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Immunolocalization and developmental expression patterns of two cathepsin B proteases (AC-cathB-1, -2) of *Angiostrongylus cantonensis*

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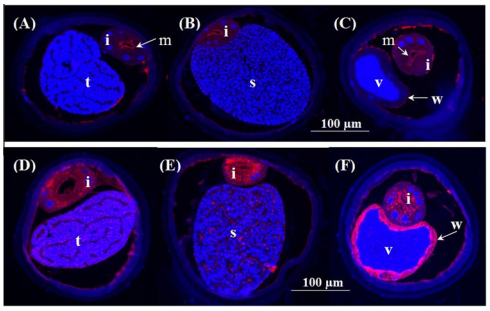
HIGHLIGHTS

- Evidences were provided for AC-cathB-1 and -2 to be the ES proteins in larvae.
- Anatomic sites and expression patterns of the two AC-cathBs were varied.
- Only AC-cathB-2 was detected in genital system, especially in wall of genital tracts.
- AC-cathB-2 might be involved in germ cells development and maturation.
- AC-cathB-1, -2 differ in substrate specificities and precise physiological functions.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study we have investigated the anatomic sites of expression and developmental expression patterns of two cathepsin B-like cysteine proteases (AC-cathB-1, -2) of *Angiostrongylus cantonensis*. The immunolocalization results revealed that native AC-cathBs were found present in the L1 and L3 larvae, female and male adults, and the AC-cathBs were localized mainly on the digestive tract of *A. cantonensis* and expressed at varied levels and in different patterns in the internal tissues according to their developmental stage. Consistent with the infective stage of L3 is a much more intense staining of AC-cathBs in the esophagus compared with the intestine. In contrast to L3, more abundant signals were located to the intestine of adults, suggesting that nutrition digestion likely to be the main function of the protease at this point. AC-cathBs fluorescent signals were present in excretory pore, excretory tube in lateral cords, and muscular esophagus of larvae, further supported the AC-cathB-1, -2 likely to be released by *A. cantonensis* as excretory/secretory products. Additionally, only the protein AC-cathB-2 was detected in the reproductive system, especially in the wall of vas deferens, uterus, and oviduct of the parasites, whether the AC-cathB-2 has some function in germ cells development and maturation need to be further characterized. Although the anatomic sites and expression patterns were different in larvae and adults and the corresponding function might not the same, AC-cathB-1 and -2 involved in the host-parasite interaction in addition to digestive function.

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1. Introduction

Cysteine proteases have been shown to be one of the most abundantly expressed protease families in the gastrointestinal tracts of parasitic helminths. These proteases are often developmentally regulated throughout the complex parasitic life cycles, are frequently up-regulated in actively feeding stages and have been shown to be commonly expressed in organs or organelles involved in feeding (Jasmer et al., 2004). Haematophagous nematodes express proteases of different mechanistic classes in their intestines, many of which have proven or putative roles in degradation of haemoglobin and other proteins involved in nutrition, however, most of the proteases described previously are expressed in the intestine and many of them do not appear in excretory/secretory (ES) products, implying that they act locally in the intestine (Williamson et al., 2003). The ES products and the characterization of cathepsin B protease in different stages of development in *Angiostrongylus cantonensis* has been described using Signal-P (Fang et al., 2010), RT-PCR, and western blot analyses (Ni et al., 2012), however, there are no data that directly support the cathepsin B protease (AC-cathB-1, -2) were released by the parasite as ES products and the anatomic location of the proteases in *A. cantonensis* remains unclear. Here we look at the anatomical localization and expression characterization of two cysteine proteases (AC-cathB-1, -2) in order to assist us in understanding better the role of cathepsin B in the *A. cantonensis*.

2. Materials and methods

2.1. Nematode and rat materials

The life cycle of *A. cantonensis* was maintained in the laboratory by routine passage through mice and the intermediate snail host *Pomacea canaliculata*. The *A. cantonensis* maintained in Sprague–Dawley (SD) rats at the Parasitological Research Laboratory of Xiamen University was 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 faeces by using a 500 mesh sieve at 45 d after the rat infection. Fresh positive rat feces, adding water into a paste, were applied in the surface of lettuce, the apple snails 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 nematodes of each stage were then preserved in a RNA store solution and kept under −80 °C until use. SD rats and immunized mouse were maintained in the Laboratory Animal Center, Xiamen University and the study was conducted adhering to the guidelines for animal husbandry and also approved by Ethic Committee from Xiamen University.

2.2. RNA extraction and reverse transcription

Two primer pairs were synthesized (Sangon, Shanghai, China) according to GenBank accession No. for cathepsin B-like cysteine protease genes 1, 2 (*AC-cathB-1*, *AC-cathB-2*) of *A. cantonensis* (Ni et al., 2012). Forward primer MEcathB-1F (5′-CGGGGTACCTCAGA AGACAACGACAAT-3′) and reverse primer (MEcathB-1R 5′-ACGC GTCGACACAGACAATGAAGTGGAA-3′) for *AC-cathB-1* (**HQ110099**); Forward primer MEcathB-2F (5′-CGGGGTACCGTCTCGGCAG-CATCTTGGC-3′) and reverse primer (MEcathB-2R 5′-ACGCGTC-GACATTTCGGTTCTCCGGCAA-3′) for *AC-cathB-2* (**HQ110100**) with restriction enzyme of *Kpn* I and *Sal* I (underlined), respectively.

Total RNA were extracted from adult *A. cantonensis* using RNAprep pure Tissue Kit (Tiangen, China) according to the manufacturer's protocols; RNA integrity was verified by electrophoresis on 1% formaldehyde-agarose gels. One microgram of RNA was then converted to the first strand cDNA in a reaction volume of 20 μL using the 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) with oligo-d (T) primer following the instruction described by the manufacturer.

2.3. Construction of expression plasmid of cathepsin B protease (AC-cathB-1, -2) cDNA

The first strand cDNA derived from adult *A. cantonensis* was served as a template to amplify a cDNA of cathepsin B proteinase (AC-cathB-1, -2) gene by RT-PCR. PCR experiment was performed in a final reaction volume of 50 μL that consisted of 2 μL of the product of reverse transcription, 10 × *Ex Taq* Buffer 5 μL (TaKaRa, Dalian, China), 8 μL dNTPs mixture (2.5 mM each), 0.5 μL *TaKaRa Ex Taq* polymerase (5 U/μL), 6 μl $MgCl_2$ (25 mM), Primer pairs MEcathB-1F and MEcathB-1R (4 μL each), and autoclaved ddH_2O. Amplification was performed under the following conditions: pre-denaturing at 95 °C for 5 min; 38 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; and a final extension at 72 °C for 7 min. The same system were carried out in the PCR reaction of *AC-cathB-2* as adopted for *AC-cathB-1*, in addition to the different primers (MEcathB-2F and MEcathB-2R) and the annealing temperatures (56 °C). 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 using EZ-10 Spin Column DNA Gel Extraction Kit (Sangon, Shanghai, China). The purified DNA was subcloned into a pMD18-T vector (TaKaRa, China). After PCR screening the inserts and plasmids digestion, positive plasmids were sent to Invitrogen (Invitrogen, Shanghai, China) for sequencing.

For construction of the expression vector of cathepsin B protease pET32a-*Ac-CathB-1* and pET32a-*Ac-CathB-2*, the right plasmid was digested by *Kpn* I and *Sal* I and cloned into the expression vector of pET-32a (Invitrogen, Shanghai, China) between these two sites with T4 DNA ligase (TransGen Biotech, Beijing, China), then transformed into competent *Escherichia coli* BL21. The identity of the individual colonies were confirmed by PCR screening the inserts using same primers as employed for their amplification, and restriction enzymes digestion as well as the plasmids sequencing.

2.4. Expression of recombinant protein fragments and polyclonal antisera production

The transformant *E. coli* BL21 clones were grown at 37 °C to an OD_{600} value of 0.7 in LB/ampicillin (100 μg/mL), induced with 1 mM isopropylthio-β-D-galactoside (IPTG) and incubated for 5 h. The induced cells were disrupted by sonication and recombinant protein was purified using HisTrap™ HP Columns (GE Healthcare, Shanghai, China) according to the manufacturer's protocol.

Three mouse each was injected intramuscularly with 60 μg of recombinant protein mixed with an equal volume of Freud's complete adjuvant, followed by three additional boost injections of 60 μg of recombinant protein in the same Freud's incomplete adjuvant at 1 week intervals. Blood was taken from the mouse after the last boost 10 d before the immunization, the polyclonal mouse anti-*A. cantonensis* antisera were stored at −20 °C until used.

2.5. Western blot analysis

For Western blots, 5 μL of recombinant polypeptide were separated through 12% SDS–PAGE, and electro-transferred to nitrocellulose membrane for western blotting according to standard