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-CXE*s are highly similar to those of other crustaceans. Tissue specific-expression analysis showed that both *ES-CXE*s 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-CXE*s as well as changes in the activities of carboxylesterase family genes. Our results suggest that *ES-CXE*s 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 (OD260/280). The RNAs with OD260/280 = 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 (XWBIOL, Beijing, China), ligated into a pMD19-T-vector (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 ddH2O, were 0.002 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 (8.52×10⁻³ μg/L for β-cypermethrin, 4.08×10⁻⁴ μg/L for avermectin and 5.00×10⁻⁵ g/L for trichlorfon). Three crabs from each container were
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−ΔΔCt) 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). The reaction for all target genes and CXEs was statistically analyzed by using single-factor ANOVA. The activities of CXE family genes at different time points after pesticide treatment were determined by spectrophotometry using the Carbamoylcholine Esterase 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

Table 1 Primers used in the present study

| Primers      | Sequence (5'-3')                  | Primer description      |
|--------------|-----------------------------------|-------------------------|
| ES-CXE3-S'R1 | TGACACCCCAAGACGTCGTGGTG          | 5' RACE primer for first round |
| ES-CXE3-S'R2 | CTCTGTCAGCCACACCTCGCC           | 5' RACE primer for second round |
| ES-CXE3-F1   | CAGAAAGGAGCAGAGAAGA             | 3' RACE primer for first round |
| ES-CXE3-F2   | GTCGAGTAATCTAAGTCGGT            | 3' RACE primer for second round |
| ES-CXE4-S'R1 | TACGGGCGCTGGGTCCTTGGAA          | 5' RACE primer for first round |
| ES-CXE4-S'R2 | CGACCCCTCAAGCTTCTCCAGG          | 5' RACE primer for second round |
| ES-CXE4-F1   | CTACACGACAATCTTGGAGGC          | 3' RACE primer for first round |
| ES-CXE4-F    | GACTGTCAGGAGGAGGAACCC           | 3' RACE primer for second round |
| ES-CXE4-R    | GTGGTCAGGAGTCGTCAG             | RVS primer for ES-CXE3 expression |
| ES-CXE4-R    | GCCACGGAGAGTGGGTGAAA          | FWD primer for ES-CXE3 expression |
| β-actin-R    | CTCTGTCGTCGTGATCCACATC         | RVS primer for β-actin expression |
| β-actin-F    | GCATCCACGAGACACTTACA          | 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          |

CXE Family Activity Assays

The activities of CXE family genes at different time points after pesticide treatment were determined by spectrophotometry using the Carbamoylcholine Esterase 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.
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 (AAATA) 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 (AAATA) 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)|x|S|x|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*, *Necocaridina 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× 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-CXE 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).
clustered with the β-esters and non-lepidopteran JHEs from insects. Previous studies showed that β-esters 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 (Arresse 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

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).
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 CXE gene will increase the detoxification rate 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 show 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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