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Identification and characterization of a Cystatin gene from Chinese mitten crab *Eriocheir sinensis*

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ABSTRACT

Cystatins are a superfamily of proteins as reversible inhibitor of cysteine proteinases which play essential roles in a spectrum of physiological and immunological processes. In this study, a novel member of Cystatin superfamily was identified from Chinese mitten crab *Eriocheir sinensis* (designated EsCystatin) by expressed sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of EsCystatin was of 1486 bp, consisting of a 5′-terminal untranslated region (UTR) of 92 bp, a 3′ UTR of 1034 bp with a polyadenylation signal sequence AATAAA and a polyA tail, and an open reading frame (ORF) of 360 bp encoded a polypeptide of 120 amino acids with the theoretical isoelectric point of 5.48 and the predicted molecular weight of 13.39 kDa. A signal Cystatin-like domain (Gly^{25} to Lys^{112}) was found in the putative amino acid sequences of EsCystatin. Similar to other Cystatins, the conserved central $Q^{70}VVSG^{74}$ motif was located in the Cystatin-like domain of EsCystatin. But EsCystatin lacked of signal peptide and disulphide bond. The EsCystatin exhibited homology with the other known Cystatins from invertebrates and higher vertebrates, and it was clustered into Cystatin family 1 in the phylogenetic tree. The mRNA transcripts of EsCystatin were mainly expressed in hemolymph, gill, hepatopancreas, gonad and muscle, and also marginally detectable in heart. After *Listonella anguillarum* challenge, the relative expression level of EsCystatin in hemolymph was down-regulated to 0.6-fold ($P < 0.05$) at 3 h post-challenge. Subsequently, it was up-regulated to 3.0-fold ($P < 0.01$) at 24 h. Afterwards, EsCystatin mRNA transcripts suddenly decreased to original level. After *Pichia pastoris* GS115 challenge, its mRNA expression level in hemolymph was up-regulated to the peak at 3 h (2.8-fold of that in blank ($P < 0.01$)). The cDNA fragment encoding the mature peptide of EsCystatin was recombined and expressed in *Escherichia coli* Rosetta-gami (DE3). The recombinant EsCystatin displayed a promoter inhibitory activity against papain. When the concentration of EsCystatin protein was of 300 μg mL^{-1}, almost 89% of papain activity could be inhibited. These results collectively suggested that EsCystatin was a novel member of protein in Cystatin family, was a potent inhibitor of papain and involved in immune response versus invading microorganisms.

1. Introduction

Cystatins are a superfamily of proteins as reversible inhibitor of cysteine proteinases which play essential roles in a spectrum of physiological processes [1]. Cystatins have been widely found in vertebrates such as human [2], mouse [3], rat [4], chicken [5], and chum salmon [6]. And Cystatins have been also characterized in some invertebrates including fruit fly *Drosophila melanogaster* [7], liver fluke *Fasciola hepatica* [8], filarial nematode *Acanthocheilonema viteae* and *Brugia malayi* [9], filaria *Onchocerca volvulus* [10], Gastrointestinal Nematode Parasite *Nippostrongylus brasiliensis* [11], barber pole worm *Haemonchus contortus* [12], tick *Haemaphysalis longicornis* [13] and horseshoe crab *Tachypleus tridentatus* [14].

The known Cystatin proteins in animals have been classified into three evolutionary related protein families, namely 1, 2 and 3 [15]. Family 1 Cystatins (also known as stefins) are low molecular weight and single domain Cystatins that do not contain disulphide bridges and carbohydrate side chains [15]. Family 2 Cystatins containing Cystatin C, D, E, S and SN are secretory inhibitors and possess a single Cystatin domain, but their structures have at least two intramolecular disulphide bonds. And family 3 Cystatins (also known as kininogens) display a higher degree of structural complexity characterized with the occurrence of multiple Cystatin-like domains,

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each with two disulphide bridges at positions homologous to those in family 2 Cystatins [16]. But family 4 is mainly consisted of invertebrate origin was reported by Khaznadji et al. [8].

All Cystatins contain the conserved QxVxG motif that is generally located in the central region of protein sequence. And some other amino acids are partially conserved among Cystatins, such as a glycine residue is located at the N-terminal region, a PW motif at the C-terminal region [17,18]. Among these Cystatin families, family 1 Cystatins are mostly intracellular proteins lacking a signal peptide [19].

Cystatins are considered to be involved in the protection of cells from unfavorable proteolysis by intracellular and external cysteine proteinase and also involved in biological defense systems against invaders [20,21]. Members of Cystatin superfamily function as potent inhibitors of papain-like cysteine proteinases to regulate numerous processes that are dependent on endogenous cysteine proteinase activity [22]. For example, human Cystatins are implicated in various pathologies such as inflammation, Alzheimer's disease, viral diseases and tumor malignancy [19,23,24]. The functions of Cystatin in invertebrates were also reported. For instance, filarial nematode *Litomosoides sigmodontis* Cystatin acted as an immunomodulatory molecule in protective immune response during the course of filarial infection [25]. Cystatin secreted by roundworm nematode *B. malayi* could block conventional cysteine proteases as well as asparaginyl endopeptidase involved in the Class II antigen processing pathway in human B cells [9]. Although some Cystatins have been found in invertebrate animals, structure and function of Cystatin from crustacean remain still unknown.

Chinese mitten crab *Eriocheir sinensis* is an important economical aquatic species and widely farmed in south China. As crustacean, crabs lack of adaptive immune system and mainly depend on innate immunity to against invading microbes [26]. Investigating the roles of Cystatin in the immune response to invading microorganisms in crabs and its inhibitory activity to proteinases would provide new insights into the function of this important, widespread and functionally diverse family of proteins and give new evidence for further discussion about the Cystatin molecules in crustacean. The main objectives of the present study were: (1) to clone the full-length cDNA of Cystatin from Chinese mitten crab *E. sinensis* (designated EsCystatin); (2) to investigate the expression pattern of EsCystatin mRNA in different tissues and its temporal expression in hemolymph of crab challenged with *Listonella anguillarum* and *Pichia pastoris* GS115; and (3) to characterize the inhibitory activity of recombinant EsCystatin protein to papain and its possible function in immune defense of crab.

2. Materials and methods

2.1. Cloning of the full-length cDNA of EsCystatin

A cDNA library was constructed from hemolymph of Chinese mitten crab challenged with *L. anguillarum* and *Staphylococcus aureus*. Random sequencing of the library yielded 7535 successful sequencing reactions and these ESTs were assembled into 2943 unigenes [27]. Among them, one contig sequence (seq.filter.Contig20) was homologous to *Felis catus* Cystatin A (Expect = 7e-17), *Homo sapiens* Cystatin A (Expect = 7e-15). This contig sequence was used to clone the full-length cDNA of EsCystatin from *E. sinensis*.

Two gene specific primers P1 (5′-CGTTCAACACTTCACCGACAA-3′), P2 (5′-CCCACCGCCATCACAATA-3′) and oligo (dT)-adaptor (5′-GGCCACGCGTCGACTAGTACT$_{17}$-3′) were used to clone the 3′ end of EsCystatin cDNA by 3′ RACE technique. The obtained PCR products were cloned into pMD18-T simple vector (Takara, Japan), and transformed into competent cells of *Escherichia coli* DH5α. The recombinants were identified through blue–white color selection in ampicillin-containing LB plates and white colonies were screened by PCR with primers RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) and M13-47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′). Positive clone was sequenced to verify the full-length cDNA of EsCystatin.

2.2. Sequence analysis

The cDNA sequence and deduced protein sequence of EsCystatin were analyzed with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Signal Peptide was predicted using version 3.0 of SignalP. The putative domain was predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Analysis of protein motifs was performed using the PROSITE database (ScanProsite, http://www.expasy.org/prosite). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used to create the multiple sequence alignment. An unrooted phylogenetic tree was constructed based on the deduced amino acid sequences of EsCystatin and other Cystatins by the neighbor-joining method embedded in Mega 4 program. The reliability of the branching was tested by bootstrap re-sampling (1000 pseudo-replicates).

2.3. Crabs, immune challenge and hemolymph collection

E. sinensis, averaging 50 ± 5 g in weight were collected from a farm in Qingdao, China, and kept in filtered aerated freshwater at 20–25 °C for a week before processing. For the challenged groups, fifty crabs were suspended respectively in high density of *L. anguillarum* (10^7 CFU mL^{-1}) and *P. pastoris* GS115 (10^7 CFU mL^{-1}). Five individuals were randomly sampled at 1.5, 3, 6, 12, 24 and 48 h in the challenge groups during experiment. The untreated crabs were used as blank group.

The hemolymph from blank and challenged groups was collected using syringe from the last walking leg, and quickly added to anticoagulant solution (glucose, 2.05 g; citrate, 0.8 g; NaCl, 0.42 g; double distilled water was then added to a total volume of 100 mL) [28]. Samples were immediately centrifuged at 800×*g*, 4 °C for 10 min to harvest the hemocytes. The hemocytes pellets were immediately used for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen, America) according to the manufacture's protocol (Biostar, China).

2.4. Real-time PCR analysis of EsCystatin mRNA expression

The total RNA was extracted from above-mentioned hemolymph and various tissues (hemolymph, gonad, heart, muscle, hepatopancreas and gill from five adult crabs as parallel samples) by following the manufacturer's instructions (Invitrogen, America). The cDNA first strand synthesis was carried out based on Promega M-MLV RT Usage information (Promega, America). cDNA mix was diluted to 1:100 and stored at −80 °C for subsequent fluorescent real-time PCR.

The mRNA expression of EsCystatin in different tissues and its temporal expression in hemolymph of immune challenged crabs were measured by fluorescent real-time PCR. Two EsCystatin gene specific primers P3 (5′-CCCACCGCCATCACAATAC-3′) and P4 (5′-GGGTTTCCCGAGTCGTTCT-3′) were used to amplify a product of 278 bp from cDNA, and the PCR product was sequenced to verify the specificity of RT-PCR. Two β-actin primers, P5 (5′-GCATCCACGAGACCACTTAC-3′) and P6 (5′-CTCCTGCTTGCTGATCCACATC-3′) were used to amplify a 266 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding samples.

Real-time PCR amplification was carried out in an ABI 7300 Real-time Thermal Cycler according to the manual (Applied Biosystems,