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Cathepsin B-like and hemoglobin-type cysteine proteases: Stage-specific gene expression in *Angiostrongy cantonensis*

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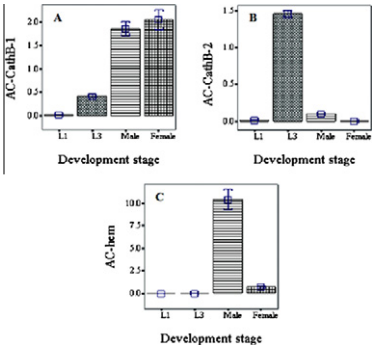
HIGHLIGHTS

- Two cathepsin B-like and one hemoglobin-type cysteine protease gene was cloned.
- Varied transcription patterns of the proteases were observed in different stages.
- AC-cathBs play an important role in host invasion; AC-hem activated AC-cathB-1.
- AC-cathB-2 is similar to cathB of *Parelaphostrongylus tenuis*, AC-hem resemble legumain of *Haemonchus contortus*.
- Ac-cathBs contain all of the conserved regions of cathepsin B from other parasites.

GRAPHICAL ABSTRACT



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ABSTRACT

Three cysteine protease genes, cathepsin B-like enzyme gene 1, 2 (*AC-cathB-1*, *AC-cathB-2*) and hemoglobin-type cysteine protease gene (*AC-hem*) were isolated and described from *Angiostrongylus cantonensis* adult. The deduced amino acid sequence of Ac-cathB-1 and AC-cathB-2 contain all of the conserved regions of cathepsin B. AC-cathB-2 is similar to a host intrusion-related cysteine protease B from *Parelaphostrongylus tenuis*, and the AC-hem shares high similarity to legumain from *Haemonchus contortus*. AC-cathB-1 was expressed significantly higher in L1 as compared with AC-hem, the AC-cathB-2 followed; AC-cathB-2 transcripts in L3 were found increased rapidly and obviously abundant, suggesting that AC-cathB-1 and AC-cathB-2 may play an important role in intermediate and final host invasion, separately. The cysteine protease genes were more or less expressed in adult stage excepted for AC-cathB-2. As the *AC-cathB-1* and *AC-hem* highly expressed in adult worms, suggesting AC-hem may activate AC-cathB-1 which involved in the host invasion and feeding process.

1. Introduction

Cysteine proteases, a superfamily of hydrolytic enzymes, have numerous functions in parasites. Proteases are utilized by helminth parasites at several stages of their lifecycles. Helminth parasites employ these enzymes during skin penetration, and to evade host immune responses through digestion of host immune effector molecules (Tort et al., 1999). In some study, it has been established that cysteine proteases are essential for pathogenicity of *Leishmania tropica* amastigote in mammalian host (Mahmoudzadeh-Niknam and McKerrow, 2004). Also, many helminths are bloodfeeders and rely upon hemoglobin obtained from the vertebrate host as a significant source of nutrition (Baig et al., 2002). A number of proteases have been proposed to play a role in the digestion of

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hemoglobin, including cathepsin L-, D-, C-, and B-like cysteine proteases. The role of the cathepsin B-like cysteine proteases as hemoglobinases in blood feeding parasites have been well established in some helminths, and suggested in others (Brindley et al., 1997; Jasmer et al., 2001).

Cathepsin B have been widely characterized in a number of parasites (Caffrey and Ruppel, 1997; Shompole and Jasmer, 2001; Baig et al., 2002; Caffrey et al., 2002) and have been found to be of great importance in their survival (Aldape et al., 1994; Que et al., 2002; Buxbaum et al., 2003; Skelly et al., 2003; Correnti et al., 2005; Cunningham et al., 2010). Because of their central role in tissue invasion, feeding and immune modulation, cysteine proteases are a focus of attention in the investigation for potential vaccines against parasites (Dalton and Mulcahy, 2001; Buxbaum et al., 2003; Abdulla et al., 2007).

In the development process of organism, only a small part of the genes are expressed and gene expression varied at different developmental stages or in different tissues (Liang and Pardee, 1992). As we have previously reported three cysteine protease genes, cathepsin b-like enzyme gene 1, 2 (*AC-cathB-1*, *AC-cathB-2*) and hemoglobin-type cysteine protease gene (*AC-hem*) found in *Angiostrongylus cantonensis* based on the ESTs sequence (Fang et al., 2010), and therefore we speculated that a variety of cathepsin B may exist in different developmental stages and have different functions. So, it is important to study cysteine protease to aid understanding how *A. cantonensis* breaking through the "blood-intestinal barrier" of the host. In addition, no studies have yet been performed with respect to the expression characteristics of cysteine protease, which is likely to be an indispensable part of the tissue degrading and feeding activity in *A. cantonensis*. In the present paper, we isolated and characteristics of cathepsin B-like and hemoglobin-type cysteine protease from *A. cantonensis*.

2. Materials and methods

2.1. Nematodes

The *A. cantonensis* maintained in Sprague Dawley rats (SD rat) which managed and housed in the Xiamen University Laboratory Animal Center (specific pathogen free) were used. Adult *A. cantonensis* were collected from the pulmonary artery of rats after mercy killing. The first stage larvae (L1) of *A. cantonensis* were collected from feces by using a sugar flotation technique at 45 days after the rat infection. Fresh positive rat feces, adding water into a paste, were applied in the surface of lettuce, the apple snails, *Pomacea canaliculata* were then fed on a diet of lettuce and infected consequently. After 3 weeks of being infected, the snails were cut into small pieces and digested overnight in digestive fluid (0.7% pepsin in 0.5% HCl). Third stage larvae (L3) were collected under a dissecting microscope. The worms were then preserved in a RNAstore solution (TIANGEN, China) and kept under −80 °C until use.

2.2. RNA isolation and converted to cDNA

Total RNA of adult nematodes of *A. cantonensis* were extracted by β-mercaptoethanol isolation protocol using RNAprep pure Tissue Kit (TIANGEN, China) according to the manufacturer's instructions; Total RNA of larvae were isolated by using Stool RNA Kit (OMEGA, China) according to the manufacturer's instructions. Adding 35 μL of extracted RNA, 4 μL RNase-free buffer RDD, and 1 μL RNase-free Dnase I stock solution in a RNase-free centrifuge tube, the residual genome was digested by incubating for 10 min at 24 °C. RNA integrity was verified by electrophoresis on 1% formaldehyde-agarose gels. The treated samples were incubated 65 °C for 10 min to inactivate of DNase I. Ten microliter of total RNA isolated from *A. cantonensis* was converted to cDNA using1st Strand cDNA Synthesis Kit (Takara, China), also following the manufacturer's protocols.

2.3. Molecular cloning of the cysteine proteases gene of A. cantonensis

2.3.1. Rapid amplification of cDNA 3′-ends (3′ RACE)

Based on the ESTs sequence (GenBank accession No. DN190193, DN190226, DN191098) of the three cysteine protease genes, cathepsin B-like enzyme gene 1, 2 (*AC-cathB-1*, *AC-cathB-2*) and hemoglobin-type cysteine protease gene (*AC-hem*) found in *A. cantonensis* (Fang et al., 2010), the following specific primers were designed respectively: *cathB-1F* (5′-ATCATGTTGGGCATTCGG-3′), *cathB-1R* (5′-GCATTTCGGTGTTGGGTA-3′); *cathB-2F* (5′-AGTCGTCCTGTGGCTCCT-3′), *cathB-2R* (5′-GTCCTTTGGGCACTTCTT-3′); *hem-F* (5′-ACATTCTTCTTCACCCTTCT-3′) and *hem-R* (5′-CGAGTCGCTTTGTAGTC-3′). Primer specificity was confirmed by PCR and sequenced completely in both directions (data not shown).

The 3′-end sequence of the *AC-cathB-1* was amplified by using a commercially available 3′-Full RACE Core Set Ver 2.0 (Takara, China) according to the manufacturer's protocol. Briefly, 2 μL of total RNA isolated from adult *A. cantonensis* was converted to cDNA, the 3′-end of the *AC-cathB-1* transcript was amplified using a combination of *AC-cathB-1*-specific primer *cathB-1F* and 3′RACE outer primer.

PCR assays were performed in a final reaction volume of 50 μL that consisted of 2 μL of the product of reverse transcription, 0.5 μL *Taq* polymerase (5 U μL^{-1}) (TaKaRa, China), 1× cDNA dilution buffer II 2 μL (TaKaRa, China), 10× PCR buffer 3 μL (TaKaRa, China), 4 μL dNTPs mixture (2.5 mM each) (TaKaRa, China), 4 μL $MgCl_2$ (25 mM) (TaKaRa, China) and 1.5 μL *cathB-1F* and 1.5 μL 3′RACE Outer Primer (10 μM each) and ddH_2O. Amplification was performed under the following conditions: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; and 72 °C for 7 min).

The same system were carried out in the 3′RACE reaction of *AC-cathB-2* and *AC-hem* as adopted for *AC-cathB-1*, in addition to the different primers (*cathB-2F* and *hem-F* separately for *AC-cathB-2* and *AC-hem*) and the annealing temperatures (59 and 53 °C for *AC-cathB-2* and *AC-hem* respectively).

PCR products were checked on 1.0% (w/v) agarose gels and visualized using ethidium bromide and UV illumination to confirm product size. The PCR products were purified by using EZ-10 Spin Column DNA Gel Extraction Kit (Sangon, China). The purified DNA was subcloned into a pMD18-T vector (TaKaRa, China), then transformed into competent *Escherichia coli* DH5α. Product was bidirectionally sequenced (Invitrogen, Shanghai) and blasted the GenBank Database.

2.3.2. Rapid amplification of cDNA 5′-ends (5′ RACE)

The 5′-end sequence of the *AC-cathB-1* was amplified by using a commercially available 5′-Full RACE Core Set Ver 2.0 (Takara, China) according to the manufacturer's protocol. Briefly, 43.5 μL of total RNA isolated from adult *A. cantonensis* was converted to cDNA, the 5′-end of the *AC-cathB-1* transcript was amplified using a combination of *AC-cathB-1*-specific primer *cathB-1R* and 5′RACE outer primer.

PCR assays were performed in a final reaction volume of 50 μL that consisted of 2 μL of the product of reverse transcription, 0.5 μL *Taq* polymerase (5 U μL^{-1}) (TaKaRa, China), 1× cDNA dilution buffer II 2 μL (TaKaRa, China), 10× PCR buffer 3 μL (TaKaRa, China), 4 μL dNTPs mixture (2.5 mM each) (TaKaRa, China), 4 μL $MgCl_2$ (25 mM) (TaKaRa, China) and 1.5 μL *cathB-1R* and 5′RACE Outer Primer (10 μM each) and dH_2O. Amplification was performed under the following conditions: 95 °C for 5 min; 35 cycles