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Role of transglutaminase in immune defense against bacterial pathogens via regulation of antimicrobial peptides

You-Ting Zhu [1], Dan Li [1], Xing Zhang, Xue-Jie Li, Wei-Wei Li**, Qun Wang*

Laboratory of Invertebrate Immunological Defense & Reproductive Biology, School of Life Science, East China Normal University, Shanghai, China

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ABSTRACT

Transglutaminase (TGase) is critical for blood coagulation, a conserved immunological defense mechanism among invertebrates. Here, a 3248-bp (full-length) TGase cDNA in *Eriocheir sinensis* (*EsTGase*) was cloned, with a 2274-bp open reading frame (ORF) encoding a 757 amino acid protein containing two transglut domains, one TGase/protease-like homolog domain and a KGD (Lys-Gly-Asp) motif. Phylogenetic analysis demonstrated that *EsTGase* appeared earlier in evolution compared with TGases of other crustaceans and mammals. *EsTGase* mRNA was mainly detected in hemocytes and up-regulated post-challenge with bacteria (*Vibrio parahaemolyticus* and *Staphylococcus aureus*), suggesting an immune function for this gene. Moreover, the *EsTGase* activity in hemocytes challenged with *V. parahaemolyticus* and *S. aureus* was decreased significantly. RNA interference of *EsTGase* down-regulated expression of immune-related genes *CrusEs2*, *EsLecG* and *Es-DWD1* with or without bacteria stimulation *in vitro*. Furthermore, absence of *EsTGase* led to higher bacterial counts in the hemocyte culture medium. Thus, *EsTGase* is an important component of the crab immune response and is involved in the regulation of certain immune-related genes, particularly those encoding anti-microbial peptides.

1. Introduction

Blood coagulation or hemolymph clotting is a key component of the immune response, which is the first line of defense and an integral part of the overall invertebrate immune system (Loof et al., 2011). After injury, open wounds represent an ideal entry port for bacteria to invade an open (invertebrates) or closed circulatory system (vertebrates). Therefore, these sites confer great risk of systemic infections. To prevent bacterial dissemination from the site of injury and the loss of hemolymph (invertebrates) or blood (vertebrates), wound sealing must occur quickly. Thus, remodeling of tissue integrity at the wounded site should be considered an important part of the innate immune system. This requirement is particularly important for invertebrates, which only have an open circulatory system, whereas a closed system may provide greater protection since it is restricted (Loof et al., 2011; Theopold et al., 2004). Furthermore, lacking of adaptive immunity in invertebrates places a greater importance on the immobilization of intruders during clotting (Theopold et al., 2004).

Mechanisms of hemolymph clotting have been proposed based on horseshoe crabs, (*Limulus polyhemus* and *Tachypleus tridentatus*) and crayfish (*Pacifastacus leniusculus*) as model organisms (Cerenius and Söderhäll, 2011). Clotting of horseshoe crab hemolymph has been thoroughly characterized and involves the activation of one of two proteolytic cascades, factors C and G (Cerenius and Söderhäll, 2011). Ultimately, both cascades lead to the cleavage of coagulogen into coagulin, which polymerizes to form an insoluble polymer. In this process, transglutaminase (TGase) released from hemocytes increases clot stability through cross-linking different clot components including coagulin. Crustaceans including crayfish and shrimps rely upon the crosslinking of soluble proteins, which show sequence similarity to storage proteins (vitellogenins). Individual vitellogenin-like molecules are cross-linked by a TGase leading to polymers, which form the clot matrix (Cerenius et al., 2010; Hall et al., 1999). Hemolymph clotting, in insects and crustaceans, occurs by the polymerization of a plasma protein catalyzed by a Ca^{2+}-dependent TGase, which is released from the hemocytes under foreign particle stimulus or tissue

* Corresponding author. School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai, China.
** Corresponding author. School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai, China.
E-mail addresses: Liweiwei85@gmail.com (W.-W. Li), qwang@bio.ecnu.edu.cn (Q. Wang).
[1] These authors contributed equally to this work.

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damage (Hall et al., 1999; Ho et al., 1992; Ichinose et al., 1990). The TGase, during this process, forms the ε-(γ-glutamyl)-lysine cross-links between glutamine and lysine of the clottable protein (CP) (Kopáček et al., 1993). This polymerization step has been conserved through evolution (Wang et al., 2001).

TGases and CPs have been documented to be involved in the hemolymph clotting system of shrimp. TGase has been cloned and localized in crayfish (Wang et al., 2001) and further characterized in tiger shrimp, *Penaeus monodon* (Huang et al., 2004). Another type of TGase was found to be involved in the blood coagulation system of tiger shrimp (Chen et al., 2005). Subsequent biochemical assays were utilized to investigate the involvement of TGase in coagulating plasma CP (Yeh et al., 2006). In crayfish, TGase has been demonstrated to affect hematopoiesis in addition to coagulation. Knockdown of TGase was shown to affect the cell morphology and induce cell spreading (Lin et al., 2008). In kuruma shrimp, *Penaeus japonicas*, silencing of TGase resulted in altered gene expression and caused significant down-regulation of the expression of crustin and lysozyme (Fagutao et al., 2012), suggesting that release of antimicrobial peptides (AMPs) in shrimp depends on activation of the shrimp coagulation system or particularly on TGase activity. Furthermore, TGase-depleted shrimp were observed to have lower hemocyte counts and higher total bacterial counts in hemolymph (Fagutao et al., 2012). To date, TGases have been identified in various crustacean species (Cerenius and Söderhäll, 2011; Chen et al., 2005; Huang et al., 2004; Liu et al., 2011, 2007; Yeh et al., 2006, 2009, 2013), but its role in the immune system of crustaceans is still poorly understood.

The Chinese mitten crab, *Eriocheir sinensis*, is one of the most important crustacean species and widely cultivated in Southeast Asia (Yang and Zhang, 2005). However, frequent outbreaks of diseases have caused decreased production and catastrophic economic losses in the past decade (Wang and Gu, 2002). Diseases of *E. sinensis* are usually caused by various bacteria in the aquatic environment, and close examination of the immunological host defense would reveal expression of AMPs as part of the strong arsenal of immune reactions (Li and Xiang, 2013; Rowley and Powell, 2007). Based on the observations in kuruma shrimp (Fagutao et al., 2012), regulating the expression of AMPs via TGase and enhancing the antimicrobial activity of crustaceans may be potential approaches to defending against various bacteria. To test that hypothesis in this study, a novel full-length TGase cDNA of Chinese mitten crab, *E. sinensis*, was cloned (abbreviated *EsTGase*), and the tissue distribution and expression profile of this gene post-bacterial challenge were determined. Based on results using RNA interference (RNAi), the relationship between *EsTGase* and different immune-related genes as well as the antimicrobial effect of *EsTGase* were analyzed.

2. Materials and methods

2.1. Experimental animals and sample collection

Healthy adult Chinese mitten crabs (n = 50, 100 ± 20 g wet weight) were collected from the Xin'an agricultural market in Shanghai, China. Animals were acclimated for one week at 20–25 °C in filtered, aerated freshwater before study.

Prior to dissection, healthy crabs were placed in an ice bath (5 min) until lightly anesthetized. Tissues of gills, hepatopancreas, brain, stomach, muscle, intestine and hemocytes were collected, snap frozen in liquid nitrogen and stored at −80 °C for tissue transcriptional analysis. For cloning and expression analysis, tissues from seven individuals were pooled and ground with a mortar and pestle prior to extraction. After the sampling site was wiped with 70% ethanol, hemolymph was collected from the non-sclerotized membrane of the posterior walking leg from individual crabs by using a sterile 10-ml syringe loaded with 5 ml of pre-cooled (4 °C) sterile anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate; 26 mM citric acid and 10 mM EDTA, pH 4.6; passed through a 0.22 μm filter) at a 1:1 ratio. Samples were immediately centrifuged at 300× g for 10 min at 4 °C to collect the hemocytes.

For bacterial challenge, *Vibrio parahaemolyticus* (BYK00036) and *Staphylococcus aureus* (BYK0113) from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China) was cultured overnight in Luria-Bertani (LB) medium, collected by centrifugation at 5000 × g for 3 min, washed with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) three times, resuspended in PBS and plated for colony counting. After adjusting to 1×10^6 CFU/ml, the bacterial suspension (100 μl) was injected into each crab intramuscularly at the non-sclerotized membrane of the posterior walking leg with similar mock PBS injections in the control crabs. Hemocytes were collected at 0, 2, 4, 6, 12, 24 and 48 h post-challenge with bacteria (at least five crabs at each time point) using the method mentioned above for analyzing the expression profiles and protein activity of *EsTGase* in hemocytes. In total 320 crabs were used in this study.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA samples from different tissues and hemocytes of bacteria-challenged *E. sinensis* were extracted using Trizol® reagent (RNA Extraction Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The total RNA concentration and quality were evaluated by measuring absorbance at 260 nm by spectrophotometry and agarose-gel electrophoresis, respectively.

For full-length cDNA cloning, total RNA (5 μg) was reverse transcribed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA). For reverse transcription PCR (RT-PCR) and quantitative real-time RT-PCR (RT-qPCR) analysis, total RNA (4 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (TaKaRa, Shiga Japan).

2.3. Cloning of full-length EsTGase cDNA

Original EST sequences of *E. sinensis* TGase (EsTGase) was obtained from the hemocyte library. Partial EsTGase cDNA sequences were extended using 5′ and 3′ RACE (SMARTer® RACE cDNA Amplification kit, Clontech, Mountain View, CA, USA), and gene-specific primers (Table 1) were designed based on the original EST sequence. The 3′ RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first-strand cDNA reaction as the template, 5 μl of 10 × Advantage 2 PCR buffer, 1 ml of 10 mM dNTPs, 5 μl (10 μM) of gene-specific primers (EsTGase-3′ RACE, Table 1), 1 μl of Universal Primer A Mix (UPM; Clontech), 34.5 μl of sterile deionized water (RNase free, TaKaRa) and 1 U of the 50 × Advantage 2 polymerase mix (Clontech). For the 5′ RACE, UPM was used as the forward primer in PCR reactions in conjunction with the reverse gene-specific primers (EsTGase-5′ RACE, Table 1). PCR amplification conditions for both the 3′ and 5′ RACE were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. PCR amplicons were size separated and visualized on an ethidium bromide stained 1.2% agarose gel. Amplicons of expected sizes were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), inserted into a pZeroBack/Blunt vector (Tiangen, Beijing, China) and transformed into TOP10 *Escherichia coli*. Positive clones containing inserts of an expected size were two-way sequenced