DEGRADATION OF HUMAN MATRIX METALLOPROTEASE-9 BY SECRETORY METALLOPROTEASES OF *ANGIOSTRONGYLUS CANTONENSIS* INFECTIVE STAGE

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Abstract. *Angiostrongylus cantonensis* infection is the major cause of eosinophilic meningitis. Successful migration and evasion of the immune system by infective-stage larvae (L3) rely heavily on secreted proteases, which activate human pro-matrix metalloprotease (MMP-9) into active MMP-9. This study showed that the proteases in excretory-secretory (ES) products of *A. cantonensis* third stage larvae degraded recombinant and native human proMMP-9 in a dose- and time-dependent manner. Protease inhibitory assays showed that metalloproteases were the key enzymes involved in the degradation of human proMMP-9. To assess the effects of ES products on inflammation, ES products were incubated with THP-1 human monocytic cells, which showed induction of MMP-2 and not MMP-9 production. These results indicated that degradation of human MMP-9 was due to metalloproteases present in ES of *A. cantonensis* L3, which may be involved in suppressing the host's immune response to allow parasite migration to the host central nervous system.

Keywords: *Angiostrongylus cantonensis*, eosinophilic meningitis, matrix metalloprotease-9, excretory-secretory products, proteases, protease inhibitors

INTRODUCTION

Eosinophilic meningitis is commonly caused by infection with *Angiostrongylus cantonensis*, which occurs when non-permissive and permissive hosts ingest snails or vegetables contaminated with infective-stage larvae (L3) (Li *et al*, 2008). The larvae pass into the small intestine, penetrate the intestinal wall, and then migrate to the brain, subarachnoid space, and lungs. In non-permissive hosts, including humans, the 5th-stage larvae (L5) are killed by host immune responses before migration to the final destination where development is completed (Alicata and Ben, 1965). Eosinophils are recruited as the major effector cells into the infected

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area where they eliminate parasites by releasing several mediators, including eosinophil peroxidase, lipase, and plasminogen. However, these mediators can also destroy host tissues, causing permanent nerve and brain damage, or even death.

Migration of *A. cantonensis* L3 into the brain is associated with secreted proteases, which the parasite uses to penetrate the intestinal wall. Serine proteases have been identified as the important enzymes involved in this penetration (Lee and Yen, 2005). Cathepsin B has been identified as being expressed by L4 and L5 stages residing in the hosts' brains. Cathepsin B also induces dendritic cell maturation, which may be involved in the invasion of the central nervous system (CNS), and modulation of host immune response (Han *et al*, 2011).

Matrix metalloproteases (MMPs) are predominant proteases in excretory/secretory (ES) products of *A. cantonensis* L3 stage (Lai *et al*, 2005). However, their roles in the parasite life cycle and in pathogenesis, have not been elucidated. In other organisms, proteases contained in the frass (fine powdery material insects pass as waste after digestion) of cockroaches and house-dust mites appear to activate the conversion of human proMMP-9 to the active form (Hughes and Page, 2007). This may explain the clinical manifestations and severity of some allergies (Page *et al*, 2006; Hughes and Page, 2007). In eosinophilic meningitis, MMP-9 levels were up-regulated in both cerebrospinal fluid (CSF) and blood, and could be detected by gelatin zymography (Tsai *et al*, 2008). A study using an experimental non-permissive model showed that MMP-9 levels are highly expressed in macrophages and eosinophils associated with eosinophilia in the CSF, and inflammation of the subarachnoid space (Lee *et al*, 2004). MMP-9 is a zinc endopeptidase, whose functions include degradation of extracellular matrix (fibrillin, elastin, gelatin and collagen type IV, V, XI and XVI), activation of procytokine (Yu and Stamenkovic, 2000), and leukocyte migration (Gong *et al*, 2008). Activation or inhibition of MMP-9 clearly affects host's biology, physiology, and immune response.

In this study, we focused on the interaction between ES products of *A. cantonensis* L3 and human proMMP-9. The ES products were incubated directly with recombinant and native human proMMP-9 to determine their effects on proMMP-9, and family-specific protease inhibitors were used to identify the type of proteases involved. The effects of ES products on immune cells also were investigated using a monocytic THP-1 cell line in order to clarify the action of proteases in inflammatory responses.

MATERIALS AND METHODS

Parasites

Third-stage larvae (L3) were obtained from naturally infected *Achatina* spp (land snail) and maintained by cyclic infection of Wistar rats (*Rattus norvegicus*) and *Biomphalaria glabrata* (freshwater snail). Rats were maintained at the Laboratory Animal Science Center, Faculty of Tropical Medicine, Mahidol University, according to the ethics protocol for animal use (FTM-ACUC 005/2004). L3 were obtained by digesting gastropod tissues in acid pepsin solution (0.7% pepsin, 0.7% HCl) for 1 hour at 37°C. Host tissue debris was eliminated using the Baermann technique (Walters and Andersen, 1973) and subsequently sedimented several times in 0.85% normal saline solution (NSS).

Preparation of parasite antigens

Parasites at L3 stage were washed

twice with culture medium [RPMI-1640 (Gibco, Grand Island, NY), supplemented with 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco)] and then cultured for 7 days (maximum) under an atmosphere of 5% CO_2, 10% O_2, and N_2. Parasite viability was checked daily under a microscope, and culture process was stopped when viability was < 70%. Culture media were collected daily and replaced with fresh media. The collected media were pooled, dialyzed against phosphate-buffered saline (PBS) pH 7.4, and concentrated using a 5 kDa cut-off ultrafiltration (Amicon, Beverly, MA). The ES products were sterilized by passing through 0.2 µm syringe filter (Pall Life Sciences, Ann Arbor, MI), and protein concentration was determined by Bradford assay (Thermo Scientific, Rockford, IL) and stored at -70ºC until analysis.

Gelatin zymography of parasite antigens

ES products of *A. cantonensis* L3 were mixed at a ratio of 1:1 with a non-reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 30% glycerol, 0.01% bromophenol blue), incubated at room temperature for 5 minutes and then analyzed by 10% SDS-PAGE containing 0.2% gelatin (Sigma Chemical, St Louis, MO) (Laemmli, 1970). Each gel was washed twice at room temperature for 1 hour with 2.5% Triton X-100 and twice with 10 mM Tris-HCl pH 7.5 for 10 minutes. The gel was incubated in substrate buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.05% Brij35) at 37ºC for 16-18 hours. Then, the gel was stained with 0.5% Coomassie brilliant blue R-250 (USB Corporation, Cleveland, OH). Proteins with gelatinase activity produce clear bands against a blue background.

Protease inhibitory assays were performed by incubating parasite antigens in substrate buffer containing different protease inhibitors (Table 1) followed by electrophoresis as described above (Lai *et al*, 2005).

Table 1

Inhibitor	Protease family inhibited	Concentration
E64	Cysteine	10 mM
EDTA	Metallo	10 mM
Pepstatin	Aspartyl	5 µg/ml
PMSF	Serine	5 mM

Interaction analysis between parasite antigens and recombinant human proMMP-9

Recombinant human proMMP-9 (rh-proMMP-9; R&D Systems, Minneapolis, MN) (40 ng) was incubated with 0-80 ng of *A. cantonensis* L3 ES products at 37ºC for 2 hours. For time-dependence study, ES products were incubated with 40 ng of rh-proMMP-9 at 37ºC for 5-120 minutes. Reactions were stopped by adding 6x reducing buffer (62.5 mM Tris-HCl pH 6.8, 30% glycerol, 0.01% bromophenol blue, 10% SDS, 0.6 M DTT) and heating at 100ºC for 5 minutes. Samples were then separated by 4-16% gradient SDS-PAGE and then transferred onto PVDF membranes (Pall) using a semi-dry blotting system (ATTO, Tokyo, Japan). Blotted membranes were incubated with primary rabbit anti-human MMP-9 antibodies (Millipore Corporation, Temecula, CA) followed by secondary anti-rabbit IgG-HRP antibodies (Biorad Laboratories, Hercules, CA). Signals were developed by enhancement chemiluminescence method (Thermo Scientific). Each assay was performed in triplicate.

Analysis of the effects of parasite antigen on native human MMP-9

Culture medium of human monocyte

THP-1 cell line containing MMP-9 (5 µl) was used to incubate with 0-40 ng of ES products at 37°C for 2 hours. The reaction products were analyzed by 4-16% gradient SDS-PAGE and blotting onto PVDF membrane. MMP-9 was detected by immunoblot assay as described above. Each assay was performed in triplicate.

Determination of MMP-9 in human monocyte THP-1 cell line

THP-1 cells (ATCC) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under 5% CO_2 atmosphere. Cells ($1x10^6$ /ml) were seeded into a 24-well culture plate in serum-free RPMI-1640 medium and were then treated in the absence or presence of 200 ng-20 µg ES products at 37°C under 5% CO_2 atmosphere for 24 hours. Cells then were harvested by sedimenting at 1,000*g* for 5 minutes, followed by lysis with lysis buffer [50 mM HEPES pH 7.6, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100] and measurement for protein concentration using Bradford assay kit (Thermo Scientific). Three µg of lysate were analyzed for presence of MMP-9 by gelatin zymography as described above. Each assay was performed in triplicate.

Bioinformatics analysis of *A. cantonensis* metalloprotease

A search for *A. cantonensis* metalloproteases was conducted using NCBI database (http://www.ncbi.nlm.nih.gov) in order to discover amino acid sequences conserved residues similar to related orthologs using Clustal W 2.0.12 program (Mobyle@Pasteur) and multiple sequence alignments were displayed employing BOXSHADE 3.21 programs http://www.ch.embnet.org/software/BOX_form.html) and phylogenetic tree was created by

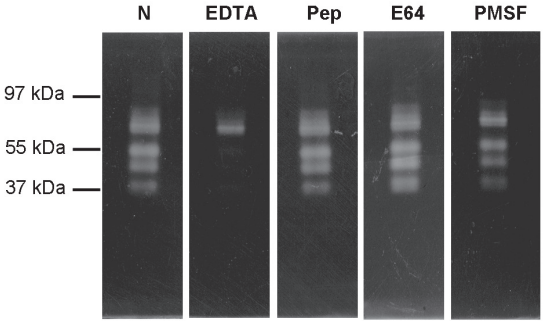

Fig 1–Gelatin zymogram of ES products of *A. cantonensis* L3 larvae. Clear bands indicate gelatinase activity. N, ES products only; EDTA, with 10 mM EDTA; Pep, with 5 µg/ml pepstatin; E64, with 1 µM E64; PMSF, with 5mM PMSF.

PHYLIP 3.67: Drawgram program (Mobyle@Pasteur). GenBank accession numbers of helminthic metalloproteases used for alignment and phylogenetic analyses were as follows: *A. cantonensis* (AEB96398), *Ancylostoma caninum* (AAK62032), *Strongyloides stercoralis* (AAK55800), *Onchocerca volvulus* (AAV71152), *Trichinella spiralis*-1 (XP_003366164), *T. spiralis*-2 (XP_003374877), *T. spiralis*-3 (XP_003367402), *T. spiralis*-4 (XP_003369704), and *T. spiralis*-5 (XP_003369054).

RESULTS

Protease activity in ES products of *A. cantonensis* L3

Gelatin zymography was used to identify proteases in ES products of *A. cantonensis* L3. Four clear bands demonstrating protease activity were identified with sizes of 35, 45, 55 and 65 kDa. For inhibition assay, zymogram gel was incubated with different types of protease inhibitors (Table 1), showed that gelatinase activity of the ES products was most sensitive to inhibition by EDTA, a metalloprotease inhibitor except the 65 kDa band (Fig 1).

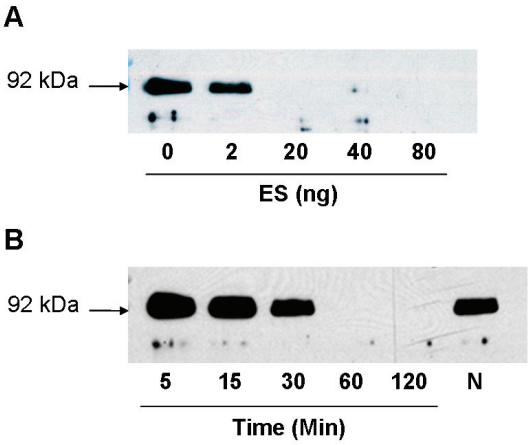

Fig 2–Degradation of recombinant human proMMP-9 by ES products of *A. cantonensis* L3 larvae. A) Incubation of 40 ng of rh-proMMP-9 with ES products for 2 hours at 37°C. Degradation of rh-proMMP-9 was detected by western blot analysis. Arrow indicates proMMP-9 of 92 kDa. B) Incubation of 20 ng of ES products with 40 ng of rh-proMMP-9 for 5-120 minutes at 37°C. N, the absence of ES products.

Serine (PMSF), aspartic (pepstatin) and cysteine (E64) protease inhibitors did not affect gelatinase activity. Thus the majority of proteases in ES products belonged to the metalloprotease family.

Degradation of recombinant human pro-MMP-9 by ES products of *A. cantonensis* L3

The notion that the ES products of *A. cantonensis* L3 can activate human pro-MMP-9 into active MMP-9 was tested by incubating recombinant human proMMP-9 with ES products, which showed a dose response degradation of rh-proMMP-9 over the range of 2-20 ng of ES products (Fig 2A). Using 20 ng of ES products, rh-proMMP-9 was degraded after incubation for 30 minutes and was completely digested at 60 minutes (Fig 2B). These results indicated that ES products degraded rh-proMMP-9 and not convert the latter to the active form.

The degradation of rh-proMMP-9 was inhibited by 10 mM EDTA (metalloprotease inhibitor) but not by other inhibitors, namely E64 (cysteine protease inhibitor), pepstatin (aspartic protease inhibitor), and PMSF (serine protease inhibitor) (Fig 3). Combinations of the four types of protease inhibitors did not inhibit degradation of rh-proMMP-9, except in the presence of EDTA. ES products (20 ng) also was able to degrade native human proMMP-9 (data not shown).

Effect of *A. cantonensis* L3 ES products on monocytic THP-1 cell line

A. cantonensis L3 ES products did not induce production of MMPs in THP-1 cells, even at 2,000 ng/ml (Fig 4), while the same protein concentration of crude L3 antigens (CWA) induced MMP-2 synthesis (72 kDa), but not MMP-9.

DISCUSSION

During a parasite life cycle, several functions are associated with the generation proteases, including encystation (Delcroix *et al*, 2006; DuBois *et al*, 2008; Moon *et al*, 2008), excystation, nutrient uptake, host tissue invasion (Delcroix *et al*, 2006; McGonigle *et al*, 2008), migration (McGonigle *et al*, 2008), and evasion of host immune system (Baxt *et al*, 2008; Swenerton *et al*, 2011). This study showed that the ES products of *A. cantonensis* L3 exhibited four different species with gelatinase activity, most of which were inhibited with EDTA, a metalloprotease inhibitor, in agreement with previous findings that matrix metalloproteases are the major enzymes involved in digestion of gelatin substrate (Lai *et al*, 2005). *Strongyloides stercoralis* infective stage larvae secrete metalloproteases to facilitate skin penetration, which is inhibited

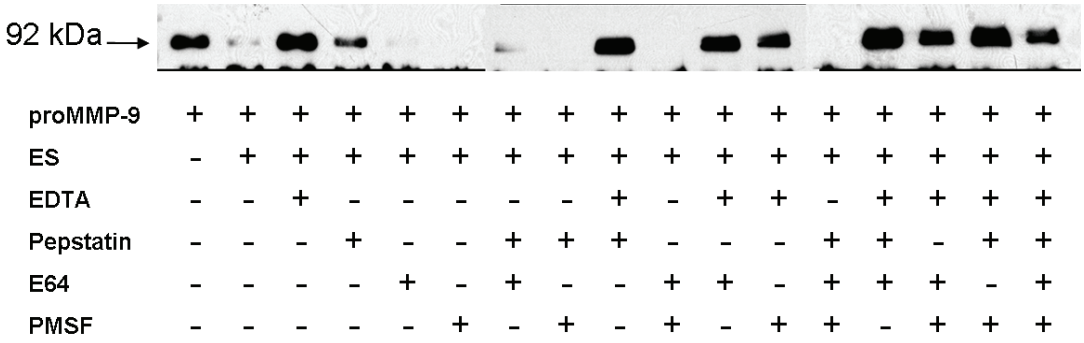

Fig 3–Effect of protease inhibitors on degradation of rh-proMMP-9 by *A. cantonensis* ES products. ES products (20 ng) were incubated with EDTA (10 mM), pepstatin (5 µg/ml), E64 (1 µM) or EDTA (10 mM), and combination of 2, 3, and 4 inhibitors for 30 minutes at 37°C, followed by addition of rh-proMMP-9 (40 ng) and incubation for an additional 1 hour at 37°C. Degradation of rh-proMMP-9 was determined by western blot analysis.

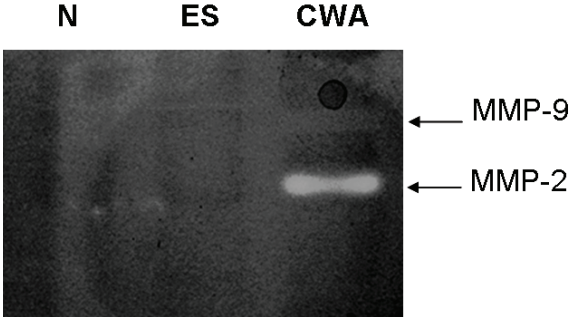

Fig 4–Effect of *A. cantonensis* L3 larvae ES products on expression of MMP in THP-1 monocytic cells. THP-1 cells were incubated with 2,000 ng/ml ES products (ES) or crude *A. cantonensis* L3 antigen (CWA) (2,000 ng/ml) for 24 hours at 37°C. Intracellular MMP was extracted and MMP-2 and MMP-9 contents were determined by gelatin zymogram. N, non-treated control. Arrow indicates proMMP-9 (92 kDa) and proMMP-2 (72 kDa).

by metalloprotease inhibitors (McKerrow *et al*, 1990). As for *A. cantonensis*, matrix metalloproteases have been suggested having a role in penetration of the gut wall (Lai *et al*, 2005). However, Lee and Yen (2005) showed that suppression of metalloprotease activity only results in 20% inhibition of penetration of the intestinal wall, with 51% inhibition in the presence of serine protease inhibitor. This suggests that *A. cantonensis* L3 metalloproteases are involved in digesting the host extracellular matrix, but not in the penetration of intestinal wall.

It has been suggested that ES products from *A. cantonensis* infective stage larvae could convert proMMP-9 into active MMP-9, like proteases from cockroach feces (Hughes and Page, 2007). However, this study found that the proteases in ES products did not activate, but rather degraded proMMP-9 in a dose- and time-dependent manner. MMP-9 has been characterized as pleiotropic protease, involved in degradation of extracellular matrix, tissue remodeling, cancer metastasis, pro-cytokine and chemokine activation, leukocyte migration, and inflammation (reviewed by Van den Steen *et al*, 2000; Renckens *et al*, 2006; Page-McCaw *et al*, 2007). MMP-9 is needed in innate immune response, where it enhances the efficiency of leukocytes (Rencken *et al*, 2006). MMP-9 knockout mice are more susceptible to *E. coli* peritonitis than normal wild-type

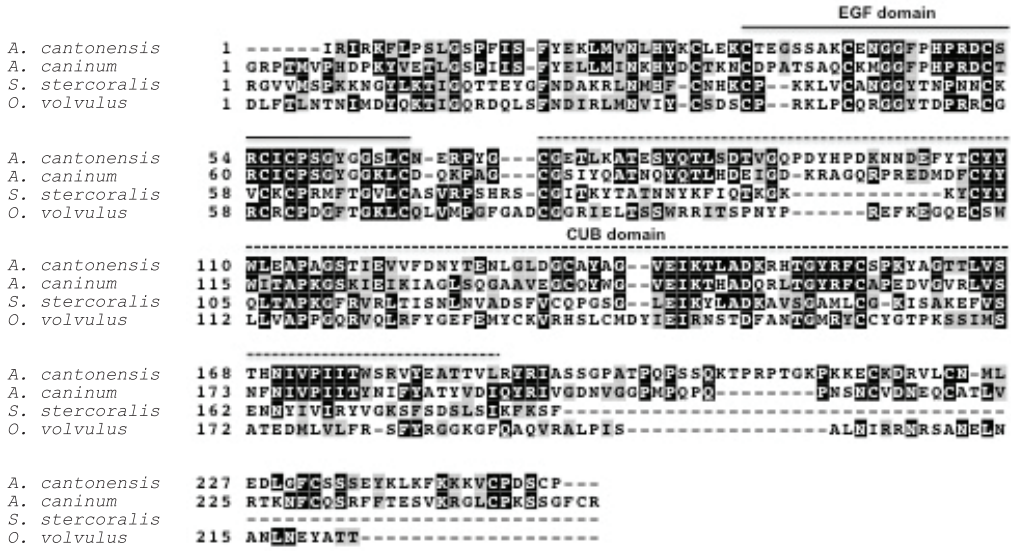

Fig 5–Alignment of the deduced metalloprotease amino acid sequence of *A. cantonensis* (AEB96398), *Ancylostoma caninum* (AAK62032), *Strongyloides stercoralis* (AAK55800), and *Onchocerca volvulus* (AAV71152). The boxes indicate conserved residues; black boxes identical residues, and grey boxes similar residues. EGF-like and CUB motifs are indicated by solid and dash line, respectively.

mice, and impair recruitment of leukocytes (Renckens *et al*, 2006). Moreover, MMP-9 acts as an inflammatory chemokine, functioning as a chemo-attractant to induce neutrophil migration to an inflammation site (Van den Steen *et al*, 2000). The degradation of human proMMP-9 by *A. cantonensis* L3 proteases in ES products may be an immune evasion mechanism, which the parasite uses to suppress the host immune response. This mechanism may benefit infective-stage parasite survival from the host immune system during migration to the brain.

The protease type responsible for degrading rh-proMMP-9 was metalloprotease as characterized by protease inhibitory assay. Three species (35, 45 and 55 kDa) with gelatinase activity were inhibited by 10 mM EDTA only, but one band (65 kDa) was resistant to all four inhibitors used. However, *A. cantonensis* L3 ES products degraded human proMMP-9, which was inhibited by EDTA, indicating that metalloproteases were responsible. Many pathogens have metalloproteases with inhibitory activity against host immune response. *Leishmania* sp carry leishmanolysin, a metalloprotease, on their surface to destroy the host's anti-microbial peptides, which induce apoptosis in the protozoa (Kulkarni *et al*, 2006). The metalloproteases found in the secretory products of *Necator americanus*, a hookworm, are associated with proteolysis of eotaxin, an eosinophil-selective chemokine (Culley *et al*, 2000) that recruits eosinophil to the site of infection. *A. cantonensis* metalloproteases deposited in NCBI database (AEB96398), when compared with orthologs, shows a conserved epidermal growth factor-like (EGF) and a complement C1r/C1s, Uegf, Bmp1 (CUB) domains at the COOH-terminus (Fig 5). Both domains are specific signatures of the astacin subfamily of metalloproteases. The CUB

domain is used for oligomerization, recognition, and binding of substrates (Gomez Gallego *et al*, 2005). However, the amino acid sequence in the database lacks the NH_2-terminus, which in other helminthic metalloproteases contains Zn^{2+} binding and Met-turn motifs, and the active site. Phylogenetic analysis showed that the *A. cantonensis* metalloprotease is closely related to other helminthic metalloproteases, including *A. caninum*, *S. stercoralis*, and *O. volvulus* (data not shown).

Treatment of monocytic THP-1 cells with *A. cantonensis* L3 ES products could induce the expression of MMP-2 but not MMP-9. In *Nippostrongylus brasiliensis*, L3-stage ES products suppress the migration of neutrophils and production of several inflammatory mediators, *viz*., adhesion molecules, cytokines, chemokines, and nitric oxide (Zhao *et al*, 2009). The immunomodulatory molecule, ES-62, present in ES products of the rodent filarial nematode, *Acanthocheilonema viteae*, is able to ameliorate the severity of inflammatory disease (Harnett and Harnett, 2006). The anti-inflammatory and immunomodulatory functions of *A. cantonensis* ES products have not yet been identified, and requires further study.

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