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Short communication

Expression characterization and activity analysis of a cathepsin B from Pacific abalone *Haliotis discus hannai*

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ABSTRACT

Cathepsin B (EC 3.4.22.1) is a member of lysosomal cysteine protease and has a papain-like fold. In mammals, it is involved in protein degradation and other physiological processes including immune response. However, little is known about the function of cathepsin B in mollusks. In this study, we identified and analyzed a cathepsin B homolog (HdCatB) from Pacific abalone (*Haliotis discus hannai*), an economically important mollusk species cultured in East Asia. HdCatB is composed of 336 amino acid residues and its mature form is predicted to start at residue 86. HdCatB possesses typical domain architecture of cathepsin B and contains a propeptide region and a cysteine protease domain, the latter containing the four active site residues (Q108, C114, H282, and N302) that are conserved in many different organisms. HdCatB shares 40–60% overall sequence identities with the cathepsin Bofa number of vertebrates and invertebrates and is phylogenetically very close to mollusk cathepsin B. Quantitative real time RT-PCR analysis revealed that *HdCatB* expression occurred in multiple tissues and was upregulated by bacterial infection. Recombinant HdCatB purified from *Escherichia coli* exhibited apparent protease activity, which was optimal at 45 °C and pH 6.0. These results indicate that HdCatB is a bioactive protease that is likely to be implicated in the immune response of abalone during bacterial infection.

1. Introduction

Lysosomes and lysosomal hydrolases constitute a vital system in intracellular clearance. A substantial body of evidence indicates that this system is linked to various functions in physical and pathological processes, including immune response [1–4]. Cathepsins are a group of lysosomal proteases, most of which (cathepsins B, C, F, H, K, L, O, S, V, W, and X) contain a cysteine residue at the active site and belong to the C1 family of papain-like enzymes [5]. Cysteine cathepsins are synthesized as inactive zymogens and their activation requires removal of an N-terminal propeptide region. This proregion has various functions *in vivo*, such as facilitating folding of newborn enzymes, inactivating the protease domain and stabilizing the enzyme against denaturation before its trans location to the lysosome [6].

Cysteine cathepsins possess typical papain-like folds, which consist of two domains designated as L- (left) and R- (right) [7,8]. The two domains contact each other and enclose a water-filled channel. At the surface of the protein, L- and R-domain diverge and form a V-shape active site cleft [5,8]. The structural variations of this cleft result in different catalytic properties: in endopeptidase cathepsins (F, L, K, S and V), a long cleft goes across the whole interface, while in exopeptidase cathepsins (B, C, H and X), the cleft is shaped by additional structural features to reduce the proportion of binding sites [1,9]. In case of cathepsin B, a characteristic "occluding loop" at the "rear" of the cleft exerts essential functions in the exopeptidase activity of the enzyme, mainly through two sequential histidines within the loop [10]. Besides exopeptidase activity, cathepsin B can also function as endopeptidase. It exhibits dipeptidylcarboxypeptidase, carboxypeptidase or endopeptidase activity depending on pH as well as the type of substrate [11]. This variation of proteolytic activities is determined by different structural features of the protein [8]. Besides the physiological role in intracellular protein turnover, cathepsin B has been implicated in many pathological states, such as inflammatory response, cancer, Alzheimer's disease and apoptosis [12–15].

In mollusks, a few cathepsin B sequences have been reported [16,17], and only two from bivalves, i.e., oyster *Crassostrea gigas* and clam *Meretrix meretrix*, were functionally characterized [18,19]. In the present work, we reported for the first time a cathepsin B homolog (HdCatB) from gastropod abalone (*Haliotis discus hannai*), a commercially valuable marine mollusk species cultured in China,

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Japan, and Korea [20,21]. We characterized the enzyme activity of recombinant HdCatB and analyzed the transcriptional expression of *HdCatB* under normal physiological conditions as well as after bacterial infection. Our results pointed to a possible role for HdCatB in the innate immunity of abalone.

2. Materials and methods

2.1. Animals

Pacific abalone (*H. discus hannai*) of 3 years age, averaging 76 ± 4.5 mm in shell length, were collected from an abalone farm and kept in aerated sea water (20 °C) for 7 days before experimental manipulation.

2.2. Cloning of HdCatB

Total RNA was isolated from hemocyte using the HP Total RNA kit (Omega Bio-tek, USA). The RNA was used for the construction of cDNA library with the Super SMART PCR cDNA Synthesis Kit (Clontech, USA) according to manufacturer's instructions. One thousand and five hundred clones of the library were randomly selected and subjected to DNA sequence analysis; one clone was discovered to contain the full length cDNA of *HdCatB* with 5′- and 3′- untranslated regions (UTRs). The nucleotide sequence of HdCatB has been deposited in GenBank database under the accession number KC171017.

2.3. Sequence analysis

Nucleotide and amino acid sequences were analyzed with the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System (ExPASy). Domain architecture analysis was performed using the simple modular architecture research tool (SMART) version 4.0 and the conserved domain database (CDD) of NCBI. The molecular weight (Mw) and theoretical isoelectric point calculations were carried out with EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was implemented with the ClustalX program. Phylogenetic analysis was performed using MEGA 4.0 with Neighbor-joining (NJ) algorithm.

2.4. HdCatB expression under different conditions

For *HdCatB* expression under normal physiological conditions, digestive gland, foot muscle, hemocyte, mantle, gill, and adductor muscle were taken aseptically from four abalone and used for total RNA extraction with the HP Total RNA kit (Omega Bio-tek, USA). One micro gram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real time reverse transcriptase-PCR (qRT-PCR) was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [22]. The expression level of *HdCatB* was analyzed using comparative threshold cycle method ($2-\Delta\Delta CT$) with elongation factor-1-α (EF1A) as the internal control. The PCR primers for *HdCatB* are RTF1 (5′- TGGCGGCACCTGAATACTA-3′) and RTR1 (5′-GCCCAACAGACGCTTGAA-3′); the primers used to amplify the *EF1A*gene are EF1AF(5′-TGCTGTCTGATCGTTGCCT-3′) and EF1AR (5′-GCTGTCCATCTTGTTGATTCCA-3′). Melting curve analysis was operated at the end of each PCR to verify that only one amplicon was amplified. All data are given in terms of mRNA levels relative to that of *EF1A* and expressed as means plus or minus standard errors of the means (SE).

For *HdCatB* expression under pathogenic conditions, the bacterial pathogen *Vibrio anguillarum* was cultured in LB medium and resuspended in PBS to 2×10^8 colony forming units (CFU)/mL. Abalone were divided randomly into two groups (24 abalone/group) and injected intramuscularly with100 μL of bacteria or PBS (control). Abalone (four at each time point) were killed at 1.5 h, 3 h, 6 h, 12 h, 24 h and 48 h post-infection, and tissues (hemocyte and digestive gland) were taken under aseptic conditions and used for qRT-PCR as described above. For hemocyte, α-tubulin was used as internal control with the PCR primers TUBF (5′-CGACTCCTTCAACACCTTCTTCA-3′) and TUBR (5′-TTTGCGGCATCTTCCTTTCC). For digestive gland, β-actin was used as internal control with the PCR primers ACTBF (5′-GGTATCCTCACCCTCAAGTACCC-3′) and ACTBR(5′-GGGTCATCTTTTCACGGTTGG-3′).

2.5. Plasmid construction

To construct the vector for HdCatB expression, the cDNA sequence corresponding to the mature form of HdCatB (residue 86–336), i.e., the fragment lacking the signal peptide and propeptide, was amplified with PCR primers HdCatB-F (5′-GATATCATGCTGCCAGAGAACTTTGATGC-3′; underlined, EcoRV site) and HdCatB-R (5′-GATATCCAGTTTGGGGTCTCCAGC-3′; underlined, EcoRV site). The PCR products were ligated with the T-A cloning vector pEASY-T1 (Tiangen, Beijing, China). Then the recombinant plasmid was digested with EcoRV to retrieve the 0.8 kb fragment, which was inserted into pET259 [23] at the SwaI site, resulting in the expression vector pETHdCatB.

2.6. Purification of recombinant protein

Escherichia coli BL21 (DE3) (Bioteke, Beijing, China) was transformed with pETHdCatB. The transformants were cultured in LB medium at 37 °C to mid-log phase, and the expression of His-tagged recombinant HdCatB (designated as rHdCatB) was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM. After growth at 22 °C for an additional 10 h, the cells were harvested by centrifugation, and rHdCatB was purified using Ni-NTA agarose (QIAGEN, Valencia, CA, USA) under denaturing conditions as recommended by the manufacturer. The protein was reconsituted as reported previously [24], and the reconstituted protein was concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie brilliant blue. The concentration of the obtained protein was measured using the Bradford method with bovine serum albumin as the standard.

2.7. Analysis of the proteolytic activity of rHdCatB

The proteolytic activity assay was performed based on the method of Fricker et al. [25]. In brief, rHdCatB was diluted in assay buffer (pH 6.0), and dithiothreitol (DTT) was added to the buffer to a final concentration of 0.4 mM. After incubation at 30 °C for 1 h, the protein solution was further diluted to a series of concentrations in assay buffer. The diluted protein or buffer along (control) was mixed with 1 mM cathepsin B specific substrate Z-Arg-Arg-p-nitroanilide (Sigma–Aldrich, St. Louis, MO, USA). The mixture was incubated at 30 °C for 2 h, and absorbance at 410 nm was determined. The effects of pH and temperature were determined in the same fashion except that the assays were performed at various temperature (20 °C–70 °C with 5 °C intervals) and pH (3.5–8.0 with 0.5 intervals).