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RESEARCH PAPER

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Cloning and Expression of Two Carboxylesterases, and Their Activity Modulation in Chinese Mitten crab *Eriocheir sinensis* under Pesticide Exposer

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Abstract

Carboxylesterases (*CXEs*) belong to a family of multifunctional enzymes. They metabolize drugs, environmental toxicants, and carcinogens, and inhibit bacterial pathogenesis. In this study, the full-length cDNAs of *ES-CXE3* (2,444 bp) and *ES -CXE4* (2,385 bp) were cloned from the Chinese mitten crab, *Eriocheir sinensis*. Sequence analysis showed that both *ES-CXE* sequences contained the catalytic triplet structure characteristic of the *CXEs* superfamily. Alignment and phylogenetic analyses revealed that the two *ES-CXEs* are highly similar to those of other crustaceans. Tissue specific-expression analysis showed that both *ES-CXEs* were highly expressed in the hepatopancreas. Real-time fluorescence quantitative PCR showed that the maximum expression levels of the *ES-CXE3* and *ES-CXE4* genes in the hepatopancreas of *E. sinensis* exposed to low doses of β-cypermethrin, avermectin and trichlorfon were 10×, 8×, 6× and 600×, 110×, 250× higher than relative to those of the control group, respectively, and that enzyme activities steadily increased and were significantly higher than that of the control group. Therefore, treatment with these insecticides may induce the expression of both *ES-CXEs* as well as changes in the activities of carboxylesterase family genes. Our results suggest that *ES-CXEs* might play vital roles for insecticide detoxification in *E. sinensis*

Introduction

Carboxylesterases (*CXEs*) are ubiquitous aliphatic esterases in animals, plants, and microorganisms (Jeon *et al.,* 2011). They have a catalytic triad structure and a near-*N*-terminus glycosylation site, which maintains enzyme activity and stabilizes the active sites (Zhang, 2014). Carboxylesterases belong to a superfamily of multifunctional enzymes that participate in signal transmembrane transduction, metabolic detoxification of organophosphorus insecticides and other pest control products, and lipid synthesis and decomposition (Teng and Sun, 2003; Zhang *et al.,* 2012).In insects, insect resistance to organophosphorus, carbamate, and deltamethrin is correlated with the *in vivo* enhancement of the metabolic activity of *CXEs*, achieved mainly by *CXE* gene amplification, regulation of *CXE* gene expression, and *CXE* gene mutation (Li *et al.,* 2007; Dou *et al.,* 2010; Grigoraki *et al.,* 2016).

Eriocheir sinensis is also known as the river crab and it is an economically important crustacean cultivated in China (Shen *et al.,* 2017). With the rapid increase of the *E. sinensis* aquaculture industry, numerous diseases have recently evolved (Shen *et al.,* 2015). Both abiotic and biotic stressors are intensifying in aquaculture. Numerous diseases have recently evolved and many pest control products have been used (Geng, 2010). In agricultural production, insecticides are used to kill crop pests. Pesticide residues may enter aquatic ecosystems via surface runoff, rainwater scour,

and domestic wastewater. These inputs may also pollute aquaculture water sources (Xu and Liu, 2017). Pesticide residues might therefore cause various crustacean diseases. Trichlorfon is often used as an agricultural pesticide, in order to control parasites on the surface of aquatic (Chang et al, 2010); avermectin has good effect on parasite control of shrimp and crab (Kovecses et al, 2002); Beta-cypermethrin is a commonly used pyrethroid insecticide. It is mainly used in aquaculture to clear algae in ponds and kill parasites on the surface of crustaceans (Wendt-Rasch et al, 2003). The three insecticides are less toxic to mammals, but are extremely toxic to aquatic animals (Tatjana et al, 2006). As an important detoxification enzyme, there are litter known about pesticide resistance mechanisms of *CXEs* in *Eriocheir sinensis.* Based on the study of insect *CXEs* gene in the metabolism and detoxification of pesticides and Shen et al. (2017) on the up-regulation of carboxylesterase gene expression in Chinese mitten crabs with hepatic pancreatic necrosis (HPND), the two carboxylesterase sequences obtained from the transcriptome data of the laboratory were studied for their molecular characteristics and expression patterns, which laid a foundation for studying the mechanism of metabolic detoxification.

Materials and Methods

Experimental Animals and RNA Isolation

Chinese mitten crabs were obtained from a breeding pond in Yandu District, Yancheng City, Jiangsu Province, China, and raised in a container (97 × 48 × 63 cm) equipped with a pump oxygen system to simulate their natural growth environment. The animals received commercial feed and water temperature was maintained at 20.0 ± 1.0 °C. Before the onset of the experiment, the animals were maintained in the container for 1 week to become acclimated to the environment.

Total RNAs were extracted from tissue samples using TRIzol reagent (Beijing Cwbiotech Company, Beijing, China) according to the manufacturer's instructions. While RNA integrity was verified by electrophoresis on a 1.5% agarose gel, RNA purity was quantified by reading its absorbance at 260 and 280 nm ($OD_{260/280}$). The RNAs with $OD_{260/280}$ = 1.8-2.2 were stored at −80 °C until use in subsequent analyses**.**

Cloning of Full-length *CXE* cDNAs

The full-length cDNAs of two *ES-CXEs* were obtained by rapid amplification of cDNA ends (RACE) according to the SMARTer® RACE 5' Kit User Manual and 3'-Full RACE Core Set v. 2.0 (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). Partial cDNA sequences putatively encoding *ES-CXEs* were obtained from previously collected hepatopancreas transcriptome data (Shen *et al.,* 2017). Gene-specific primers were designed using these partial cDNA sequences (Table 1). The final PCR products were purified using a gel extraction kit (XWBIO, Beijing, China), ligated into a pMD19-Tvector (TaKaRa Bio Inc.), and transformed into competent *Escherichia coli* cells. The positive transformants were selected and sequenced in both directions. The sequencing results were used to assemble full-length cDNA sequences of the *ES-CXEs*.

Bioinformatics Analysis

The open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to search for ORFs in the obtained two sequences. The NCBI basic local alignment search tool (BLAST) was used to verify the similarity of the deduced amino acid sequences of the two *CXEs* (http://blast.ncbi.nlm.nih.gov/). The molecular mass and isoelectric point (PI) of each *CXE* were estimated by the Compute PI/Mw tool (http://www.expasy.ch/tools/pi_tool.html). Amino acid multiple sequence alignment was performed using DNAMAN v. 5.2.2 and multiple sequence comparison tools (http://www.bio-soft.net/sms/). A phylogenetic tree was constructed in MEGA v. 6.0 using the maximum likelihood (ML) method, based on the putative amino acid sequences of the identified *CXEs* and other related *CXEs*, and branch support was based 1,000 bootstrap repetitions.

CXE mRNA Expression in Various Tissues

Three vigorous, healthy, mature crabs of both sexes were used to determine the tissue distributions of the identified *ES-CXEs*. Samples of the following tissues were collected from each crab for RNA isolation: hepatopancreas, gills, heart, muscle, intestine, accessory sex gland, ovary, and seminal vesicle. The tissues were freshly dissected, frozen in liquid nitrogen, and stored at −80 °C until used for total RNA extraction.

Induced *CXE* Expression and Enzyme Activity under Three Different Pesticide Treatments

Twenty -five healthy and vigorous crabs, each weighing 5.5 **±** 0.5 g, were randomly assigned to one of four treatment groups (β-cypermethrin, avermectin, trichlorfon and no pesticide added; further information on these three pesticides is given in Table 2), each with three replicates, and placed into one of 12 different containers (97 × 48 × 63 cm). The concentrations of the three aforementioned pesticides, diluted with ddH_2O, were0.002 μg/L, 0.05 g/L, and 0.001 μg/L, respectively, in each container. The concentration set for each of the insecticides was based on the safe concentration obtained from laboratory semi-lethal concentration experiments (8.52×10^{-5} μg/L for β-cypermethrin, 4.08×10^{-4} μg/L for avermectin and 5.00×10^{-4} g/L for trichlorfon). Three crabs from each container were

Table 1 Primers used in the present study

Primers	Sequence (5'-3')	Primer description
ES-CXE3-5'R1	TGCACCACCACCAGTACCACGTCGT	5' RACE primer for first round
ES-CXE3-5'R2	CTCCTGTAGCCACCACCACCATCGC	5' RACE primer for second round
ES-CXE3-3'F1	CAGAAAGAGCCAGCAGAAGGA	3' RACE primer for first round
ES-CXE3-3'F2	GTCGTAGTAACTTAGCTGGTG	3' RACE primer for second round
ES-CXE4-5'R1	TGACGGGGCTTCGGGGTCCTTGAA	5' RACE primer for first round
ES-CXE4-5'R2	CGCCCCTTCAACGTTTTCTCCAGG	5' RACE primer for second round
ES-CXE4-3'F1	CTAATCAGACATCTTGAGGC	3' RACE primer for first round
ES-CXE3-R	GAGCGTCAGGAAGGGGAAACC	RVS primer for ES-CXE3 expression
ES-CXE3-F	GAGTCATGACCTCACCACCGC	FWD primer for ES-CXE3 expression
ES-CXE4-R	GTTGGTCCAGAGCGTCGTCA	RVS primer for ES-CXE4 expression
ES-CXE4-F	GCGACCGAGAGTGGGTGAAC	FWD primer for ES-CXE4 expression
β-actin-R	CTCCTGCTTGCTGATCCACATC	RVS primer for β-actin expression
β-actin-F	GCATCCACGAGACCACTTACA	FWD primer for β-actin expression

Table 2 Information on the three tested insecticides

Insecticide	Company	Concentration	Registration number
Avermectin	Shanxi Shouai Animal Pharmaceutical industry	1%	PD20040372
Trichlorfon	Nantong Jiangshan pesticide chemical industry	90%	Veterinary drug GMP No. 04031
β-cypermethrin	Zhejiang Welda chemical industry	4.5%	PD84108-5

sacrificed at 0, 3, 6, 12, 24 and 48 h after pesticide administration. The hepatopancreas of each crab was collected for RNA isolation and *CXEs* family activity assays.

Real-time Fluorescence Quantitative PCR

The mRNA expression patterns of the cloned *ES-CXEs* in the various tissues and their expression levels at different time points after pesticide treatment were examined by real-time fluorescence quantitative PCR (qRT-PCR), using primers (Table 1) designed according to the full-length cDNAs of *ES-CXEs*. The internal reference gene *β-actin* (GenBank Accession no. HM053699.1) was used to calibrate the cDNA template. The qRT-PCR was performed with the ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The reaction conditions were as follows: denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s; and 60 °C for 1 min.

Three replicates were prepared for each sample. The comparative Ct method ($2^{-\Delta\Delta Ct}$) as described by Livak (2008) was used to calculate the relative expression of each target *CXE* gene in different tissues and in the hepatopancreas after pesticide treatment. Specificity of the amplification for all target genes and *β-actin* was confirmed by a melting curve analysis performed on SDS software (Applied Biosystems Inc., Foster City, CA, USA). Data were statistically analyzed with SPSS v. 18.0 (IBM Corp., Armonk, NY, USA) using single-factor analysis of variance (ANOVA) considering P <0.05 as the significance threshold. Data are expressed as means ± standard deviation.

***CXE* Family Activity Assays**

The activities of *CXE* family genes at different time points after pesticide treatment were determined by spectrophotometry using the Carboxylesterase Activity Assay Kit (Beijing Solarbio Company, Beijing, China) and the method measured and activity calculation was performed according to the Lai, et al (2018). Data were statistically analyzed with SPSS v. 18.0 (IBM Corp.) using single-factor ANOVA and considering P <0.05 as the significance threshold. Data are expressed as means ± standard deviation.

Statistical Analysis

The statistical analysis was performed using SPSS v. 18.0 (IBM Corp.) (* indicated P < 0.05, ** indicated P < 0.01). Data are expressed as means ± standard deviation, and the sampling points for the different treatments were analyzed by using single-factor ANOVA.

Results

cDNA Cloning of *CXEs*

Full-length of two *ES-CXE* cDNAs were isolated from the hepatopancreas of the Chinese mitten crab. Because two juvenile hormone esterase-like (JHE-like) *CXE* genes have been reported (Xu *et al.,* 2017), the two *ES-CXE* genes cloned in the present study were named *ES-CXE3* and *ES-CXE4*. Sequence analysis revealed that

the full-length cDNA *ES-CXE3* sequence obtained from the hepatopancreas of Chinese mitten crab by RACE was 2,446 bp **(**GenBank Accession No. MH201556). It consisted of a 5'-untranslated region (UTR) of 150 bp, a 3'-UTR of 526 bp with a polyadenylation signal (AATAA) and a Poly-A tail, and an ORF of 1,770 bp. This ORF encoded 589 amino acids with an estimated mass of 65.38 kDa and a predicted PI of 5.45 (Supplement Figure S1 (a)). The full-length cDNA sequence of *ES-CXE4* was 2,384 bp **(**GenBank Accession No. MH291557). It consisted of a 5'-UTR of 72 bp, a 3'-UTR of 536 bp with a polyadenylation signal (AATAA) and a Poly-A tail, and an ORF of 1,776 bp encoding 591 amino acids with an estimated mass of 65.09 kDa and a predicted PI of 4.79 (Supplement Figure S1 (b)). The amino acid identity between *ES-CXE3* and *ES-CXE4* was 74%.

Aminoacid Homology and Phylogenetic Relationships

The deduced amino acid sequences of the two *ES-CXEs* were aligned with related *CXEs* derived from several insect and crustacean species. Multiple alignments revealed that both *ES-CXEs* contained domains typical of the *CXE* family proteins (Thomas *et al.,* 1999), including three amino acid residues of the catalytic triad serine (S), glutamic acid (E), and histidine (H), RF and GG regions, and a catalytic *N*-terminus region. A carboxylesterase-specific glycine (G)×S×G, which includes the S residue of the catalytic triad, was conserved in both *ES-CXE3* and *ES-CXE4* (Figure 1). Alignment and phylogenetic analyses revealed that the amino acid identity between ES-CXE3 and *Portunus trituberculatus* was the highest, about 47.97%. The amino acid identity between *ES-CXE4* and *Portunus trituberculatus* was the highest, about 48.07%.

The evolutionary relationships between these two *ES-CXEs* and those from insects and other crustaceans were evidenced in the phylogenetic tree constructed based on the multiple amino acid sequence alignment. This phylogenetic tree showed that the two *ES-CXEs* belonged to the same crustacean *CXE* group as the JHE-like *CXE* proteins from *Pandalopsis japonica*, *Neocaridina denticulata,* and *Portunus trituberculatus* (Figure 2).

Tissue Distribution of *ES-CXEs*

Relative expression levels obtained from the qRT-PCR used to test the tissue distribution of the *ES-CXEs* (Figure 3) indicated that *ES-CXE3* was highly expressed in the hepatopancreas, muscle, testes, and accessory gonadal glands. However, its expression levels were low in the heart, gills, and ovaries. Although *ES-CXE4* was also prominently expressed in the hepatopancreas, its expression levels were nearly zero in the heart, gills, and ovaries. Generally, *ES-CXE3* expression levels were higher than those of *ES-CXE4* in the testes and accessory gonadal glands.

***ES-CXE* Expression Pattern Analysis after Pesticide Treatment**

Induction of *ES-CXE* expression was determined in the hepatopancreas following exposure to β-cypermethrin, avermectin, or trichlorfon (Figure 4). The expression levels of both *ES-CXEs* significantly increased in the hepatopancreas following pesticide treatment. Twelve hours after the β-cypermethrin treatment, *ES-CXE3* and *ES-CXE4* expression levels were 10× and 600× higher in the experimental group than in the control group, respectively. Twenty-four hours after the avermectin treatment, *ES-CXE3* and *ES-CXE4* expression levels were 8× and 110× higher in the experimental group than in the control group, respectively. Six hours after the trichlorfon treatment, *ES-CXE3* and *ES-CXE4* expression levels were 4× and 250× higher in the experimental group than in the control group, respectively.

Analysis of *CXEs* Family Activity Change Patterns after Pesticide Treatment

The enzyme activities of the *CXEs* in the hepatopancreas determined following exposure to β-cypermethrin, avermectin, or trichlorfon (Figure 5, because there was no evident change in patterns at the six time points, no data is presented for the blank group) were significantly higher than that of the control group. The highest activities of *ES-CXEs* in the hepatopancreas under the β-cypermethrin, avermectin, or trichlorfon, were 8×, 9×, and 6× that of the control group, respectively.

Discussion

In the present study, two cDNAs encoding *ES-CXEs* were cloned from *E. sinensis* in our laboratory according to a transcriptome database. Multiple alignment analysis revealed that both *ES-CXEs* contain motifs typical of the *CXE* family proteins (Thomas *et al.,* 2015; Xu *et al.,* 2017). Previous studies proposed that JHE-like *CXEs* from *P. trituberculatus*, *P. japonica*, *E. sinensis* and *N. denticulata* have esterase activity (Lee *et al.,* 2011; Sin *et al.,* 2015; Tao *et al.,* 2017; Xu *et al.,* 2017). Multiple alignment analysis indicated that the sequences of *ES-CXE3* and *ES-CXE4* resemble those of the JHE-like *CXEs*. Therefore, *ES-CXE3* and *ES-CXE4* might have esterase activity.

Based on sequence similarities and substrate specificities, insect *CXEs* with catalytic activity can be assigned to five subfamilies: α-esterases, β-esterases, JHEs, acetylcholinesterases, and integument esterases (Oakeshott *et al.,* 2005). In the present study, however, *ES-CXE3* and *ES-CXE4* genes cloned from *E. sinensis*, and JHE-like *CXEs* from other crustaceans, were classified as crustacean *CXEs*. This classification differs from existing traditional ones and suggests a new *CXE* race. According to the phylogenetic tree, crustacean *CXEs* were

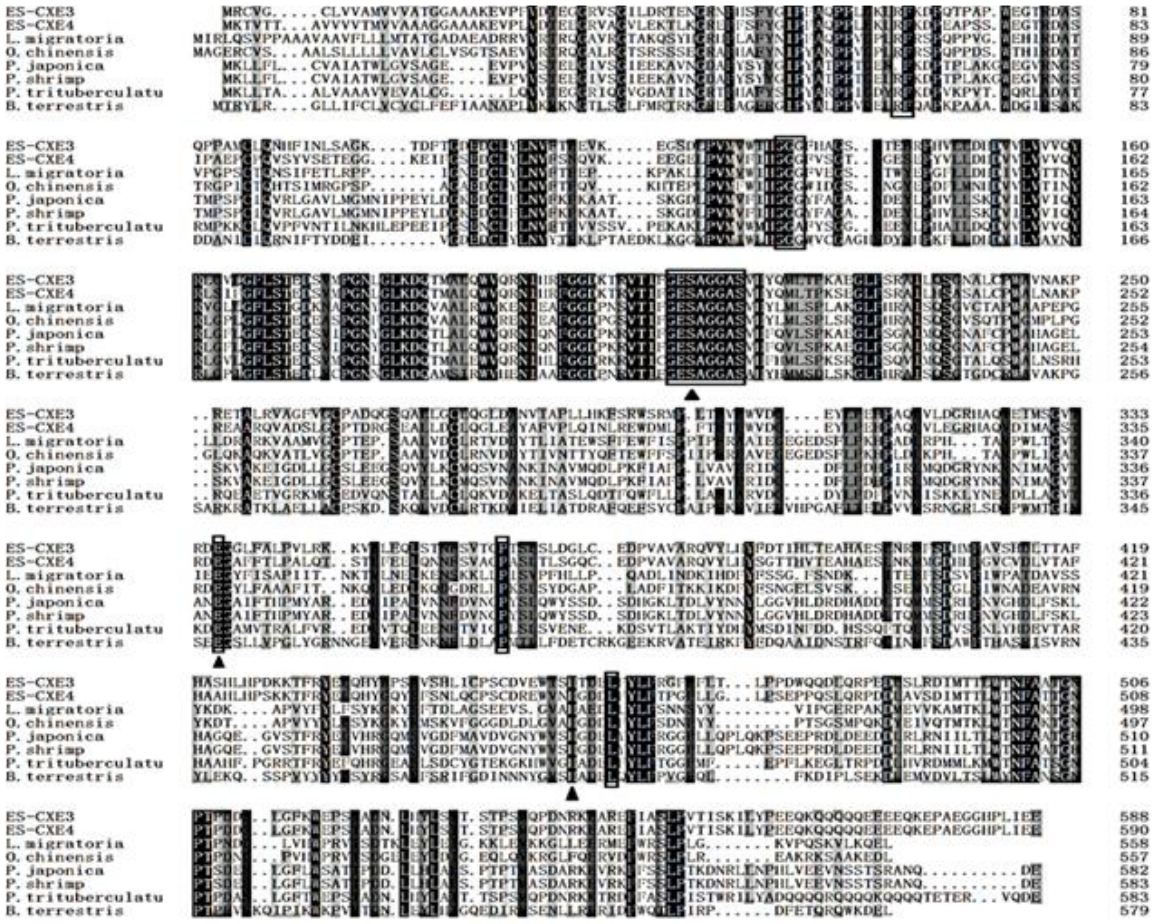

Figure 1. Multiple alignment of *ES-CXE3* and *ES-CXE4* amino acid sequences from a selection of related species. GenBank accession numbers by species: *Pandalus borealis* (HQ406776), *Portunus trituberculatus* (ALT10384.1), *Pandalopsis japonica* (ADZ9996217.1), *Locusta migratoria* (AHJ81347.1), *Oxya chinensis* (AJP62564), *Bombus terrestris* (XP003399739.1), and *Eriocheir sinensis* (MH201556, MH201557). The solid black triangle represents the residues of the catalytic triad (S, E, and H) at the bottom**.** Boxes indicate residues or motifs characteristic of carboxylesterases.

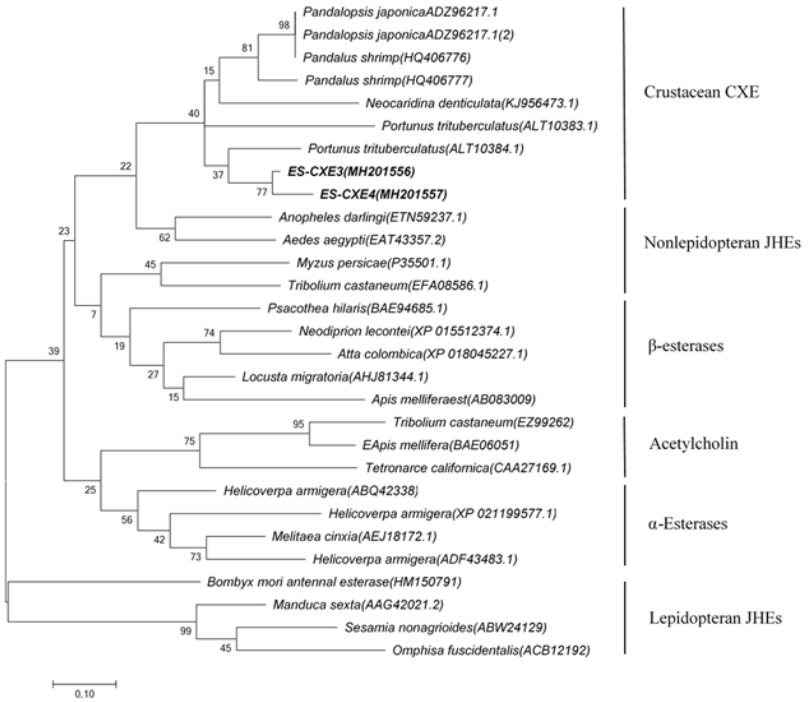

Figure 2. Phylogenetic analysis of the deduced *ES-CXEs* amino acid sequences relative to other carboxylesterases. MEGA v. 6.0 was used to construct the phylogenetic tree, based on maximum likelihood and using 1,000 bootstrap replications. GenBank accession numbers are shown in the tree.

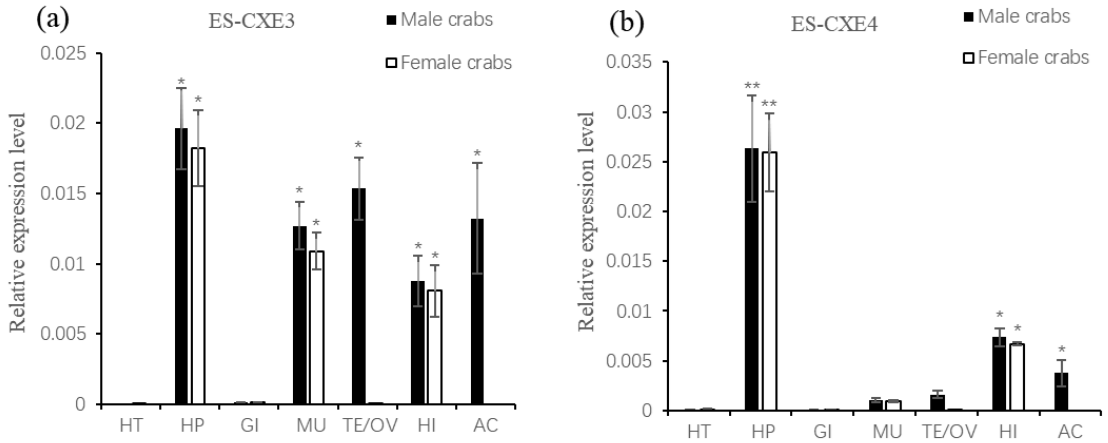

Figure 3. Tissue distribution analysis of *ES-CXE3* (a) and *ES-CXE4* (b). Relative expressions were normalized to the *β-actin* reference gene. HT, heart; HP, hepatopancreas; GI, gill; MU, muscle; TE, testis; HI, hindgut; OV, ovary; AC, accessory gonadal gland**.** Bars represent mean ± standard error of the mean (n = 3). Asterisks represent values statistically different (* *P* <0.05, ** *P* <0.01).

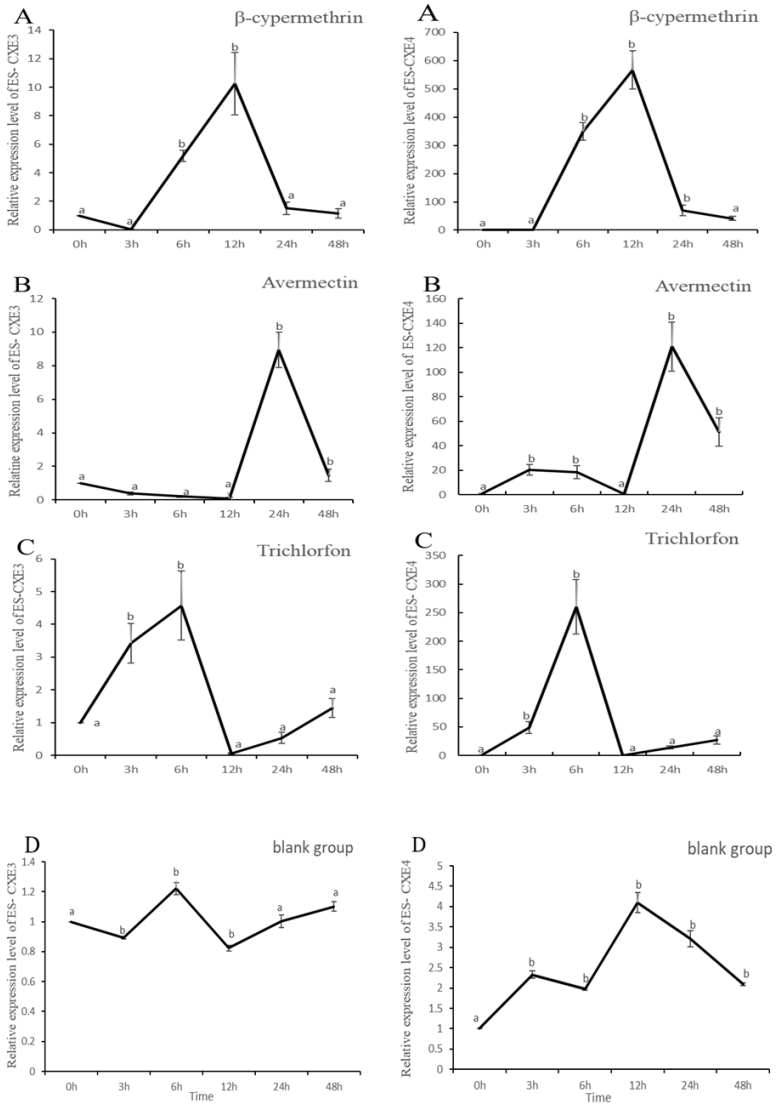

Figure 4. qRT-PCR analysis of the relative *ES-CXEs* expression levels in the hepatopancreas after treatment with β-cypermethrin (A), avermectin (B), and trichlorfon (C), blank(D) (mean ± standard error of the mean; n = 3)**.** The *β-actin* gene expression was used as an internal control. *ES-CXE* expression levels determined at the first time point were used as references. Means with different lowercase letters are significantly different (*P* <0.05).

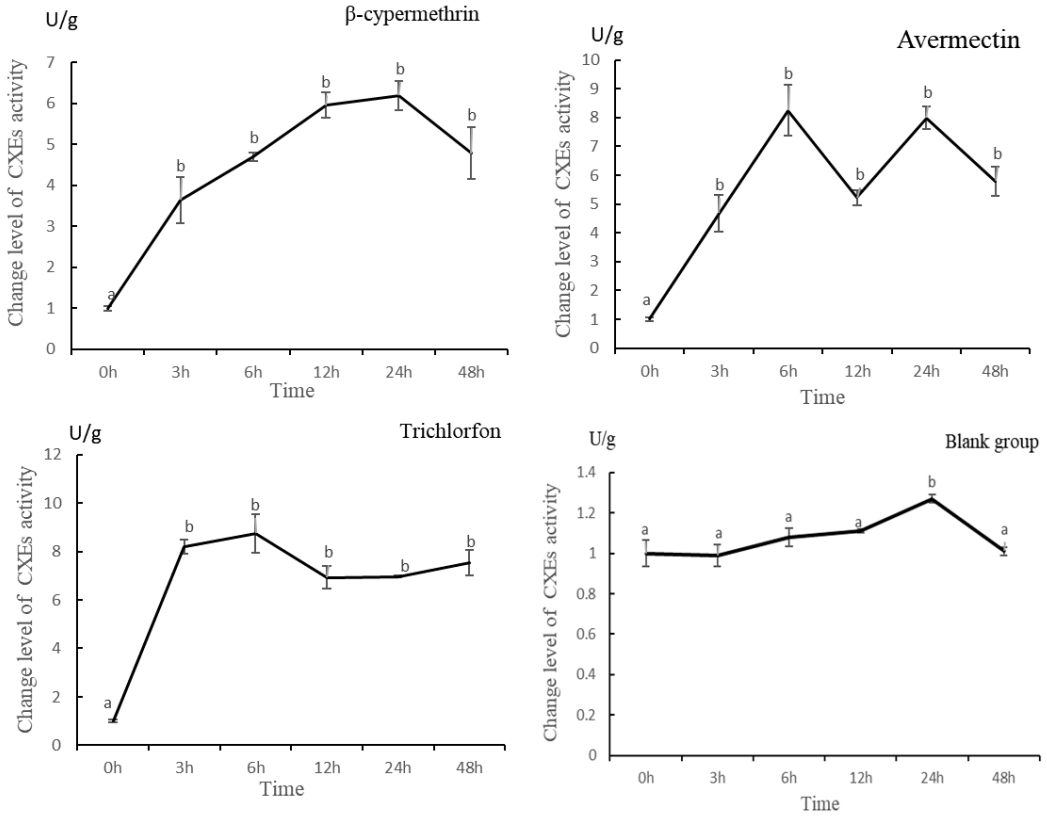

Figure 5. Carboxylesterase activity patterns in the hepatopancreas after treatment with β-cypermethrin, avermectin, trichlorfon, and blank group (mean ± standard error of the mean; n=3). Means with different lowercase letters are significantly different (P<*0.05*).

clustered with the β-esterases and non-lepidopteran JHEs from insects. Previous studies showed that β-esterases mediate the metabolism of many pesticides and other heterologous substances (Oakeshott *et al.,* 2005). In addition, *JHE* genes have been associated with the development and application of late-model insecticides (Ren *et al.,* 2014). Therefore, the two *ES-CXEs* might mediate insecticide metabolism.

Previous research has shown that the *CXEs* from *P. trituberculatus, P. japonica*, *N. denticulata*, and *E. sinensis* participate in hormone metabolism (Lee *et al.,* 2011; Tao *et al.,* 2017; Xu *et al.,* 2017). The phylogenetic tree obtained here evidenced that JHE-like *CXEs* clustered into a crustacean *CXEs* group along with the two *ES-CXEs* analyzed in the present study, but a systematic classification of crustacean *CXEs* is still needed. In addition, two full-length *ES-CXE* DNA sequences have been previously cloned and characterized (Xu *et al.,* 2017) to validate the probable function in pheromone and JH degradation. Although it is not indicated in the phylogenetic tree, all four *ES-CXEs* belong to the crustacean *ES-CXEs* group.

Studies on insect *CXEs* have revealed that lipid bodies are the main sites for protein metabolism and enzyme synthesis. The main functions of lipid bodies are energy storage and detoxification (Arrese and Soulages, 2010; Zhang, 2014). Cytochrome P450, glutathione S-transferase, and *CXEs*, the three major detoxifying enzymes in insects (Taylor and Radic, 1994), are all highly expressed in lipid bodies (Arrese and Soulages, 2010). The hepatopancreas of Crustacea resembles insect lipid bodies, as it is the main site for the metabolism of endogenous and exogenous compounds (Lima, 2013; Tao *et al.,* 2017). Therefore, the hepatopancreas might be the major tissue source of crustacean *ES-CXEs*. This hypothesis was confirmed in studies of *P. trituberculatus*, *P. japonica*, and *E. sinensis* (Ren *et al.,* 2014; Tao *et al.,* 2017; Xu *et al.,* 2017). The present study showed that the expression levels of the *ES-CXEs* were higher in the hepatopancreas than in other tissues. In *P. trituberculatus* and *P. japonica*, the ovaries also present high *CXE* expression levels (Lee *et al.,* 2011; Tao *et al.,* 2017). However, in the present study, *ES-CXE3* and *ES-CXE4* were only slightly expressed in the ovaries. Moreover, the relative expression of *ES-CXE3* was more widespread than that of *ES-CXE4*. Therefore, *ES-CXE3* might have more metabolic functions than *ES-CXE4*.

Carboxylesterase mediates insecticide resistance by increasing the hydrolysis of these substances, creating barriers, or altering their enzyme affinities (Li *et al.,* 2007; Lima, 2013). Increases in *CXE* mRNA expression levels may enhance enzyme activity and, consequently, insecticide resistance. Moreover, this higher activity of *CXEs* in the body also enhances detoxification and the metabolism of exogenous

compounds, and the resistance to insecticides (Feng *et al.,* 1999; Liu *et al.,* 2015). In the present study, the expression levels of the two *ES-CXEs* were higher in the hepatopancreas of *E. sinensis* exposed to the insecticides than in the control group, the tested insecticides induced *ES-CXE3* and *ES-CXE4* expression. Moreover, the activities of *CXEs* increased steadily with exposure time. Therefore, the two *ES-CXEs* identified in our transcriptome analysis are involved in the detoxification of three pesticides, which have metabolic detoxification effects. Comparing the relative expression level of two *ES-CXEs* and the trend of esterase activity, it can be seen that the change of expression amount shows a tendency to fluctuate up and down, while the activity of enzyme enzymes basically keeps rising, presumably due to the stimulation by insecticides. The interaction between genes, through mutual stimulation and inhibition, plays a role in continuous regulation, and the increase in the expression of *CXEs* gene will increase the detoxification enzyme activity of carboxylesterase, thus showing a continuous upward trend. Whatever, based on the changes observed on the activity of *CXEs*, these enzymes are more resistant to trichlorfon than to other insecticides**.** In addition, the expression level of *ES-CXE4* was significantly higher than that of *ES-CXE3*. Therefore, *ES-CXE4* might play a more important role in metabolic detoxification than *ES-CXE3*. Infection of the freshwater Chinese mitten crab *E. sinensis* with HPND has been a major problem in the crab-cultivation Chinese Province of Jiangsu since 2015. Hepatopancreatic injury caused by environmental toxicants is believed to be one of the main causes of HPND. However, the etiology of HPND is unknown. In our previous study (Shen *et al.,* 2017), the expression level of the *ES-CXE* gene was significantly higher in *E. sinensis* with HPND than in *E. sinensis* without HPND**,** which is in line with the results obtained in the present study. Taken together, the results of the present and previous related studies indicate that pesticide use might be associated with crab HPND during *E. sinensis* breeding.

In summary, full-length sequences of two *ES-CXE* genes from Chinese mitten crab were cloned and characterized, and tissue-specific expression levels shows both *ES-CXEs* were highly expressed in the hepatopancreas. treatment with these insecticides may induce the expression of both *ES-CXEs* as well as changes in the activities of carboxylesterase family genes. We believe that this study will provide insight on the pesticide resistance mechanisms associated with the *CXEs* in Chinese mitten crab.

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References

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. Annual Review of Entomology. 55, 207-225. http//doi:10.1146/ annurev-ento- 112408-085356

Chang, Ch. Ch., Lee, P. P., Liu, Ch. H., Cheng, W. T. (2006). Trichlorfon, an organophosphorus insecticide, depresses the immune responses and resistance to Lactococcus garvieae of the giant freshwater prawn *Macrobrachium rosenbergii*. Fish & Shellfish Immunology. 20, 574-585. http://doi:10.1016/j.fsi.2005.06.012

Dou, W., Xiao, L. S., Niu, J. Z., Jiang, H. B., &Wang, J. J. (2010). Characterization of the purified glutathione Stransferades from two psocids Liposcelis bostrychophila and L. entomophila. Agricultural Sciences in China. 9 (7), 1008-1016. http// doi: 10.1016/S1671-2927(09)60184-X

Feng, Q. L., Ladd, T. R., Tomkins, B. L., Sundaram, M., Sohi, S. S., Retnakaran, A., Davey K.G., & Palli, S. R. (1999). Spruce budworm (Choristoneura fumiferana) juvenile hor- mone esterase: hormonal regulation, developmental expression and cDNA cloning. Molecular and Cellular Endocrinology. 148 (1), 95-108. http// doi: 10.1016/S0303-7207(98)00228-7

Geng, X. (2010). Studies on detection method and toxicity of deltamethrin in river crabs, Eriocheir sinensis. (MD Thesis). Nanjing Agricultural University, Nanjing, China.

Grigoraki, L., Balabanidou, V., Meristoudis, C., Miridakis, A., Ranson, H., & Swevers, L. (2016). Functional and immunohistochemical characterization of cceae3a, a carboxyle- sterase associated with temephos resistance in the major arbovirus vectors aedes aegypti and ae. albopictus. Insect Biochemistry and Molecular Biology, 74, 61-67. http//doi:10.1016/j.ibmb.2017.05.003

Jeon, J., Kim, S., Lee, H., Cha, S., Lee, J., Yoon, S., Koo, B., Lee, C., Choi, S., & Kang, S. (2011). Novel metagenome-derived craboxylesterase that hydrolyzes β-lactam antibiotics. Applied and Environmental Microbiology. 77, 7830-7836.

Kovecses, J. (2002). Avermectins: Potential environmental risks and impacts on freshwater ecosystems in Quebec. Fibers & Polymers. 11(3), 455-463. http://doi:10.1007/s12221-010-0455-4

Lai, Ch. G., Gao, H. H., Zhai, Y. F., Chen, H., Liu, Q., Dang, H. Y., Yu, Y., & Zheng, F. Q. (2018). Effect of sublethal concentrations of three pesticides on the activity of detoxifying enzymes in *Drosophila melanogaster* and *Drosophila suzukii*. Journal of Environmental Entomology. 40(3): 676-683.

Lee, S. O., Jeon, J. M., Oh, C. W., Kim, Y. M., Kang, C. K., Lee, D. S., Mykles, D. L., & Kim, H. W. (2011). Two juvenile hormone esterase-like carboxylesterase cDNAs from a *Pandalus shrimp* (Pandalopsis japonica): cloning, tissue expression, and effects of eyestalk ablation. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 159, 148-156. http// doi: 10.1016/j.cbpb.2011.03.004 ·

Li, X., Schuler, M. A., & Berenbaum, M. R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annual Review of Entomology. 52,

231-253. http//doi:10.1146/annurev.ento.51.110104.151104

Lima, A. V. B. (2013). Characterization of esterase patterns in hepatopancreas of three species of Macrobrachium (Palaemonidae). Biochem. Syst. Ecol. 47, 132-138. http// doi: 10.1016/ j.bse.2012.10.006

Livak, K J. (2008). Analyzing real-time PCR data by the comparative Ct method. Nature Protocol, 3 (6), 1101-1108. http://www.nature.com/doifinder/10.1038/nprot.2008.73

Liu, S., Feng, M. F. & He, M. Z. (2015). Identification and expression profiles of *Cnapha- locrocis medinalis* craboxylesterase genes. Chinese Journal of Applied Entomology. 52 (3), 662-670.

Oakeshott, J. G., Claudianos, C., Campbell, P. M., Newcomb, R. D. & Russell R. J. (2005). Biochemical genetics and genomics of insect esterases. In: Gilbert, L.I., Iatrou, K., Gill, S.S.(Eds), Comp. Mol.Insect Sci. Elsevier, Oxford, pp. 309-381. http// doi: 10.1016/ B0-44-451924-6/00073-9

Ren, N. N., Xie, M., You, Y. Ch. & You M. Sh. (2014). An overview on the study of insect carboxylesterases (COEs) and the COE-mediated resistance to insecticides. Journal of Fujian Agriculture and Forestry University (Natural Science Edition). 43 (3), 337-334.

Shen, H., Ma, Y., & Hu, Y. (2015). Near-full-length genome sequence of a novel reovirus from the Chinese mitten crab, \r, *eriocheir sinensis*. Genome Announcements, 3(3), e00447-15. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4440949/

Shen, H. Sh., Zang, Y. N., Song, K., Ma, Y. Ch., Dai, T. H. & Ali S. (2017). A meta-transcriptomics survey reveals changes in the microbiota of the Chinese mitten crab *Eriocheir sinensis* infected with hepatopancreatic necrosis disease. Frontiers in Microbiology.8, 732-739. http// doi: 10.3389/ fmicb. 2017. 00732

Sin, Y. W., Kenny, N. J., Qu, Z., Chan, K. W., Cheong, S. P., Leunq, R.W., Chan, T. F., Bendena, W. G., Chu, K. H., Tobe, S. S. & Hui, J.H. (2015). Identification of putative ecdysteroid and juvenile hormone pathway genes in the shrimp *Neocaridina denticulate*. General and Comparative Endocrinology. 214, 167-176.

Tao, T., Xie, X., Liu, M., Jiang, Q., & Zhu, D. (2017). Cloning of two carboxylesterase cdnas from the swimming crab *portunus trituberculatus*: molecular evidences for their putative roles in methyl farnesotae degradation. Comparative Biochemistry & Physiology Part B Biochemistry & Molecular Biology, 203, 100-107. http//doi:10.1016/j.cbpb.2016.10.001

Tatjana, T., Nevenka, K. (2006). Abamectin in the aquatic environment. Ecotoxicology. 15(6): 495-502. http://doi:10.1007/s10646-006-0085-1

Taylor, K. P. & Radic, M. Z. (1994). The cholinesterases: from genes to proteins. Annu Rev Pharmacol Toxicol, 34(34), 281-320.http// doi:10.1146/ annurev.pa. 34. 040194. 001433

Teng, X. & Sun, M. J. (2003). Advanees on carboxylesterase researeh. Chinese Bulletiu of Life Sciences,15, 31-35.

Thomas, B., Church, W. T., & Hammock, B. (2015). Homology model of juvenile hormone esterase from the crop pest, heliothis virescens. Proteins-structure Function & Bioinformatics, 34(2), 184-196.

Wendt-Rasch, L., Pirzadeh, P., Woin, P. (2003). Effects of metsulfuron methyl and cypermethrin exposure on freshwater model ecosystems. Aquatic Toxicology. 63, 243-256. http://doi:10.1016/S0166-445X(02)00183-2 http//doi:10.1002/(SICI)1097-0134(19990201)34:2<184::AID-PROT4>3.0.CO;2-8 ·

Xu, Ch. & Liu, Y. (2017). Accumlation and elimination of deltamethrin in Megalobramaa amblycephala. Journal of Northwest A&F University (Natural Science Edition). 45(12): 31-37.

Xu, Y., Zhao, M., Deng, Y., Yang, Y., Li, X., Lu, Q., Ge, J., Pan, J. & Xu, Z. (2017). Molecular cloning, characterization and expression analysis of two juvenile hormone esterase-like carboxylesterase cdnas in Chinese mitten crab, *Eriocheir sinensis*. Comparative Biochemistry & Physiology Part B Biochemistry & Molecular Biology, 205, 46-53. https://doi.org/10.1016/j.fsi.2015.04.022

Zhang, M. W., Liu, Y. & Li, H. (2012). The research progress of Microbial Esterases. Journal of Guangdong University of Education. 32 (3): 66-71.

Zhang, Z. W. (2014). Cloning and Functional Analysis of Carboxylesterase Gene Bmbe2 in the Silkworm, Bombyx mori (MD Thesis). Chongqing University, Chongqing, China.

Zhang, J., Li, D., Ge, P., Guo, Y., Zhu, K. Y., & Ma, E., (2014). Molecular and functional characterization of cdnas putatively encoding carboxylesterases from the migratory locust, locusta migratoria. Plos One, 9(4), e94809. http//doi:10.1371/journal. pone.0094809