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Vitellogenin regulates antimicrobial responses in Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

Vitellogenin (Vtg) is traditionally regarded as a key supplier of nutrients and energy during the early development of embryos and larvae, but accumulating evidence suggests that Vtg is also involved in innate immune defense. Whether Vtg is involved in innate immunity in *Eriocheir sinensis*, and its functions, remain largely unknown. In this study, a cDNA representing the vitellogenin1 gene from *E. sinensis* (*Es-vtg1*) was cloned. The full-length *Es-vtg1* cDNA comprised 7939 nucleotides, encoding an open-reading frame of 2567 amino acid residues. Bioinformatic analysis showed that the domains of *Es*-Vtg1 have been conserved during evolution. Quantitative real-time PCR and western blotting showed that *Es-vtg1* was highly expressed in ovary and hepatopancreas. Moreover, bacteria could induce the high-level expression of *Es*-Vtg1. *Es*-Vtg1 plays important roles in immunological defense, including binding to bacteria, inhibiting bacterial proliferation, and regulating the expression of antimicrobial peptides. Collectively, these results demonstrate that *Es*-Vtg1 plays critical roles in antimicrobial function.

1. Introduction

Vitellogenin (Vtg), the precursor of vitellin, which functions as a major supplier of necessary nutrients and energy during the early development of embryos and larvae [1], is found in most oviparous animals including insects, crustaceans, fish, etc. [2–6]. Vtg was first reported in insects as one of the vitellogenic blood proteins [7]; it is a phospholipoglycoprotein with different subunits and ranges in molecular weight from 300 to 600 kDa [8]. Different isoforms of the *vtg* gene are found in vertebrates [9–13] and invertebrates [14,15], and its expression profile varies, for example in the fat bodies of insects [16,17], the liver of most vertebrates [18–20], and the ovary and hepatopancreas of crustaceans [21–23], which suggests potentially different functions. Intriguingly, in recent years, Vtgs have also been found to be involved in immune responses in different species, especially in innate immune defense in crustaceans.

Initially, the vitellogenin superfamily was reported to be involved in defense reactions in sea urchin [24], then Vtg was found to have hemagglutinating and antimicrobial activities in the protochordate amphioxus *Branchiostoma japonicum* [25]. Meanwhile, Vtg was found to counter the infectivity of infectious pancreatic necrosis virus [26]. Later, it was reported that Vtg could bind to *Escherichia coli*, *Staphylococcus aureus*, *Pichia pastoris* and microbial pathogen-associated molecular patterns (PAMPs), after which Vtg could enhance phagocytosis by opsonization and disrupt bacterial cell walls [27,28]. Furthermore, it was shown that injection of lipopolysaccharide or lipoteichoic acids (LTA) was able to upregulate the expression of Vtg at both the transcriptional and translational levels in zebrafish, and Vtg was shown to be able to bind to bacteria and inhibit their growth in a dose-dependent manner [29].

Chinese mitten crab, *Eriocheir sinensis*, an important economic aquatic animal in southeast Asia [30], is threatened by frequent outbreaks of diseases that lead to decreased production and economic loss [31]. Some of these diseases are caused by bacteria, and studies of immunological host defense have revealed that antimicrobial peptides (AMPs) are essential factors in immune reactions of crustaceans [32,33]. In the present work, the full-length vitellogenin cDNA from *E. sinensis* was obtained and named *E. sinensis-vitellogenin1 gene* (*Es-vtg1*), and the levels of transcription and translation were determined. In addition, we

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undertook bioinformatic analysis, and explored the expression patterns of *Es-vtg1* on pathogen challenge and the antibacterial functions of *Es*-Vtg1 in innate immune defense, including regulation of AMPs.

2. Materials and methods

2.1. Experimental animals and sample preparation

Healthy adult female Chinese mitten crabs were purchased from the local agricultural market in Shanghai, China. Crabs were acclimated for 7 days at room temperature in freshwater. All the animal experiments in this study were approved by the Ethics Committee of Laboratory Animal Experimentation at East China Normal University. Before dissection, crabs were anesthetized in ice for about 10 min. Tissues including heart, muscle, stomach, intestine, gill, hepatopancreas, hemocytes and ovary were collected and frozen quickly in liquid nitrogen, and stored at −80 °C. *E. sinensis* primary hemocytes were cultured and collected according to a previous study [34].

2.2. RNA extraction and cloning of full-length cDNA

Total RNA was extracted from *E. sinensis* using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and quality of the total RNA were detected using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose-gel electrophoresis, respectively. For first-strand cDNA synthesis, 5 μg total RNA was reverse transcribed using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's protocols. Meanwhile, for cloning the full-length *Es-vtg1* gene, 5 μg total RNA from ovary was extracted and reverse transcribed using a SMARTer® RACE 5′/3′ Kit (Clontech, Japan) following the instructions of the manufacturer. Gene-specific primers for 3′- and 5′- RACE were designed based on the *E. sinensis vtg* expressed sequence tag and synthesized by Sangon (Shanghai, China) (Table 1). The PCR conditions were: initial denaturation at 94 °C for 4 min; followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and elongation at 72 °C for 2 min; with a final extension at 72 °C for 7 min. The products of PCR and RACE-PCR were purified and inserted into the pEASY-T1 vector (TransGen, China) for sequencing. Subsequently, the full-length cDNA sequence of *Es-vtg1* was assembled using DNAMAN software and submitted to GenBank with accession number MF043589.

Table 1
Primer sequences used in this study.

Primer	Sequence (5′-3′)	Direction
cDNA cloning		
5′In-vtg1	AGCATGAGGGCTGTGCGGTTGGTC	reverse
5′Ou-vtg1	TGGAGCAACGAGGAACGGCATCT	reverse
3′In-vtg1	TCAAGCCCAACATGGAAGTGAA	forward
3′Ou-vtg1	GAAACCTACTATGCGTGGATGCC	forward
Vtg1-1 F	GATGCCGTTCCTCGTTGC	forward
Vtg1-1 R	AATGTTTGTCAGCAGGGAGC	reverse
Vtg1-2 F	ACAAACATTCTCATTCCCGATA	forward
Vtg1-2 R	GCTCAGGTGTTTTAATGAAAGT	reverse
Vtg1-3 F	AAGGGCCTGTTACTTTCATTA	forward
Vtg1-3 R	TCCACGTCGCGGAACGGGGAC	reverse
Vtg1-4 F	ACTCCTTCAGGATTGCGTC	forward
Vtg1-4 R	CGGTCTTGATCAGCTGGTA	reverse
Vtg1-5 F	CCAACGAACAGTCTGGTAC	forward
Vtg1-5 R	CGGGGCTTATGGAAGGAGT	reverse
qRT-PCR		
Vtg1 F	CACCTCCGCCTATGATGAC	forward
Vtg1 R	TTCGGGTAGACCTTGATGC	reverse
β-actin F	GCGAGAAATCGTGCGAGACAT	forward
β-actin R	CCGAGGAAGGAAGGCTGGAAGAG	reverse
Prokaryotic expression		
Vtg1 OF	CGGAATTC ATCCCCTCCAACAAGA	forward
Vtg1 OR	CCGCTCGAG**TTA**GAGGTTTGTGGCGTTC	reverse
RNAi		
siVTG F	GCAGGCUACUUCAAGAAAUTT	forward
siVTG R	AUUUCUUGAAGUAGCCUGCTT	reverse

Note: *Eco*RI and *Xho*I restriction sites are marked by underlining; the stop codon is in bold.

2.3. Bioinformatic analysis of Es-vtg1

The obtained sequence was analyzed against the NCBI database using the online search tool BLASTX (http://www.ncbi.nlm.nih.gov/) and the open reading frame (ORF) was identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Tools at SMART (http://smart.embl-heidelberg.de/) and ExPASy (http://prosite.expasy.org/prosite.html/) were used to predict the structure and functional domains of *Es*-Vtg1. Meanwhile, the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of signal peptide cleavage sites in *Es*-Vtg1. Multiple sequence alignment of *Es*-Vtg1 was performed using the ClustalX 2.0 program and DNAMAN software. A phylogenetic tree based on Vtg proteins was constructed using MEGA 4.1 software by the neighbor-joining (NJ) method with 1000 replications.

2.4. Quantitative real-time PCR (qRT-PCR) of Es-vtg1

2.4.1. Expression of Es-vtg1 in different tissues

Total RNA was collected from different tissues and the first-strand cDNA was produced. Specific primers for *Es-vtg1* (Vtg1 F and Vtg1 R; Table 1) and *β-actin* (*β*-actin F and *β*-actin R; Table 1) were used for qRT-PCR. Expression of *Es-vtg1* in different tissues was analyzed by qRT-PCR using a CFX96™ Real-Time System (Bio-Rad). SYBR Premix Ex *Taq* (TaKaRa) was used with the reaction protocol: 95 °C for 30 s; and 40 cycles each of 95 °C for 5 s and 58 °C for 30 s, followed by 95 °C for 10 s, and a 0.5°C/5s incremental increase from 65 °C to 95 °C The samples were normalized to the expression of the *β-actin* gene and gene expression data were analyzed using CFX Manager™ software, and quantified using the comparative CT method ($2^{-\Delta\Delta Ct}$ method) based on Ct values for *Es-vtg1* and *β-actin* [35]. Each sample had three repetitions and the data obtained from qRT-PCR analysis were analyzed by one-way analysis of variance (ANOVA) and post-hoc Duncan's multiple range tests.

2.4.2. Expression of Es-vtg1 after bacterial challenge

For bacterial challenge, *Vibrio parahaemolyticus* (BYK00036) and *Staphylococcus aureus* (BYK0113) from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China) were used as bacterial suspensions (1×10^6 CFU/ml, heat-killed at 72 °C for 20 min), and injected into cultured primary hemocytes. The concentration of hemocytes was 1×10^5 cells/ml in each 60-mm dish (Corning, Corning, NY, USA). Sterile phosphate-buffered saline (PBS) was added as a control. The hemocytes were collected 0, 2, 4, 8, 16, 24, 36 and 48 h post-challenge (in triplicate at each time point), and then the total RNA was extracted. Expression of *Es-vtg1* in hemocyte samples after bacterial challenge was analyzed by qRT-PCR, in triplicate for each sample. The primers and reaction conditions were as described in section 2.4.1.

2.5. Es-vtg1 RNA interference (RNAi) in vitro

For RNAi experiments, siRNA for *Es-vtg1* and GFP (as a control) were synthesized by GenePharma (Shanghai, China). The primers