ISJ 21: 25-33, 2024 ISSN 1824-307X

RESEARCH REPORT

Sequence features and expression profiles of a novel α-amylase gene from Chinese mitten crab *Eriocheir sinensis*

S-R Shao[1,2], J-J Hu[1,2,3], M-Q Wang[1,2,3*]

[1]MOE Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Qingdao 266003, China
[2]Key Laboratory of Tropical Aquatic Germplasm of Hainan Province, Sanya Oceanographic Institute, Ocean University of China, Sanya 572024, China
[3]Hainan Yazhou Bay Seed Laboratory, Sanya 572024, China

 Accepted March 8, 2024

Abstract

Amylase is the main digestive enzyme in crustaceans, and plays extremely important roles in metabolism, growth and development. Some studies have also shown that amylase may play a role in the immune defense system of aquatic organisms. In this study, a novel α-amylase gene (designated as EsAMY) was cloned and identified from Chinese mitten crab *Eriocheir sinensis*. The complete cDNA sequence of EsAMY contained a 5' untranslated region (UTR) of 39 bp, a 3' UTR of 70 bp with a polyA tail, and an open reading frame (ORF) of 1554 bp encoding a polypeptide of 517 amino acids with the predicted molecular weight of 56.9 kDa. The deduced amino acids sequence of EsAMY contained conserved cysteine site, active catalytic site, calcium binding site and chloride binding site, which was similar with previously identified α-amylase genes in other species. Quantitative real-time PCR (qPCR) analysis showed that EsAMY transcripts were detectable in all the tested tissues, with the highest mRNA expression levels in hemocytes. Both *Aeromonas hydrophila* and polybrominated diphenyl ether-47 (BDE-47) stimulation could significantly induce the mRNA expression of EsAMY gene in hemocytes, and its responses to *A. hydrophila* was more intense than those of BDE-47. These results indicate that EsAMY is a new member of the amylase family and may be involved in the immune response of Chinese mitten crab to both invasive microorganisms and external pollutant stimulation.

Key Words: α-Amylase; *Eriocheir sinensis*; innate immunity

Introduction

The nutrients required for the growth, development and reproduction of marine invertebrates are basically derived from the digestion of food or stored nutrients by digestive enzymes (Lemoine *et al*., 1997). Amylase is a general term for a class of digestive enzymes that hydrolyze glycosides in starch and glycogen. According to the different isomerism types of enzymatic hydrolysates, they are usually divided into three categories: α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and γ-amylase (EC 3.2.1.3) (Cupta *et al*., 2003). Among these categories, α-amylase is the most common type, and is an important carbohydrate hydrolase in the digestive gland of aquatic organisms (Huang *et al*., 2016). The strength of its secretory function directly affects the digestion ability of organisms to food, thus affecting other physiological processes such as growth and reproduction (Deng *et al*., 2018). Therefore, α-amylase plays an important role in carbohydrate metabolism in the digestive system of marine invertebrates. At present, α-amylase gene has been identified from a variety of marine invertebrates. For example, the α-amylase activity of a variety of liver extracts were determined in crustaceans, which demonstrated that α-amylase activity is high in some shrimps and crabs (Vanwarmhoudt *et al*., 1995). Studies on fish have revealed that the level of α-amylase activity is related to intestinal filling and nutritional conditions of animals. The level of α-amylase in fish is higher when the fish is full, and the juvenile fish is higher than the adult fish (Garuso *et al*., 2008). Based on the study on correlation among α-amylase gene polymorphism, growth traits and digestive characteristics of *Crassostrea gigas*, it was also found that α-amylase gene had a significant effect on the feeding and absorption efficiency of oysters, which in turn affected the growth of oysters (Huvet *et al*., 2008). In addition, it

Corresponding author:
Mengqiang Wang
MOE Key Laboratory of Marine Genetics and Breeding
Ocean University of China
Qingdao 266003, China
E-mail: wangmengqiang@ouc.edu.cn

was found that the polymorphism of α-amylase gene was significantly correlated with the growth of oysters and considered that the molecular markers of amylase gene had potential application value in breeding (Purdence *et al*., 2006).

In addition to its role in digesting starch components, α-amylase is also found to be involved in immunosuppression and responses to hormones (Slater *et al*., 1993). Some studies have shown that amylase can respond to the stimulation of external stress factors. For example, a study investigated the effect of environmental salinity on the expression of α-amylase in the hepatopancreas of *Neohelice granulate*, and showed that salinity acclimation had a significant effect on the expression of α-amylase and total amylase activity (Asaro *et al*., 2018). In addition, studies have shown that α-amylase is involved in the defense against pathogens by destroying the cell membrane structure of *Porphyromonas gingivalis*, which leads to cell death (Furukawa *et al*., 2013).

Eriocheir sinensis is an important marine economic species with the advantages of fast growth, strong adaptability, delicious taste and high nutritional value. With the continuous expansion of intensive farming scale, a variety of diseases caused by bacteria, viruses and other microorganisms frequently appear in crabs, causing huge economic losses (Li *et al*., 2008). Since crabs only have innate immunity, the various diseases cannot be prevented or treated by vaccines (Wang *et al*., 2016). Therefore, understanding the innate immune defense mechanisms of crabs may help develop better disease control strategies in species breeding. The intestine is one of the significant organs in the digestive system, accumulated experimental studies have shown that local intestinal immunity is an important factor in maintaining the overall health of aquatic animals. For example, studies have revealed the relationship between the gastrointestinal tract microbiota and intestinal health by activating lymphocyte T and B cells in epithelial cells, indicating that intestinal immunity is one of the crucial ways to solve the problem of aquatic diseases (Dawood, 2021). It has also been reported that the intestinal immune genes of *E. sinensis* may be involved in the regulation of intestinal bacterial community, revealing that the intestinal bacterial community in *E. sinensis* was closely related to intestinal immunity (Gai *et al*., 2009).

Therefore, this study takes amylase gene as the entry point to explore the function of intestinal genes in the innate immunity of Chinese mitten crab, in order to more effectively and deeply understand the important role of amylase genes in immune defense. The main purpose of this study is to: (1) obtain the full-length cDNA sequence of α-amylase gene (named as EsAMY) from Chinese mitten crab, and analyze its sequence characteristics, (2) investigate the tissue distribution of EsAMY mRNA transcripts and their temporal expression profiles after *Aeromonas hydrophila* or polybrominated diphenyl ether-47 (BDE-47) attacked, (3) predict the potential functions of EsAMY in the defense system of crab.

Table 1 Primer sequences used in the experiments

Primer	Sequence (5'-3')	Brief information
adaptor primer	GGCCACGCGTCGACTAGTAC	Anchor primer for RACE
adaptor primer-oligo (dT)	GGCCACGCGTCGACTAGTACT_{17}VN	cDNA synthesis for 3'-RACE
adaptor primer-oligo (dG)	GGCCACGCGTCGACTAGTACG_{10}HN	Anchor primer for 5'-RACE
EsAMY-RACE-F1	CAACTCGGGATCCGGCGATATCGAGAA	Gene specific primer for RACE
EsAMY-RACE-F2	AGATTAAGGACTACTTGAACAAGCTGA	Gene specific primer for RACE
EsAMY-RACE-R1	GAGATCTGCACGCCAGCAAAGCCTCTA	Gene specific primer for RACE
EsAMY-RACE-R2	GTCCCACTGGGCATTGGCGGACCCCAA	Gene specific primer for RACE
EsAMY-CDS-F	ATGATCCGAGTGGTCGATGCTGCG	Gene specific primer for CDS
EsAMY-CDS-R	TTACAGCTTGGAGTTGGCGTGGAT	Gene specific primer for CDS
EsEF-1α-qRT-F	CGAGGTGATGAGATTAAGGTGTG	Internal control for real-time PCR
EsEF-1α-qRT-R	CAAAGGAAATTGTTAGCGAGTGAC	Internal control for real-time PCR
EsAMY-qRT-F	GATAGTTACAACACCCTCAGCC	Gene specific primer for real-time PCR
EsAMY-qRT-R	CCACCAGTCGTTCATGTCC	Gene specific primer for real-time PCR
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer for sequencing

Materials and methods

Experimental crabs, stimulation assay, and samples collection

Healthy adult Chinese mitten crabs were collected from an aquatic farm in Yancheng, China, with an average body weight of 50 ± 5 g. They were cultured in aerated seawater at 20-25 °C for two weeks before processing. To determine the tissue distribution of EsAMY mRNA transcripts, nine tissues were collected from five untreated Chinese mitten crabs to study the tissues-specific expression of EsAMY, including gill, gonad, heart, hematopoietic tissue, hemocytes, hepatopancreas, muscle, optic stalk and stomach. To determine the immune responses of EsAMY, approximately 450 crabs were employed for the stimulation assay. The crabs were randomly divided into three groups: two experimental groups and a control group. The crabs of control group received no special treatment. The two experimental groups were immersed in *A. hydrophila* at a final concentration of 1.0×10^7 CFU/mL or BDE-47 at a final concentration of 0.8 mg/L. Samples were taken at 0 h, 3 h, 6 h, 12 h, 1 d, 2 d, 3 d, 4 d and 5 d post stimulation, with 5 replications at each time point and each replication was a mixture of 3 individuals. Hemolymph was collected from the last leg of each of the five groups of Chinese mitten crabs using a sterile syringe preloaded with equal volume of anticoagulant buffer (NaCl 510 mM, glucose 115 mM, citric acid 200 mM, trisodium citrate 30 mM and EDTA·2Na 10 mM, pH 7.3), then centrifuged at 800 × g at 4 °C for 10 minutes and immediately frozen in liquid nitrogen then stored at -80 °C until RNA isolation.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from different tissues using RNAiso Plus (9108, Takara, Japan). RNA degradation and contamination were monitored on 1.5% agarose gel. The quality and quantity of RNA was examined using NanoDrop 2000c (Thermo Fisher, USA). The first strand cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (RR470A, Takara, Japan) with RNA samples and adaptor primer-oligo (dT) as template and prime. The reactions were performed at 37 °C for 15 min, heated to 85 °C for 5 s, and then a homopolymeric tail was added using Terminal Deoxynucleotidyl Transferase (2230A, Takara, Japan).

Cloning and sequencing of EsAMY cDNA

All of the *E. sinensis* EST sequences in NCBI database were annotated using BlastX, and an EST (GenBank Accession Number FG359429) homologous to previous identified amylase genes was selected for further gene cloning (Jiang *et al*., 2009). The full-length cDNA of EsAMY was cloned by the rapid amplification of cDNA ends (RACE) technique using Premix Ex Taq Hot Start Version (RR030A, Takara, Japan). The 5'-terminal-region of EsAMY cDNA was determined by 5'-RACE, primer EsAMY-RACE-R1 were used for the first round of PCR, and EsAMY-RACE-R2 was used for the second round (Table 1). The 3'-terminal-region of EsAMY cDNA was determined by 3'-RACE, primer EsAMY-RACE-F1 were used for the first round of PCR, and EsAMY-RACE-F2 was used for the second round (Table 1). The coding sequence (CDS) of EsAMY was amplified and confirmed by two other gene-specific primers EsAMY-CDS-F/R (Table 1) using Premix Taq (LA Taq Version 2.0, RR900A, Takara, Japan). The PCR product was gel-purified, inserted into pMD-18T cloning vector (6011, Takara, Japan), and transformed into *Escherichia coli* strain DH5α (CB101, Tiangen, China). The positive recombinants were identified through anti-Amp selection and PCR screening and sequenced with M13-47 and RV-M primers. All the PCR amplification was performed in a TP600 PCR Thermal Cycler (Takara, Japan).

Bioinformatics analysis of EsAMY

The search for nucleotide and amino acid sequence homologs was conducted with Blast algorithm at National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Multiple sequence alignment and phylogenetic analysis of EsAMY and other reported amylase genes were performed using ClustalW multiple alignment program (www.genome.jp/tools-bin/clustalw) and multiple alignment show program (SMS, www.bioinformatics.org/sms/multi_align.html).
Protein motif features were predicted by the Simple Modular Architecture Research Tool (SMART, smart.embl-heidelberg.de). The presence and location of signal peptides were predicted using SignalP 4.1 (services.healthtech.dtu.dk/services/SignalP-4.1).
The calculated molecular mass and theoretical isoelectric point (pI) were predicted using the Expert Protien Analysis System (ExPASy, www.expasy.org) service. MEGA 11.0 software were used to compare the amino acid sequence and construct the unrooted Neighbor-Joining (NJ) phylogenetic tree. To obtain the confidence value of phylogenetic analysis, the Bootstrap test was repeated for 1000 times.

Quantitative real-time PCR analysis of EsAMY mRNA expression

Quantitative real-time PCR (qPCR) was used to detect the transcriptional level of EsAMY gene in different tissues of *E. sinensis* and its temporal expression profiles in hemocytes stimulated by *A. hydrophila* or BDE-47. The primers of qPCR were designed with PerlPrimer 1.1.21 software. The efficiency of each primer pair was analyzed with serial two-fold dilutions of cDNA (1, 1/2, 1/4, 1/8, 1/16 and 1/32) to determine that all pairs of primers have similar efficiency (Gu *et al*., 2023), which were 97.6% for EsEF-1α-qRT-F/R, and 98.3% for EsAMY-qRT-F/R, respectively. All qPCR reactions were performed with TB Green Premix Ex Taq (Tli RNaseH Plus) with ROX (RR420A, Takara, Japan) using about 100 ng cDNA as template and 0.2 μM of each primer (Table 1), in an ABI 7500 Real-Time PCR system (Thermo Fisher, USA). The relative mRNA expression level of EsAMY gene was normalized by elongation factor 1α (EF-1α), and obtained by comparative C_T ($2^{-\Delta\Delta Ct}$) method (Wang *et al*., 2011). All data were given in terms as mean ± S.D. (n = 5). The statistical analysis was performed by oneway analysis of variance (one-way ANOVA)

using SPSS Statistic 22.0 software. The p values less than 0.05 were considered statistically significant. Additionally, the mRNA expression profiles of EsAMY gene in hemocytes stimulated by *Vibrio anguillarum* strain M3, *Micrococcus luteus* strain 28001, or *Pichia pastoris* strain GS115 were also investigated with inventory samples we previously used for related experiments (Wang *et al*., 2015b), in order to understand whether the EsAMY gene responses correlate with the type of pathogen or with the immune activation in general.

Results

Sequence characterization of EsAMY cDNA

The cDNA sequence of EsAMY from *E. sinensis* was cloned and submitted to GenBank with the accession number KU301756 (Fig. 1A). The full length of EsAMY cDNA is 1663 bp, including a 39 bp 5'-untranslated region (UTR), a 70 bp 3' UTR and a 1554 bp open reading frame (ORF). The deduced peptide sequence of EsAMY contains 517 amino acids, with the calculated molecular weight of

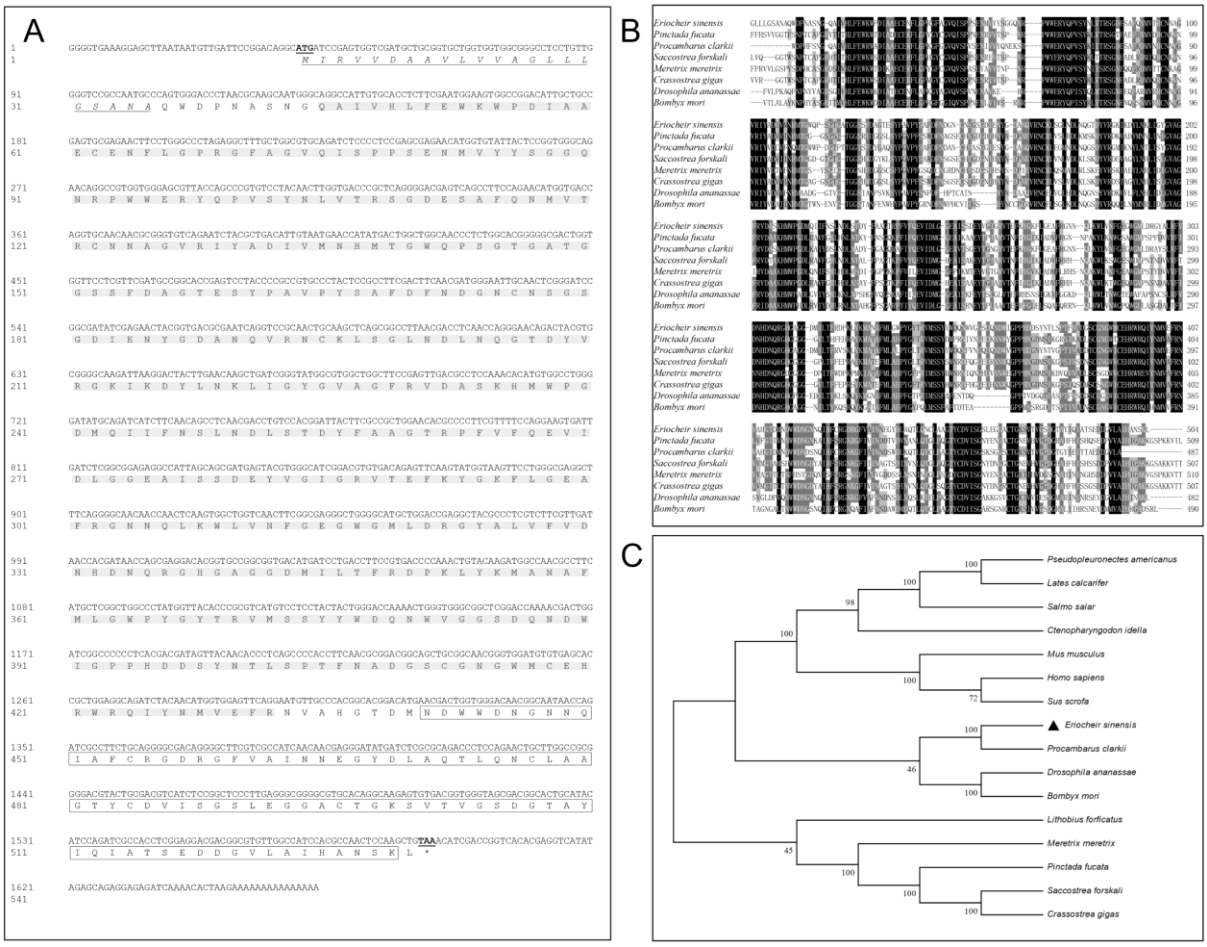

Fig. 1 Sequence features of EsAMY. A: Nucleotide and deduced amino acid sequence of EsAMY. The starting codon and the termination codon are respectively coarsened and underlined. Asterisk represents the end of protein translation. The single line part is the signal peptide. The gray shadow part is domain A, and the letter frame part is domain C. B: Multiple alignments of EsAMY with previous known amylase genes. The same amino acid residues were shaded in black, and the similar amino acids were shaded in grey. Gaps were indicated by dashes to improve the alignment. The sequences and their accession numbers are as follows: *Eriocheir sinensis* (ANG56301.1), *Pinctada fucata* (AGN55419.1), *Procambarus clarkii* (AKN79742.1), *Saccostrea forskali* (AHA11414.1), *Meretrix meretrix* (ALG64482.1), *Crassostrea gigas* (CAA69658.1), *Drosophila ananassae* (AAC35243.1), *Bombyx mori* (ACT64133.1). C: Neighbor-Joining (NJ) phylogenic tree of EsAMY constructed based on the protein sequences of amylase from different organisms. To derive confidence value for the phylogeny analysis, bootstrap trials were 1000 replicates. The black triangle indicated the amylase protein of *E. sinensis*. The numbers at the forks indicated the bootstrap value. Species and their protein accession numbers are as follows: *Pseudopleuronectes americanus* (AAF65827.1), *Lates calcarifer* (AAL84163.1), *Salmo salar* (ABD13895.1), *Ctenopharyngodon Idella* (ACX35465.1), *Mus musculus* (CAA24099.1), *Homo sapiens* (AAA52280.1), *Sus scrofa* (AAF02828.1), *Eriocheir sinensis* (ANG56301.1), *Procambarus clarkii* (AKN79742.1), *Drosophila ananassae* (AAC35243.1), *Bombyx mori* (ACT64133.1), *Lithobius forficatus* (ACA34374.1), *Meretrix meretrix* (ALG64482.1), *Pinctada fucata* (AGN55419.1), *Saccostrea forskali* (AHA11414.1), *Crassostrea gigas* (CAA69658.1)

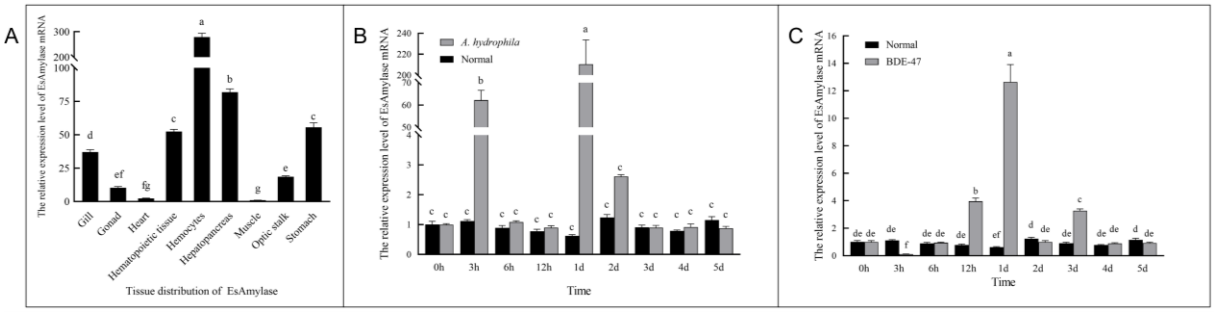

Fig. 2 Expression profiles of EaAMY. A: Relative mRNA expression level of EsAMY in different tissues. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$). B: Relative mRNA expression level of EsAMY in hemocytes challenged with *A. hydrophila*. EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$). C: Relative mRNA expression level of EsAMY in hemocytes challenged with BDE-47 (b). EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$)

56.9 kDa and the theoretical isoelectric point (pI) of 4.68. Domain analysis showed that the N-terminal of EsAMY contained a signal peptide composed of 22 amino acids. The deduced amino acid sequence of EsAMY contained a conserved A domain (from Gln32 to Arg419) and a C domain (from Asn428 to Lys516).

Multiple alignment and phylogeny relationship of EsAMY

By comparing the amino acid sequence homology of the α-amylase genes from Chinese mitten crab and other animals (Fig. 1B), it was found that EsAMY shared the highest similarity with α-amylase gene of *Procambarus clarkii* (73.7%), and the lowest with that of *Meretrix meretrix* (51.2%). In addition, a NJ phylogenetic tree based on the amino acid sequences of α-amylase from 16 different species was constructed to analyze the phylogenetic relationship of EsAMY. It was shown that EsAMY had the closest relationship with α-amylase in *P. clarkii* (Fig. 1C).

The tissue distribution of EsAMY mRNA

The tissue distribution of EsAMY mRNA in 9 different tissues of *E. sinensis* were detected by qPCR with EF-1α as internal control. EsAMY mRNA can be detected in gill, gonad, heart, hematopoietic tissue, hemocytes, hepatopancreas, muscles, optic stalk, stomach and so on (Fig. 2A). The mRNA expression level of EsAMY in hemocytes was the highest, which was 279.2-fold of that in muscle, followed by hepatopancreas (81.8-fold). The mRNA expression levels of EsAMY in stomach, hematopoietic tissue and gill were higher, which were 55.5, 52.4 and 37.1-fold of that in muscle, while the mRNA expression levels in optic stalk, gonad and heart were relatively low, and the mRNA expression level in muscle was the lowest.

The temporal mRNA expression profiles of EsAMY after challenge

The mRNA expression level of EsAMY in hemocytes significantly increased after both kinds of stimulations (Figs. 2B, 2C). The mRNA expression level of EsAMY gene in hemocytes was significantly increased at 3 h after *A. hydrophila* stimulation (increased by 62.3-fold compared with the control group, $p < 0.05$), reached the peak at 1 d (210.6-fold, $p < 0.05$), and decreased to the original level after 3 d. After BDE-47 stimulation, the mRNA expression level of EsAMY gene in hemocytes decreased first at 3 h (0.12-fold, $p < 0.05$), then significantly increased at 12 h (4.0-fold, $p<0.05$), and reached the highest level at 1 d (12.7-fold, $p<0.05$), and decreased to the original level after 4 d. While in the control group, the expression of EsAMY mRNA did not change significantly throughout the experiment. Moreover, the mRNA expression profiles of EsAMY gene in hemocytes stimulated by *V. anguillarum* strain M3, *M. luteus* strain 28001, or *P. pastoris* strain GS115 were also investigated with inventory samples we previously used for related experiments, and the mRNA expression profiles of EsAMY gene were similar with each other during the stimulation of Gram-negative bacteria, Gram-positive bacteria, or fungus (Fig. 3).

Discussion

Amylase is the main digestive enzyme in crustaceans, and plays extremely important roles in their metabolism, growth, and development (Peng *et al*., 2015). Previous studies on amylase have mostly concluded that the activity of amylase has a significant effect on the digestion and absorption efficiency of nutrients, which in turn affects the growth and development of the body (Darias *et al*., 2006; Thongsaikliang *et al*., 2014). Moreover, recent

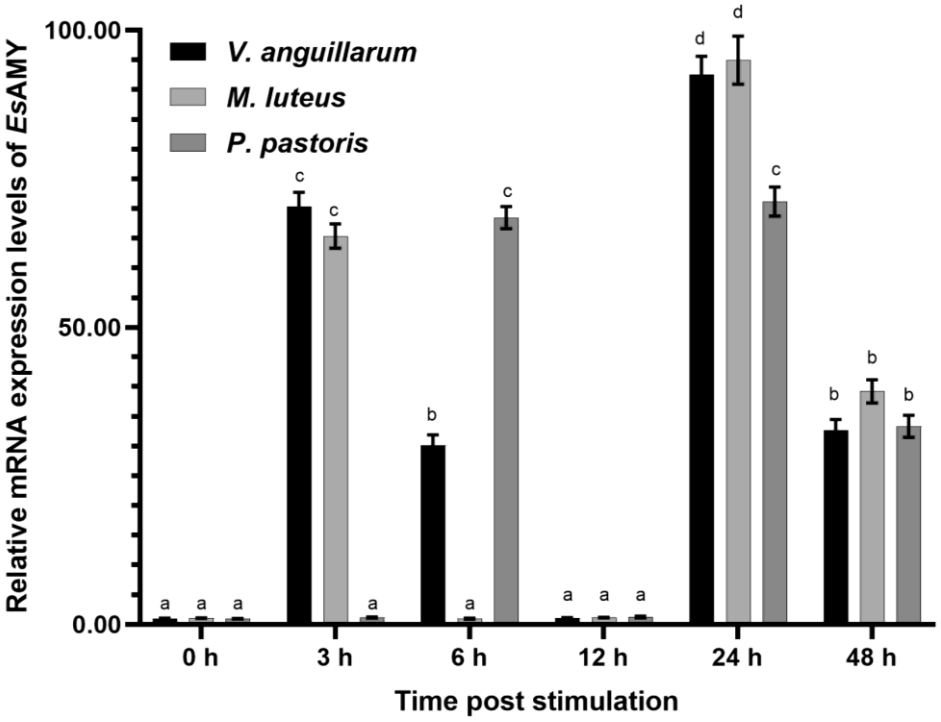

Fig. 3 Relative mRNA expression levels of EsAMY in hemocytes challenged with *V. anguillarum*, *M. luteus*, or *P. pastoris*. EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and bars with different characters were significantly different ($p < 0.05$)

studies have shown that some amylases have been highlighted to show antibacterial properties against bacteria. For example, α-amylase has the ability to bind lipopolysaccharide, and functional assays show that α-amylase can interfere with bacterial adhesion and biofilm formation of *Aggregatibacter actinomycetemcomitans* in the oral cavity (Baik., 2013). So, it can be inferred that amylase genes are likely to play a role in immune defense system of organism.

The full-length cDNA sequence of EsAMY contained two typical amylase domains, domain A and domain C. The signal peptide prediction software showed that it contained a signal peptide with 20 amino acid residues, so it is speculated that it may be a secreted protein (Vonheijne, 1990). By comparing with the α-amylase proteins of other animals, the cysteine sites, active catalytic sites, calcium binding sites and chloride binding sites in the amino acids encoded by the gene are very conserved among species. By analyzing the homology of the amino acid sequence of EsAMY with other species, it was found that the sequence similarity of amylase between *E. sinensis* and *P. clarkii* was the highest, and the molecular phylogenetic analysis showed that it had the closest relationship with *P. clarkii* and the farthest relationship with mammals, which further confirming that EsAMY belongs to the amylase family.

It has been previously reported that amylase is expressed in almost all the examined tissues in *Hyriopsis cumingii* (Ren *et al*., 2014) and *Siganus canaliculatus* (Xie *et al*., 2016), indicating that amylases may be involved in different biological processes. The study also showed that the hepatopancreas was the tissue with the highest mRNA expression level of amylase in Atlantic salmon *Salmo salar* (Froystad *et al*., 2006) and *Haliotis discus discus* (Nikapitiya *et al*., 2009). Consistent with these reports, EsAMY was expressed in each tested tissue. The widespread presence of EsAMY gene transcripts indicates that it may be involved in a variety of physiological responses of *E. sinensis* and plays an important role in its basic metabolism. The mRNA expression level of EsAMY in hemocytes was the highest, followed by hepatopancreas and stomach. In contrast to vertebrates, crustaceans have no independent immune organs. Hepatopancreas is the main organ of immunity and detoxification (Sun *et al*., 2022), and the hemocytes in invertebrate play an important role in various innate immune responses, especially in phagocytosis (Wang *et al*., 2015a). And the stomach is the main digestive organ of crustaceans (Huvet *et al*., 2012). These results suggest that it may also be an important regulatory factor in the innate immune response and digestive system of *E. sinensis*.

Besides the functions in digestion, more and more evidence showed that amylase genes might have important immune functions in organisms. In previous reports, some amylases were emphasized to be involved in the defense against pathogens, such as pentaamine and α-amylase could destroy the cell membrane structure of *P. gingivalis* cells,

and lead to bacterial cell death (Craigen *et al*., 2011). It has been reported that α-amylase could inhibit pathogens, such as *Staphylococcus aureus, Vibrio cholerae and Pseudomonas aeruginosa*, to form biofilms and it can also be effective in degradation of mature biofilm by disrupting the exopolysaccharide, so it could be used as a biofilm inhibitor in clinical applications (Lequette *et al*., 2010). In order to further investigate the role of EsAMY in immune response, we used qPCR to detect the temporal expression profiles of EsAMY mRNA in hemocytes after stimulation with *A. hydrophila* or BDE-47. Many previous studies have shown that *A. hydrophila* is one of the most common pathogenic bacteria in Chinese mitten crab (Zhu *et al*, 2002). We found that the mRNA expression of EsAMY in hemocytes increased significantly at 3 h after *A. hydrophila* stimulation, reached the maximum at 1 d, and returned to the original level at 3 d. In addition, BDE-47 is also a common source of water pollutants in recent years (Gong *et al*., 2023; Jian *et al*., 2017). After adding the pollutant BDE-47 to the water for environmental stimulation, the mRNA expression level of EsAMY gene in hemocytes decreased first at 3 h, then increased significantly at 12 h, and reached the highest level at 1 d, and returned to the original level at about 4 d. We found that the mRNA expression level of EsAMY in hemocytes increased after microbial and pollutant stimulation, further confirming the important role of EsAMY in bacterial infection and environmental stimulation. By comparing the mRNA expression of EsAMY in hemocytes, it was found that *A. hydrophila* and BDE-47 could significantly induce the expression of EsAMY gene in hemocytes, and its responses to *A. hydrophila* was more intense than those of BDE-47. This indicates that the amylase gene may be more inclined to respond to the invasion of foreign pathogens to organisms than changes in environmental conditions. It has been reported that the mRNA expression level of α-amylase B gene was significantly increased in the digestive gland of the pacific oyster *Crassostrea gigas* fed with *Alexandrium tamarense*, indicating that this toxic dinoflagellate seemed to affect the transcriptional expression of α-amylase B gene and prevent the synthesis of corresponding proteins, which means that α-amylase B gene may be involved in the digestive system of oyster and the immune response to foreign toxins (Rolland *et al*., 2012). The large amount of the mRNA expression of amylase genes in this experiment may be due to the increased demand for amylase in *E. sinensis*, which plays a protective role against external factors such as microbial invasion and environmental organic pollution, suggesting that EsAMY has an important immunomodulatory effect on *E. sinensis*. Additionally, it was reported that some immune related genes responded correlate with the type of pathogens, while the others responded correlate with the immune activation in general (Wang *et al*., 2018; Yang *et al*., 2023; Zheng *et al*., 2023). In the present research, the mRNA expression profiles of EsAMY gene in hemocytes stimulated by *V. anguillarum*, *M. luteus*, or *P. pastoris* were also investigated with inventory samples we previously used for related experiments, and the mRNA expression profiles of EsAMY gene were like each other during different stimulation, indicating the EsAMY gene response with the immune activation in general.

In summary, the full-length cDNAs of EsAMY were cloned and identified from *E. sinensis* in this study. Tissue expression analysis showed that the amylase gene was expressed in all tested tissues of Chinese mitten crab, and was highly expressed in hepatopancreas, hemocytes and stomach. After stimulation with *A. hydrophila* or BDE-47, the mRNA expression level of EsAMY gene significantly increased. Combined with previous studies, our study further confirmed that EsAMY is a typical member of the amylase family, and it is also an important regulatory factor in the innate immune responses of Chinese mitten crab.

Acknowledgement

This work was supported by the Project of Sanya Yazhou Bay Science and Technology City (SKJC-2023-01-004), the Hainan Provincial Joint Project of Sanya Yazhou Bay Science and Technology City (320LH062), and the Fundamental Research Funds for the Central Universities (202112019).

Reference

Asaro A, Martos-Sitcha J, Martinez-Rodriguez G, Mancera J, Mananes A. *In silico* analysis and effects of environmental salinity in the expression and activity of digestive alpha-amylase and trypsins from the euryhaline crab *Neohelice granulata*. C J Zool. 96: 127-139, 2018.

Baik J, Hong S, Choi S, Jeon J, Park O, Cho K, *et al*. Alpha-amylase is a human salivary protein with affinity to lipopolysaccharide of *Aggregatibacter actinomycetemcomitans*. Mol Oral Microbiol. 28: 142-153, 2013.

Caruso G, Denaro M, Genovese L. Temporal changes in digestive enzyme activities in the gastrointestinal tract of European eel (*Anguilla anguilla*) (Linneo 1758) following feeding. Mar Freshw Behav Physiol. 41: 215-228, 2008.

Craigen B, Dashiff A, Kadouri D. The Use of Commercially Available Alpha-Amylase Compounds to Inhibit and Remove *Staphylococcus aureus* Biofilms. Open Microbiol J. 5: 21-31, 2011.

Darias M, Murray H, Gallant J, Astola A, Douglas S, Yufera M, *et al*. Characterization of a partial alpha-amylase clone from red porgy (*Pagrus pagrus*): expression during larval development. Comp Biochem Physiol B Biochem Mol Biol. 143: 209-218, 2006.

Dawood M. Nutritional immunity of fish intestines: important insights for sustainable aquaculture. Rev Aquac. 13: 642-663, 2021.

Dong Y, Gao X, Sheng W, Yao H, Lin Z. Characteristics of α-Amylase gene and its association with growth traits in *Meretrix meretrix*. Invertebr Surviv J. 15: 94-103, 2018.

Froystad M, Lilleeng E, Sundby A, Krogdahl A. Cloning and characterization of alpha-amylase from Atlantic salmon (*Salmo salar* L.). Comp Biochem Physiol A Mol Integr Physiol. 145: 479-492, 2006.

Furukawa A, Nakada-Tsukui K, Nozaki T. Cysteine Protease-Binding Protein Family 6 Mediates the Trafficking of Amylases to Phagosomes in the Enteric Protozoan *Entamoeba histolytica*. Infect Immun. 81: 1820-1829, 2013.

Gai Y, Wang L, Zhao J, Qiu L, Song L, Li L, *et al*. The construction of a cDNA library enriched for immune genes and the analysis of 7535 ESTs from Chinese mitten crab *Eriocheir sinensis*. Fish Shellfish Immunol. 27: 684-694, 2009.

Gong X, Guo Y, Hu J, Bao Z, Wang M. Molecular cloning and characterization of a thioredoxin-like protein gene in rotifer *Brachionus plicatilis*. Dev Comp Immunol. 140: 104615, 2023.

Gu Y, Qu Z, Hu J, Wang M. Sequence characterization and expression analysis of a PDGF/VEGF related factor gene in swimming crab *Portunus trituberculatus*. Invertebr Surviv J. 21: 1-8, 2024.

Gupta R, Gigras P, Mohapatra H, Goswami V, Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochem. 38: 1599-1616, 2003.

Huang G, Guo Y, Li L, Fan S, Yu Z, Yu D. Genomic structure of the alpha-amylase gene in the pearl oyster *Pinctada fucata* and its expression in response to salinity and food concentration. Gene 587: 98-105, 2016.

Huvet A, Jeffroy F, Daniel JY, Quere C, Le Souchu P, Van Wormhoudt A, *et al*. Starch supplementation modulates amylase enzymatic properties and amylase B mRNA level in the digestive gland of the Pacific oyster *Crassostrea gigas*. Comp Biochem Physiol B-Biochem Mol Biol. 163: 96-100, 2012.

Huvet A, Jeffroy F, Fabioux C, Daniel J, Quillien V, Van Wormhoudt A, *et al*. Association among growth, food consumption-related traits and amylase gene polymorphism in the Pacific oyster *Crassostrea gigas*. Anim Genet. 39: 662-665, 2008.

Jian X, Tang X, Xu N, Sha J, Wang Y. Responses of the rotifer *Brachionus plicatilis* to flame retardant (BDE-47) stress. Mar Pollut Bull. 116: 298-306, 2017.

Jiang H, Cai Y, Chen L, Zhang X, Hu S, Wang Q. Functional annotation and analysis of expressed sequence tags from the hepatopancreas of mitten crab (*Eriocheir sinensis*). Mar Biotechnol. 11: 317-326, 2009.

LeMoine S, Sellos D, Moal J, Daniel J, Serrano F, Samain J, *et al*. Amylase in *Pecten maximus* (Mollusca, bivalves): Protein and cDNA characterization; quantification of the expression in the digestive gland. Mol Mar Biol Biotech. 6: 228-237, 1997.

Lequette Y, Boels G, Clarisse M, Faille C. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling 26: 753-753, 2010.

Li C, Zhao J, Song L, Mu C, Zhang H, Gai Y, *et al*. Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab *Eriocheir sinensis*. Dev Comp Immunol. 32: 784-794, 2008.

Nikapitiya C, Oh C, Whang I, Kim C, Lee Y, Kim S, *et al*. Molecular characterization, gene expression analysis and biochemical properties of alpha-amylase from the disk abalone, *Haliotis discus discus*. Comp Biochem Physiol B Biochem Mol Biol. 152: 271-281, 2009.

Peng T, Wang D, Yu Y, Liu C, Zhu B. Identification and expression of an ecdysteroid-responsive amylase from red crayfish *Procambarus clarkii*. Fish Sci. 81: 345-352, 2015.

Prudence M, Moal J, Boudry P, Daniel J, Quere C, Jeffroy F, *et al*. An amylase gene polymorphism is associated with growth differences in the Pacific cupped oyster *Crassostrea gigas*. Anim Genet. 37: 348-351, 2006.

Ren G, Tang J, Wang Y. Molecular characterization and expression pattern of an alpha-amylase gene (HcAmy) from the freshwater pearl mussel, *Hyriopsis cumingii*. Genet Mol Res. 13: 6653-6664, 2014.

Rolland J, Pelletier K, Masseret E, Rieuvilleneuve F, Savar V, Santini A, *et al*. Paralytic toxins accumulation and tissue expression of alpha-amylase and lipase genes in the Pacific oyster *Crassostrea gigas* fed with the neurotoxic dinoflagellate *Alexandrium catenella*. Mar Drugs. 10: 2519-2534, 2012.

Slater E, Hesse H, Muller J, Beato M. Glucocorticoid receptor binding site in the mouse alpha-amylase 2 gene mediates response to the hormone. Mol Endocrinol. 7: 907-914, 1993.

Sun J, Wang Y, Hu F, Hu J, Wang M. Comparative study of two novel extracellular copper/zinc superoxide dismutase (Cu-Zn SOD) genes from white shrimp *Litopenaeus vannamei*. Invertebr Surviv J. 19: 126-135, 2022.

Thongsaiklaing T, Sehawong W, Kubera A, Ngernsiri L. Analysis of the α-amylase gene sequence and the enzyme activity of Indian rock oyster *Saccostrea forskali*. Fish Sci. 80: 589-601, 2014.

Vanwormhoudt A, Bourreau G, Lemoullac G. Amylase polymorphism in crustacea decapoda-electrophoretic and immunological studies. Biochem Syst Ecol. 23: 139-149, 1995.

Vonheijne G. The Signal Peptide. J Membr Biol. 115: 195-201, 1990.

Wang M, Wang L, Guo Y, Sun R, Yue F, Yi Q, *et al*. The broad pattern recognition spectrum of the Toll-like receptor in mollusk Zhikong scallop *Chlamys farreri*. Dev Comp Immunol. 52: 192-201, 2015a.

Wang M, Wang L, Jia Z, Yi Q, Song L. The various components implied the diversified Toll-like receptor (TLR) signaling pathway in mollusk *Chlamys farreri*. Fish Shellfish Immunol. 74: 205-212, 2018.

Wang M, Wang L, Yi Q, Gai Y, Song L. Molecular cloning and characterization of a cytoplasmic manganese superoxide dismutase and a mitochondrial manganese superoxide dismutase from Chinese mitten crab *Eriocheir sinensis*. Fish Shellfish Immunol. 47: 407-417, 2015b.

Wang M, Wang L, Huang M, Yi Q, Guo Y, Gai Y, *et al*. A galectin from *Eriocheir sinensis* functions as pattern recognition receptor enhancing microbe agglutination and haemocytes encapsulation. Fish Shellfish Immunol. 55: 10-20, 2016.

Wang M, Yang J, Zhou Z, Qiu L, Wang L, Zhang H, *et al*. A primitive Toll-like receptor signaling pathway in mollusk Zhikong scallop *Chlamys farreri*. Dev Comp Immunol. 35: 511-520, 2011.

Xie D, Xu S, Wang S, You C, Li Y. Cloning, tissue expression, and nutritional regulation of the α-amylase gene in the herbivorous marine teleost *Siganus canaliculatus*. Aquaculture 454: 229-236, 2016.

Yang J, Guo Y, Hu J, Bao Z, Wang M. A metallothionein gene from hard clam *Meretrix meretrix*: Sequence features, expression patterns, and metal tolerance activities. Dev Comp Immunol. 149: 105057, 2023.

Zheng B, Wang Y, Hu J, Bao Z, Wang M. Comparative analysis of two cathepsin L genes in Asiatic hard clam (*Meretrix meretrix*): Similar in sequence features, different in expression profiles. Fish Shellfish Immunol. 133: 108527, 2023.

Zhu Y, Cao G, Xue R, Wei Y, Gong C. The characteristic of *Aeromonas hydrophial* from *Eriocheir sinensis*. Chin J Microecol. 14: 25, 2002.