridgetail white prawn *Exopalaemon carinicauda*. *Fish Shellfish Immunol* 32:1191–1197
- Lightner DV (2011) Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. *J Invertebr Pathol* 106:110–130
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25:402–408
- Mali B, Mohrlen F, Frohme M, Frank U (2004) A putative double role of a chitinase in a cnidarian: pattern formation and immunity. *Dev Comp Immunol* 28:973–981
- Okada Y, Yamaura K, Suzuki T, Itoh N, Osada M, Takahashi KG (2013) Molecular characterization and expression analysis of chitinase from the Pacific oyster *Crassostrea gigas*. *Comp Biochem Physiol B* 165:83–89
- Pan D, He N, Yang Z, Liu H, Xu X (2005) Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. *Dev Comp Immunol* 29:103–112
- Proespriawong P, Tassanakajon A, Rimphanitchayakit V (2010) Chitinases from the black tiger shrimp *Penaeus monodon*: phylogenetics, expression and activities. *Comp Biochem Physiol B* 156:86–96
- Rocha J, Garcia-Carreño FL, Muhlia-Almazán A, Peregrino-Uriarte AB, Yépiz-Plascencia G, Córdova-Murueta JH (2012) Cuticular chitin synthase and chitinase mRNA of whiteleg shrimp *Litopenaeus vannamei* during the molting cycle. *Aquaculture* 330–333:111–115
- Salma U, Uddowla MH, Kim M, Kim JM, Kim BK, Baek HJ, Park H, Mykles DL, Kim HW (2012) Five hepatopancreatic and one epidermal chitinases from a pandalid shrimp (*Pandalopsis japonica*): Cloning and effects of eyestalk ablation on gene expression. *Comp Biochem Physiol B* 161:197–207
- Söderhäll K, Smith VJ (1983) Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods. *Dev Comp Immunol* 7:229–239
- Soonthornchai W, Rungrasamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Söderhäll K, Jiravanichpaisal P (2010) Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol* 34:19–28
- Spann KM, Lester RJG (1997) Special topic review: viral diseases of penaeid shrimp with particular reference to four viruses recently found in shrimp from Queensland. *World J Microbiol Biotechnol* 13:419–426
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Tan SH, Degnan BM, Lehnert SA (2000) The *Penaeus monodon* chitinase 1 gene is differentially expressed in the hepatopancreas during the molt cycle. *Mar Biotechnol* 2:126–135
- Tian JX, Chen J, Dan J, Liao SA, Wang AL (2011) Transcriptional regulation of extracellular copper zinc superoxide dismutase from white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* and WSSV infection. *Fish Shellfish Immunol* 30:234–240
- Toranzo AE, Magarinos B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. *Aquaculture* 246:37–61
- Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E (2009) Immunity mechanisms in crustaceans. *Innate Immunol* 15:179–188
- Wang LY, Li FH, Wang B, Xiang JH (2013) A new shrimp peritrophin-like gene from *Exopalaemon carinicauda* involved in white spot syndrome virus (WSSV) infection. *Fish Shellfish Immunol* 35:840–846
- Watanabe T, Kono M, Aida K, Nagasawa H (1998) Purification and molecular cloning of a chitinase expressed in the hepatopancreas of the penaeid prawn *Penaeus japonicus*. *Biochim Biophys Acta* 1382:181–185
- Xie XX, Li HY, Xu LM, Yang F (2005) A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. *Virus Res* 108:63–67
- Xu WJ, Xie JJ, Shi H, Li CW (2010) *Hematodinium* infections in cultured ridgetail white prawns, *Exopalaemon carinicauda*, in eastern China. *Aquaculture* 300:25–31
- Zhang JQ, Sun YY, Li FH, Huang BX, Xiang JH (2010) Molecular characterization and expression analysis of chitinase (Fcchi-3) from Chinese shrimp, *Fenneropenaeus chinensis*. *Mol Biol Rep* 37:1913–1921
- Zhang YH, Feng SZ, Chen J, Qin CB, Lin HR, Li WS (2012) Stimulatory effects of chitinase on growth and immune defense of orange-spotted grouper (*Epinephelus coioides*). *Fish Shellfish Immunol* 32:844–854