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Trypsin of *Litopenaeus vannamei* is required for the generation of hemocyanin-derived peptides

Changping Li [1], Fan Wang [1], Jude Juventus Aweya, Defu Yao, Zhou Zheng, He Huang, Shengkang Li**, Yueling Zhang*

Department of Biology, Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou 515063, China

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ABSTRACT

Hemocyanin is a copper containing respiratory glycoprotein in arthropods and mollusks, which also have multiple functions *in vivo*. Recent studies have shown that hemocyanin could generate several peptides, which play important roles in shrimp innate immunity. However, how these hemocyanin-derived peptides are generated is still largely unknown. In this study, we report for the first time that the generation of hemocyanin-derived peptides was closely correlated with trypsin expression in shrimp hepatopancreas following infection with different bacteria. RNA interference (RNAi) mediated knock-down of trypsin or treatment with the serine protease inhibitor, aprotinin, resulted in significant decrease in the levels of hemocyanin-derived peptides. Moreover, recombinant trypsin (rTrypsin) was able to hydrolyse hemocynin *in vitro* with the hydrolysate having a high bacterial agglutination activity while the denatured hemocynin untreated with rTrypsin lost its agglutination activity. Taken together, our current results showed that the generation of hemocyanin-derived peptides correlates with an increase trypsin expression.

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1. Introduction

Shrimp aquaculture is an important component of modern agriculture, which has brought huge benefits to China. However, various pathogens have hampered the development of the shrimp aquaculture industry (Xiong et al., 2016). Shrimp lack adaptive immunity, with the innate immune system being the only line of defense against pathogenic bacteria and virus infection (Pope et al., 2011). Therefore, an increasing number of studies have focused on understanding the innate immune system of shrimp so as to improve the resistance of shrimps to pathogens and to efficiently culture (Shi et al., 2016).

Hemocyanin, a copper containing respiratory protein in arthropods and mollusks, is also involved in a variety of immune functions such as phenoloxidase activity, antiviral, antimicrobial, hemolytic and antitumor activity (Coates and Nairn, 2014; Zlateva et al., 1996; Yan et al., 2011; Zhang et al., 2009, 2017; Zhao et al., 2016; Zhao et al., 2012; Zheng et al., 2016). Interestingly, hemocyanin is not just an important immune molecule, as it has recently been shown that hemocyanin-derived peptides were significantly upregulated with *in vivo* pathogen challenge (Coates and Decker, 2017), suggesting that these derived peptides have important immune functions. For instance, the hemocyanin C-terminus generated a new antibacterial peptide, astacidin1 in plasma, when *Pacifastacus leniusculus* was injected with *Vibrio parahaemolyticus* (Lee et al., 2003). Similarly, Destoumieux-Garzon and colleagues separated three kinds of antifungal peptides (PvHCt, PsHCt, PsHCt) from *Penaeus vannamei* and *Penaeus stylirostris*, which had similarity with the C-terminus of hemocyanin (Destoumieux-Garzón et al., 2001). Qiu et al. idenitified two antimicrobial peptides (AMPs), FCHc-C1 and FCHcC2, from *Fenneropenaeus chinensis* heomcyanin (Qiu et al., 2014). More recently, our group identified a new 18.4 kDd hemocyanin derived peptide, HMCS4, from shrimp *Litopenaeus vannamei* injected with *V. parahaemolyticus* (Wen et al., 2016). From the foregoing, it thus seems to suggest that hemocyanin could generate various peptides in response to different pathogenic infection.

Trypsin is a serine protease found in many organisms, and cleaves peptide chains mainly at the carboxyl side of the amino

* Corresponding author. Department of Biology, School of Science, Shantou University, Shantou, Guangdong, 515063, China.
** Corresponding author. Marine Biology Institute, Shantou University, Shantou, Guangdong, 515063, China.
E-mail addresses: lisk@stu.edu.cn (S. Li), zhangyl@stu.edu.cn (Y. Zhang).
[1] These authors contributed equally to this work.

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acids lysine or arginine (Kurth et al., 1998). As a protease, trypsin is reported to play essential roles in the immune system (Patel, 2017), with some recent studies indicating that this enzyme played diverse roles in invertebrate innate immunity (Gäde and Goldsworthy, 2003). For example, hemocyanin phenoloxidase, which is one of important components of shrimp humoral immunity, was reported to be greatly enhanced via trypsin treatment (Kim et al., 2011). Similarly, trypsin purified from *Steinernema carpocapsae* could change host haemocytes, actin filaments and control haemolymph melanization (Balasubramanian et al., 2010). In the larval gut of *Heliothis virescens*, trypsin modulating ostatic factor (TMOF) is reported to control the biosynthesis of serine proteases (Nauen et al., 2001). Furthermore, people has suggest that some cysteine proteinase might be involved in the processing of the antibacterial peptides from hemocyanin (Lee et al., 2003).

Intrigued by these revelations, we sought to determine the relationship between trypsin and hemocyanin in shrimps. We had also earlier on employed the use of bioinformatics tools and found 12 potential *L. vannamei* hemocyanin-derived antimicrobial peptides, ranging from 1.5 to 1.9 kDa, by the action of potential proteases, such as trypsin, chymotrypsin, and others (data unpublished). Our results here revealed that trypsin and hemocyanin were both upregulated in shrimp hepatopancreas following infection with some pathogens. Meanwhile, hemocyanin could be hydrolyzed by trypsin both *in vitro* and *in vivo*. The present study thus expands our knowledge on hemocyanin's role in shrimp innate immunity.

2. Materials and methods

2.1. Experimental animals

Penaeid shrimps *Litopenaeus vannamei* (approximate weight of 5 g) were purchased from a local farm, Shantou Huaxun Aquatic Product Corporation (Shantou, Guangdong, China). Shrimps were immediately transferred to tanks with aerated seawater at room temperature, and acclimatized for at least 2 days before experiments. All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees at Shantou University.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from various shrimp tissues (gills, heart, hemocytes, hepatopancreas, intestine, muscle and stomach) using the RNAFAST 200 Kit (FeiJie, China). The cDNA samples were prepared using the PrimeScript™ RT reagent Kit (TaKaRa, Japan).

2.3. Tissue distribution of hemocyanin and trypsin

The tissue distribution of hemocyanin and trypsin was detected using real-time qPCR with gene specific primers (Table 1). The hemocyanin and trypsin specific primers were designed according to the hemocyanin small subunit sequence (GenBank: X82502.1) and trypsin sequence (GenBank: X86369.1). The real-time qPCR program used was set at the following conditions: 95 °C for 10 min; 40 cycles of 95 °C for 15 s; 60 °C for 20 s; a melting curve analysis from 72 °C to 95 °C. The qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Zhao et al., 2013) with the *Lv-EF-1α* gene as the internal control.

2.4. Cloning, expression and purification of recombinant trypsin

The gene coding for trypsin was amplified, using primers rTrypsin-F and rTrypsin-R (Table 1), from shrimp hepatopancreas cDNA and inserted into the vector pGEX6P-1 (Amersham). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3). The overexpressed trypsin proteins with a glutathione S-transferase tag were purified by glutathione-sepharose 4B (GE Healthcare). After the removal of the glutathione S-transferase tag by PreScission protease (GE Healthcare) cleavage, the eluted untagged recombinant trypsin (rTrypsin) was used for subsequent experiments.

Table 1
Sequence of primers and siRNAs used in this paper.

Prime	Sequence (5′-3′)
Real-time qPCR	
Trypsin-F	ATCCTCTGTGTGCTCCTTGCTGG
Trypsin-R	CATGGTAGGAGACCTCAGCGTA
HMCS-F	CCTGGCCTCATAAAGACAACA
HMCS-R	TTTTCCACCCTTCAAAGATACC
EF-1α-F	TATGCTCCTTTTGGACGTTTTGC
EF-1α-R	CCTTTTCTGCGGCCTTGGTAG
Recombinant expression	
rTrypsin-F	CCGGAATTCATGAAGACCCTCATCCTCTGTG
rTrypsin-R	CCCAAGCTTTTAAACAGCATTGGCCTTAATC
siRNA	
siTrypsin-F	GAUUAAGGCCAAUGCUGUUTT
siTrypsin-R	AACAGCAUUGGCCUUAAUCTT
si-Non-F	UUCUCCGAACGUGUCACGUTT
si-Non-R	ACGUGACACGUUCGGAGAATT

2.5. Preparation of antisera against rTrypsin

To prepare antisera against trypsin, the eluted untagged recombinant trypsin (rTrypsin) produced, was concentrated to 1 mg/ml with an ultracentrifuge filter. Equal volumes of rTrypsin (0.5 ml) and complete Freund's adjuvant (Sigma-Aldrich, USA) were mixed thoroughly and 0.1 ml each injected into 5 Kunming mice. The injection was repeated 7 days later replacing complete Freund's adjuvant with incomplete Freund's adjuvant. Mice were bleed periodically and tested for anti-rTrypsin titer and specificity. The collected anti-rTrypsin antiserum was stored at −80 °C for further use.

2.6. Challenge of shrimps and extraction of proteins

For bacteria challenge experiments, each shrimp was injected via the third and forth segment of the muscle with 0.5×10^6 CFU/g of *V. parahaemolyticus* and *Staphylococcus aureus*, and then hemolymph and hepatopancreas were extracted at different time points (0, 2, 6, 12, 24 h) as previously described (Lu et al., 2015). The hemolymph was centrifuged at 800 g for 15 min at 4 °C to sediment the hemocytes. For the proteins from hepatopancreas, extracted hepatopancreas (5 shrimp) were homogenized, centrifuged at 500 g for 10 min to pellet the cells, washed thoroughly with PBS (0.01 M, pH 7.4), lysed with lysis buffer (Beyotime, China) plus protease inhibitor PMSF for 30 min and then centrifuged at 20000 g for 15 min to collect the supernatant. The concentration of the hemolymph and hepatopancreas proteins were determined by a modified Bradford assay (Bio-Rad, USA) using BSA as standard, and used immediately for the next experiment or stored at −20 °C for later use.

2.7. SDS-PAGE and Western blot

All the extracted proteins were separated on SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane were blocked with 5% skimmed milk dissolved in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) for 2 h at

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