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Dual roles of cystatin A in the immune defense of the pacific oyster, *Crassostrea gigas*

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ABSTRACT

Cystatins are a large family of the proteins that function as reversible and tight-binding inhibitors of cysteine proteases, which consequently regulate multiple physiological activities including apoptosis and innate immunity. In the present study, we cloned a gene from *Crassostrea gigas* encoding cystatin, which is related to cystatin A superfamily. *Cg*CytA was comprised of a cystatin-like domain with two conserved glycine residues (GG) near the N-terminal and a highly conserved glutamine-valine-glycine (Q-X-V-X-G) motif in the form of QVVAG loop. Transcription analysis of *Cg*CytA indicated its constitutive expression in all tissues including mantle, gill, digestive tract, hemocytes, heart, adductor muscle, and gonads. Immune challenge with *Vibrio alginolyticus*, resulted in significant down-regulation of *Cg*CytA expression at the initial stages of infection (till 12 h post infection) and the expression of cystatin increased 48 h post infection. Protease assay demonstrated the concentration of cystatin needed to inhibit half of the maximum biological response of cysteine protease is 14.4 μg/L (IC_{50}). Furthermore, RNAi of *Cg*CytA resulted in increase of apoptotic cell population in hemocytes of *C. gigas*, suggesting protection role of *Cg*CytA from hemocytes apoptosis. Unexpectedly, knockdown of *Cg*CytA leaded to enhancement of bacterial clearance *in vivo*, implying that *Cg*CytA may negatively regulate immune defense by suppressing endogenous cysteine protease. Therefore, *Cg*CytA plays dual roles in protection of host hemocytes from apoptosis and control of bacterial clearance, which may server as one of key endogenous balancer between apoptosis and innate immunity in oyster.

1. Introduction

Cysteine proteases, play essential roles in the physiological activities of an organism including antigen presentation, bone resorption, apoptosis and protein processing, in addition to its involvement in several pathological processes such as cancer progression, inflammation and neurodegeneration [1,2]. Papain is well studied plant cysteine proteases [3] and cathepsins are a large family of lysosomal cysteine proteases involving in the bacteria defensing [4,5]. Caspases are also cysteine proteases involved in activation and implementation of apoptosis [6]. Calpains are Ca^{2+} activated cysteine proteases that cleave intracellular proteins [7,8]. They are all papain family proteins. Cysteine proteases are widely represented in various living organisms from different evolutionary groups, but are relatively less abundant in prokaryotes. In bacteria, the cysteine proteases are involved in peptidoglycan turnover and various housekeeping processes [9]. Moreover, the cysteine proteases produced by bacteria and virus act as virulence factors leading to numerous pathological conditions [10–12]. The proteolytic activity of these proteases is in turn regulated by the rate of their biosynthesis, including activation of pro-enzyme forms. Cystatins, the endogenous protein inhibitors of cysteine proteases, are considered to be protective against unwanted proteolysis, such as bacteria proteases and lysed leukocytes.

Cystatins are a large protein family characterized by the presence of at least one cystatin-like domain. They function as reversible and tight-binding inhibitors of C1 family cysteine proteinases, such as the plant-derived papain and human cathepsins B, H, and L [13,14]. Based on structural variations the cystatin superfamily can be categorized into four sub-families. All cystatins, irrespective of their family classifications, contain several conserved regions including an N-terminal glycine-containing segment, a QXVXG sequence that constitutes part of a β-hairpin loop structure, and a proline and tryptophan-containing region that forms a second hairpin loop [15–17]. Family 1 cystatins include cystatin A and B (also named stefin A and B), which are low

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molecular weight (~11 kDa) cytoplasmic proteins lacking of disulfide bonds and carbohydrate. The three-dimensional structure of a stefins consists of a five stranded antiparallel β-sheet wrapped around a five turn α-helix with an additional carboxyl-terminal strand that runs along the convex side of the sheet. Cysteine proteases can interact with the wedge shaped surface formed by the N-terminal and the two β-hairpins of stefins [15].

Proteolysis and protease inhibition play a key role in infection pathology and host defense. Recent advances in the field of protease inhibitors have drawn attention to the possible use of this collected knowledge to control related pathological processes [18]. There are several reports focusing on the biochemical characterization and enzymatic properties of cystatin [33–36]. Although cystatin A homolog have been identified from several vertebrate species [19–22], very few have been cloned from invertebrates. Here, we cloned homolog of cystatin A from the pacific oyster, *Crassostrea gigas*, and also characterized its expression profile, inhibitory activity and possible function in oyster immunity, which will benefit to us for understanding the molecular mechanism of oysters immune defense.

2. Materials and methods

2.1. Animals, tissue collection, bacterial challenge

The Pacific oysters (two years old with an average 100 mm shell length) were obtained from Qingdao, Shandong Province, China, and maintained at 22–25 °C in tanks with re-circulating seawater for one week before experiments. The oysters were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*. To analyze the tissue distribution of *Cg*CytA, total RNA was extracted from gill, mantle, adductor muscle, digestive gland, gonads and hemocytes of three healthy *Crassostrea gigas* individuals. To verify the immune response of the *Cg*CytA, one hundred oysters were randomly divided into 2 groups and placed in 2 tanks: the bacteria challenge and control group. Oysters were challenged by injecting 100 μl bacteria *Vibrio alginolyticus* (1×10^9) bacteria suspended with Phosphate buffer saline (PBS) into adductor muscles. The control groups were injected with the equal volume of PBS. Hemolymph was collected at scheduled intervals (2, 4, 6, 12, 24, 48 h after pathogenic challenge) from the pericardial cavity through the adductor muscle and immediately centrifuged (700× g for 10 min at 4 °C) to separate the blood cells from plasma. Three individuals were randomly sampled in each group at every time point after injection.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's directions, and quantified by measuring absorbance at 260 nm. The integrity of RNA was checked by agarose gel electrophoresis. Purified RNA samples were diluted to 1 μg/μl and pooled to perform cDNA synthesis using PrimerScript™ First Strand cDNA Synthesis kit (TAKARA Bio Inc. Japan) following manufacturer's protocol.

2.3. Cloning the full-length CgCytA

The partial cDNA sequence of *Crassostrea gigas* was Blast searched in Pacific oyster hemocyte EST library (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the identified EST sequence, the ORF of *Cg*CytA was obtained by Polymerase Chain Reaction (PCR) using TAKARA kit according to manufacturer's instructions. In addition, we conducted 3′RACE PCR and 5′RACE PCR using SmartRACE™ kit according to the manufacturer's instructions.

2.4. Bioinformatics analysis of CgCytA

Amino acid sequence of *Cg*CytA was aligned with cystatin A protein sequences of different species obtained from BLAST analysis using CLC Sequence Viewer 6 software. The phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) version 3.0 with the neighbor-joining method with 1000 bootstrap replicates and analyzed using Interactive Tree Of Life program, iTOL (http://itol.embl.de/). The protein domain and signal peptide were predicted with the Simple Modular Architecture Research Tool (SMART) version 4.0 program (http://smart.embl-heidelberg.de/) and SignalP (http://cbs.dtu.dk/services/SignalP) software, respectively.

Table 1
Primers used in this study.

Name	Primer sequence (5′-3′)
*Cg*CytA F1	AGTCGAATTCCTAGGCGGTCCCCGATTAAT
*Cg*CytA R1	AGTCCTCGAGCTAGAAGTAAGTGATTTCGT
*Cg*CytA F2	ATGGCGCACCAGATGTACGT
*Cg*CytA R2	CTAGAAGTAAGTGATTTCGT
*Cg*CytA qF	GAAGGACGACATTGTAGCACG
*Cg*CytA qR	GAGAAATGACGGTAAATACGAGC
β-actin F	AAGATATTGCAGCTTTAGTCGT
β-actin R	TTCTGTCCCATACCAACCAT
Cyta-dsF	GGATCCTAATACGACTCACTATAGGGTCCCCGATTAATGCCAGGG
Cyta-dsR	GGATCCTAATACGACTCACTATAGGCTAGAAGTAAGTGATTTCGT
QdsCyta-F	CGTGTGTGCTTGTTTTCTCGG
QdsCyta-R	CCTCTCTGGCTGTAGCGTCC

"F" indicates forward primer and "R" indicates reverse primer.

2.5. Transcription analysis of CgCytA by quantitative real-time PCR (qRT-PCR)

qRT-PCR analysis was used to determine the expression of *Cg*CytA mRNA in various tissues and during bacterial challenge using the gene-specific primers (Table 1). Each assay was performed in triplicate with β-actin mRNA as internal control, as it is a gene which is least affected by bacterial infection and is independent of tissue type [23]. The qRT-PCR was conducted using a LightCycler 480 (Roche) in a reaction volume of 10 μl containing 1 μl of template cDNA, 5 μl of 2×SYBR Green Mix, 0.5 μl of each primer (10 pmol/μl), and 3 μl of PCR-grade water. The qRT-PCR cycle program consist of one cycle of 95 °C for 1 min, following by 40 cycles of amplification 95 °C for 15 s, 55 °C for 15 s, 72 °C for 20 s, 85 °C for 20 s for signal collection in each cycles. The dissociation curve analysis of amplification products was performed to confirm the specificity at the end of each PCR reaction. The relative expression of *Cg*CytA gene was calculated using the $2^{-\Delta\Delta CT}$ method [24]. All data are given in terms of relative mRNA expression, expressed as means ± standard error of mean (SE).

2.6. Vector construction, over-expression and purification of recombinant CgCytA fusion protein

The cDNA fragment encoding *Cg*CytA was amplified using Extaq (TaKaRa) with primers *Cg*CytA F1, *Cg*CytA R1 with EcoR I and Xhol I. The detected signal sequence was not included in this clone. The target PCR products were purified and digested, then inserted into pET32a plasmids to construct the prokaryotic expression vector. The cloned vector was transformed into *Escherichia coli* BL21 cells and incubated in LB medium containing 100 mg/mL ampicillin at 37 °C with shaking at 200 rpm. When the culture medium OD600 reached a value of 0.5–0.6, IPTG was added to the medium to a final concentration of 1 mM, and incubated for another 6 h at 37 °C. The empty vector without insert fragment was used as negative control. The recombinant *Cg*CytA protein containing His-tag was purified using Ni-NTA Agarose columns according to the manufacturer's instructions. The purified proteins were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of recombinant *Cg*CytA-His was determined by Bradford assay [25].