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Molecular cloning and expression analysis of chymotrypsin-like serine protease from the redclaw crayfish (*Cherax quadricarinatus*): A possible role in the junior and adult innate immune systems

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ABSTRACT

A novel chymotrypsin-like serine protease (CLSP) was isolated from the hepatopancreas of the redclaw crayfish *Cherax quadricarinatus* (Cq-chy). The full-length cDNA of Cq-chy contains 951 nucleotides encodes a peptide of 270 amino acids. The mature peptide comprising 223 amino acids contains the conserved catalytic triad (H, D, and S). Similarity analysis showed that Cq-chy shares high identity with chymotrypsins from the fiddler crab; *Uca pugilator*. Cq-chy mRNA expression in *C. quadricarinatus* was shown to be: (a) tissue-related with the highest expression in the hepatotpancreas and widely distributed, (b) highly responsive in the hepatopancreas to White Spot Syndrome Virus (WSSV) challenge, and (c) differently regulated in immature and adult crayfish. In this study we successfully isolated Cq-chy. Our observations indicate that Cq-chy is differently involved in the immature and adult innate immune reactions, thus suggesting a role for CLSPs in the invertebrate innate immune system.

1. Introduction

Host defense systems in invertebrates lack an adaptive immune system and are believed to rely solely on multiple innate defense reactions to fight against invading pathogens [1]. Invertebrate phenoloxidase has been found to exist in the blood in an inactive form that is activated in a stepwise process involving serine proteinases, which have previously been activated by microbial cell wall constituents [1,2]. Prophenoloxidase-activating protein (PAP), also referred to as prophenoloxidase-activating enzyme (PPAE), is the last protease of the serine protease cascade that converts the prophenoloxidases (proPO) to phenoloxidases (PO) in *Manduca sexta* [3]. In addition, serine protease homologs (SPHs), also known as prophenoloxidase-activating factor (PPAF), are also essential for generating active PO. Serine proteinases exhibiting approximate molecular masses 30 kDa have been isolated from crayfish [4], *Drosophila melanogaster* [5] and *Bombyx mori* [6].

Serine proteases (SPs) constitute one of the largest families of enzymes in the animal kingdom [7,8] and play important roles in immune responses [9,10], food protein digestion [11], embryogenesis [12] and other biological processes in insects [13]. SPs are characterized by an active site, termed the catalytic triad, which contains H, D, and S amino acids [14]. The serine residue at the active site participates in the formation of a transient acylenzyme intermediate comprising the substrate and the protease [15]. The SP chymotrypsin, participates in immune reactions and in many other physiological functions in mammals [7]. Studies on chymotrypsins in invertebrates have mainly been focused on the digestive system in insects, which are important agriculture pests, with the aim of identifying strategies crop protection [13]. A new CLSP Scolexin in *M. sexta*, have been associated with response to bacteria, yeast, and baculovirus infection [16]. In *Drosophila*, chymotrypsin-like serine proteases are involved in antibacterial immunity [17,18]. Recently, CLSPs have increasingly been discovered in invertebrates [11,19–24]. The molecular cross-talk between the signaling pathway and a PO-dependent melanization reaction mediated by a common serine protease called Spätzle processing enzyme (SPE) was proposed in *Tenebrio molitor* [14]. Chymotrypsins have been cloned in shrimp and their polymorphisms and immune

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responses to bacteria or viruses have been analyzed [22,24,25]. However, little is known about the roles of these proteases in the immune system of the redclaw crayfish.

The Australian redclaw crayfish, *Cherax quadricarinatus* is a species native to the Northern Queensland (Australia) and the southeast regions of Papua New Guinea [26]. It is an important aquaculture species around the world that has been reared for human consumption since 1985 [27]. The worldwide shrimp/crayfish aquaculture industry has been beset by diseases mainly caused by viruses, particularly the White Spot Syndrome Virus (WSSV), and has suffered significant economic losses. WSSV is one of the most detrimental viruses and is widely disseminated in shrimp [28], including marine and freshwater crustaceans such as the American crayfish *Procambarus clarkaii*, an Australian crayfish *Cherax destructor albidus* and *C. quadricarinatus* [29–32]. In recent years, there have been many cases of natural infection of redclaw crayfish by WSSV in Zhejiang, Jiangsu and other areas of China [33–35]. Improving immunity and disease resistance in crustaceans is one of the fundamental approaches to disease prevention and control. Although studies on the epidemiology and prevention of White Spot Syndrome (WSS) as well as virus detection have been performed [31], detailed analysis of the molecular resistance mechanism to WSSV in *C. quadricarinatus* have not been reported. In this study, we cloned a CLSP, Cq-chy, from the redclaw crayfish *C. quadricarinatus*, and its transcript expression patterns were determined to ascertain how Cq-chy is involved in the innate immune defense against WSSV virus infection.

2. Material and methods

2.1. Tissue collection and nucleic acid preparation

Immature (approximately 30 d old; body weight, 3.8 ± 1.2 g; body length, 4.65 ± 1.16 cm) and adult (approximately 150 d old; body weight, 55.2 ± 12.6 g; body length, 12.65 ± 2.28 cm) *C. quadricarinatus* were purchased from the Shanghai Jinshan aquaculture farm during 2011 September to October. Crayfish were lightly anesthetized in an ice bath for 3–5 min before various organs (brain, eyestalk, gill, thoracic ganglia, heart, muscle, hemocytes, hepatopancreas, stomach, intestine, testes and ovaries) were dissected, immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted from *C. quadricarinatus* using Trizol reagent (RNA Extraction kit, Invitrogen, USA) according to the manufacturer's protocol. For cloning and expression analysis, organs from five crayfish were pooled and ground with a mortar and pestle prior to RNA extraction. Total RNA (2 μg) isolated from various organs was reverse transcribed using The Prime Script™ RT-PCR kit (TaKaRa, China). The Prime Script™ RT-PCR kit or the Prime Script Real-time PCR kit (TaKaRa, China) was used for reverse transcription-PCR (RT-PCR) or Real-time quantitative PCR (RT-qPCR) experiments, respectively.

2.2. Primer design and target fragment amplification

All the primers used in this study were synthesized by Shanghai Invitrogen Biotech Co Ltd. (Invitrogen, Shanghai) and are listed in Table 1. Degenerate primers (DP-R and DP-F) were designed by the codeHop principle [36] according to the conserved sequences from known SPs and used to amplify target fragments of the Cq-chy gene. Briefly, first-strand cDNA was used as a template and target fragments of cDNAs encoding SPs were amplified using a homology-based cloning strategy. PCR amplification was performed in a final volume of 25 μL in a Master cycler gradient (Eppendorf, Germany). The thermal cycler program consisted of 94 °C for 3 min, followed by 26 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min, and a final elongation step at 72 °C for 3 min. PCR products were separated by 1.2% agarose gel electrophoresis and then purified with a Qiagen PCR purification kit (Qiagen, Germany). The purified PCR products were cloned into the pMD18-T vector (Takara, Japan), and transformed into competent *Escherichia coli* cells. Recombinants were identified through blue/white color selection. Plasmids were extracted by alkaline lysis, and then checked by double digestion with *Eco*RI and *Hind*III. Positive clones were sequenced at Shanghai Biosune Bio Co Ltd. (Biosune, China).

Table 1
Sequences of primers.

Primers	Sequence	Code
Degenerate primers for target Cq-chy		
Forward primer	5′-GGCCGCCAGAGTCNCCGTTGCA-3′	DP-R
Reverse primer	5′-TGGCACTGGAAGTCYCCNAAGCC-3′	DP-F
Gene-specific Primers for Cq-chy cloning		
Gene-specific primer pairs for RACE	5′-AGATGAGGGAGCCGCCGCAGAAGTA-3′	Gp5-1
	5′-ATGCGATGCTATCTACGGCACTGTGACC-3′	Gp3-1
RT-PCR and RT-qPCR primers		
Cq-chy 5′ primer	5′-CGATACGGCAGAAGGCAGTTTG-3′	Q-R
Cq-chy 3′ primer	5′-GGTGGCTCTCTTCATTGACGAC-3′	Q-F
18S rRNA primers for RT-PCR and RT-qPCR		
18S rRNA Reverse	5′-GGAGGTAGTGACGAAAAATAACG-3′	18S-R
18S rRNA Forward	5′-GGAGCGTGACAGTAAGCACCATCGG-3′	18S-F

2.3. Rapid amplification of cDNA ends (RACE)

RACE technology was performed to obtain the total coding sequence. The 3′ and 5′ RACE reactions were performed using the SMARTer™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's protocol. The gene-specific primers (Gp5-1, Gp3-1; Table 1) were designed based on the obtained cDNA sequence of Cq-chy. The PCR program was performed as a touchdown PCR reaction according to the manufacturer's protocol. The amplified products were sent for DNA sequencing at Shanghai Biosune Bio Co Ltd. (Biosune, China).

2.4. Multiple sequence alignment and phylogenetic analysis

The full-length multiple alignment of the Cq-chy sequence was compared with that of CLSPs of other species using the BLAST program [37]. Amino acid sequences from various species were retrieved from the NCBI GenBank and analyzed using the ClustalW 2.0 Multiple Alignment program [38]. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA software version 4.0 [39]. The reliability of the branching was tested using bootstrap re-sampling (with 1000 pseudo replicates).

2.5. Organ distribution and RT-qPCR analysis

The organ-dependent Cq-chy mRNA expression was measured by RT-qPCR using the following method: Briefly, first-strand cDNA was prepared as described in Section 2.2. Gene-specific primers (Q-F and Q-R; Table 1) were designed based on the sequence of the cloned Cq-chy cDNA to produce an amplicon of 250 bp. All RT-qPCR reactions were performed in triplicate using extracted RNA (pooled) of the same concentration. Real-time RT-qPCR was performed in a C1000™ Thermal Cycler (BioRad CFX 96™ Real-Time System) according to the manufacturer's instructions. The final volume of each RT-qPCR reaction was 25 μL, which contained 12.5 μL 2× SYBR Premix ExTaq (TaKaRa), 1.0 μL diluted cDNA template, 10.5 μL PCR-grade water, and 0.5 μL of each primer diluted to 10 μM. PCR conditions were as follows: 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s and a 0.5 °C/5 s incremental