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Short communication

Structural characterization and expression analysis of a novel cysteine protease inhibitor from *Haliotis discus hannai* Ino

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ABSTRACT

The sequence of the cysteine protease inhibitor gene of *Haliotis discus hannai* (designated *HdCpi*) was determined using the RACE method. The full-length *HdCpi* cDNA is 1049 bp long, and contains an open reading frame of 813 bp, encoding a 271-amino-acid protein with a calculated molecular mass of 29.83 kDa and an isoelectric point of 8.57. The deduced amino acid sequence of HdCpi contains two cystatin-like domains, and each has the structural features of the cystatin family, including three evolutionarily conserved motifs known to interact with the active sites of cysteine peptidases: the Gly residue at the N-terminus (Gly^{65} and Gly^{160}), the Gln–X–Val–X–Gly motif ($Q^{106}IVSG^{110}$ and $Q^{202}VVAG^{206}$), and the less conserved motif at the C-terminus ($S^{136}W^{137}$ and $A^{254}W^{255}$). Many putative transcription-factor-binding sites involved in the immune system and cancer occur in the promoter region of *HdCpi*. Quantitative real-time RT–PCR detected *HdCpi* expression in all the tissues examined and in the gills of abalone challenged with the bacterium *Vibrio anguillarum*. *HdCpi* transcripts were expressed in the mantle, gill, digestive tract, hemocytes, and muscle, and increased *HdCpi* expression was observed after bacterial stimulation. These results suggest that HdCpi is a biologically active protease inhibitor that is likely to be involved in the antibacterial response of the abalone.

1. Introduction

Cysteine proteases are distributed in all living organisms and play key roles in diverse biological processes, including protein catabolism, antigen presentation, apoptosis, and inflammation [1]. The cysteine proteinases produced by bacteria and parasites are believed to act as virulence factors in the development of many diseases [2]. The activity of cysteine proteases can be inhibited by cysteine protease inhibitors (CPIs). Cystatins are the best-characterized CPIs and form a superfamily of structurally homologous proteins (Barrett et al., 1986). The cystatin superfamily is classified into three main protein families, based on sequence homologies. Family 1 cystatins (stefins), including stefins A and B, are about 100 amino acid residues long, and lack disulfide bridges and carbohydrate residues. Family 2 cystatins, consisting of cystatins C, D, S, SN, and SA, are single-chain proteins containing two intracellular disulfide bonds. Recently, the cystatin-related epididymal-specific gene (*Cres*) was identified in the mouse epididymis, and is considered to encode a new subgroup of the family 2 cystatins [3]. Family 3 cystatins (kininogens) display high structural complexity, with multiple cystatin-like domains that are linked by disulfide bridges [1,4]. More than 10 families of CPIs have been classified based on the amino acid sequences of their inhibitory domains, according to the MEROPS database [5]. Three sequence motifs are highly conserved in all cystatins, allowing them to bind to the catalytic sites of cysteine proteinases and inhibit their activity: a Gly in the vicinity of the N-terminal region, a central QxVxG motif in the first hairpin loop, and a less conserved PW motif in the second hairpin loop in the C-terminal region [6–8]. It has been shown that a tripartite wedge-shaped edge, constructed from the amino-terminal segment and the two loops of the cystatins, interacts directly with the active site cleft of the cysteine peptidases of the papain family [9].

Cystatins occur in all living organisms and are involved in various biological processes, including immune functions [10–12]. To date, only a few cystatins have been characterized in mollusks and these are believed to be involved in the innate immune responses of these species. In the snail *Planorbarius corneus*, a gene encoding a cystatin-like protein was upregulated in the hepatopancreas after the snail was infested with trematodes [13], and a

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family 2 cystatin was also elevated in the plasma of *Biomphalaria glabrata* following infection with *Echinostoma caproni* [14]. Recently, a family 1 cystatin (cystatin B) gene was identified in the Manila clam *Ruditapes philippinarum* [15]. Although a sequence encoding a CPI in the abalone *Haliotis diversicolor supertexta* has been submitted to the NCBI database, there has been little research into this gene. In the present study, a novel cysteine protease inhibitor (designated "HdCpi") was identified in *Haliotis discus hannai*, a commercially important marine mollusk species cultivated in the northern provinces of China [16]. We characterized HdCpi from structural and functional perspectives, and examined its potential role in the abalone immune responses by investigating its tissue distribution and its expression after the abalone was infected with a *Vibrio* pathogen. We thus hope to provide new information to advance disease control in abalone aquaculture.

2. Material and methods

2.1. Animals and bacterial challenge

H. discus hannai specimens, with an average shell length of 60 ± 2 mm and bodyweight of 27 ± 3 g, were obtained from a commercial farm and transported to the laboratory. They were acclimated in seawater tanks (10 m^3) and fed fresh seaweed (*Laminaria japonica* Aresch) daily. After they had been maintained for one week, the individuals in the experimental group were challenged with 50 μL of live *Vibrio anguillarum* (strain MVM425, cultured in 2216E medium at 25 °C overnight, the cell suspension was centrifuged at 1500 × *g* for 10 min at 4 °C and the resultant pellet was re-suspended in phosphate-buffered saline [PBS, pH 7.2], adjusted the optical density at 600 nm [OD_{600}] = 0.4), and the control group was injected with 50 μL of PBS. The injected abalone were returned to the seawater tanks and four individuals from each group were randomly sampled after 0, 1.5, 3, 6, 12, and 24 h. The gill samples were harvested by dissection and stored in RNAlater® (Omega Bio-Tek, Inc., Norcross, USA) until used for quantitative real-time RT–PCR. Five tissues (hemocytes, gill, mantle, digestive gland, and muscle) were collected from the individuals of the blank control group to analyze the tissue-specific expression of *HdCpi*.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues with the RNA Extraction Kit (Tiangen, China), and treated with RQI RNase-Free DNase (Promega, USA) to remove any contaminating genomic DNA. cDNA was synthesized from 2 μg of the total RNA with the PrimeScript™ RT Reagent Kit (TaKaRa, Japan), according to the manufacturer's instructions.

Table 1
Primers used in the experiment.

Name	Sequences (5′–3′)	Sequence information
Cpi31 (Forward)	GGCAACCAACACCAACAGCG	3′ RACE outer Primer
Cpi32 (Forward)	AACCAAAAATGGCTGCGGACAC	3′ RACE inner Primer
T_{17}AP (Reverse)	GACTCGAGTCGACATCGAT$_{(16)}$	3′ RACE Primer
Cpi-real-F (Forward)	CGCTGTCAGCAAGAGAGGTCTTTTT	Real-time RT–PCR
Cpi-real-R (Reverse)	GTCCACTTTCTCAGTCACCATCCG	Real-time RT–PCR
Hd-actin-F (Forward)	ACGAAGATGTTGCTGCGTTGGTT	Real-time RT–PCR
Hd-actin-R (Reverse)	TCGATGGGGTACTTGAGGGTGAG	Real-time RT–PCR
Hd-Cpi-GSP-1	ATACCCCCAGACCCAGATGTGTGAAGT	First outer primer for genome walking
Hd-Cpi-GSP-2	GAGAGGTCTTTTTCTGGGAGGTGTGGA	Second nested primer for genome walking
Hd-Cpi-GSP-3	CCACGGACTGTGCTCTCTTCTTCAA	Third nested primer for genome walking

2.3. Molecular cloning of the full-length HdCpi cDNA sequence

An expressed sequence tag (EST) sequence (DN856375) from an abalone digestive tract and gonad cDNA library was found to be homologous to the sequence of the identified cysteine protease inhibitor. Because the 5′ end of *HdCpi* of the gene was included in the EST, specific primers (Table 1) were designed to amplify only the 3′ end of *HdCpi* with seminested PCR. The reaction system contained 1 μL of each primer (10 mM), 1 μL of diluted cDNA, 2.5 μL of 10 × PCR buffer, 2 μL of dNTPs (2.5 mM each; TaKaRa), 0.25 μL (5 U) of Takara Ex Taq™ DNA polymerase, and 17.25 μL of distilled water. The PCR cycling program consisted of denaturation at 94 °C for 3 min, and then 35 cycles of 94 °C for 30 s, 55 °C for 50 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were purified and sequenced in both directions, and the resulting sequences were verified and subjected to a cluster analysis.

2.4. Molecular cloning of the HdCpi promoter sequence

Genomic DNA was extracted from the abalone *H. discus hannai* with the phenol–chloroform method [17]. The promoter region of *HdCpi* was determined with a genome walking strategy. A gene-specific primer (GSP) was designed based on the known sequence of the cDNA (Table 1) and four other arbitrary primers were provided with the Genome Walking Kit (TaKaRa, Japan). The genome walking reactions were performed according to the manufacturer's recommendations. The specific PCR products were cloned and sequenced.

2.5. Transcriptional analysis of HdCpi with real-time PCR

Real-time PCR was used to determine the tissue-specific *HdCpi* mRNA expression in hemocytes, gill, mantle, digestive gland, and muscle tissues of *H. discus hannai*. The temporal expression of *HdCpi* mRNA was also determined in the gills of abalone challenged with *V. anguillarum* or PBS. Disk abalone actin was selected as the internal PCR control and the actin gene transcripts were amplified with primers Hd-actin-F and Hd-actin-R (Table 1). The reactions were performed in a 20 μL reaction volume containing 12.5 μL of SYBR Green Supermix (Toyobo, Japan), 5 μL of cDNA diluted 1:20, 0.5 μL of each primer, and 6.5 μL of PCR-grade water. The real-time RT–PCR cycling program consisted of denaturation at 94 °C for 30s, 35 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The relative mRNA levels of *HdCpi* were calculated with the $2^{-\Delta\Delta C_T}$ method. The statistical analysis was performed with one-way ANOVA and Duncan's multiple range test for pairwise comparisons, using SPSS 11.5 (SPSS Inc., Chicago, IL). Differences were considered significant at $p < 0.05$.

2.6. Bioinformatic analysis

The *HdCpi* cDNA sequence was analyzed with the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast), where we searched for orthologous sequences. The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). A multiple alignment of HdCpi and cysteine protease inhibitor proteins from other organisms was constructed with the ClustalW multiple alignment program. TRANSFAC and AliBaba 2.1 software (http://www.gene-regulation.